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Tissue Folacin Stores in Rats Measured by Radioassay (40467)

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During the past few years, several radiometric techniques for measuring human serum and erythrocyte folacin have been published (1–3) which offer the advantage of being more convenient and rapid than the standard microbiologic (*L. casei*) assay. The purpose of the present study was to determine the validity and relative advantage of using a radioassay to measure tissue folacin in rats in depletion–repletion experiments.

Three experiments were performed in this study. The aim of the first experiment was to determine the relation between intake of folic acid and tissue folacin levels measured by radioassay in rats fed control and deficient diets. The aim of the second and third experiments was to compare folacin values measured by radioassay with those determined by the standard microbiological procedure.

Materials and methods. In the first experiment, female rats of Sprague-Dawley descent (Blue Spruce Farms, Altamont, NY) weighing 155.2 ± 0.9 g (±SEM) were housed individually in stainless steel wire cages, in a temperature-regulated room (21.4°) with a 12-hr light: 12-hr dark cycle. The rats were fed a 25% casein diet containing 3 mg/kg folic acid. Composition of the diet was identical to that used previously in this laboratory (4). Food and water were provided *ad libitum,* and food intake was measured at regular intervals during the study. After 3 weeks on the diet, seven rats were killed for determination of baseline serum and liver folacin concentrations. The remaining rats were then randomly divided into four dietary groups as follows: Control rats continued to receive the diet containing 3 mg/kg folic acid; group LF (low folacin) was given a diet containing 0.5 mg/kg folic acid; group 0 received a diet with no added folic acid (folacin content of this diet was 0.1 mg/kg as determined by radioassay); group O + SST received the O folic acid diet containing 0.5% succinylsulfathiazole, to determine whether suppression of intestinal synthesis of folacin would affect tissue depletion.

After 7, 21, and 42 days on the different diets, seven animals from each group were killed for determination of serum and liver folacin. Kidney folacin was determined at 42 days. Folacin repletion was examined in three rats from group O by placing them on the control diet for 14 days and then assaying liver and serum folacin.

The rats were killed by decapitation, and trunk blood was collected for subsequent separation of serum. The liver and kidneys were then flushed of blood, via the abdominal aorta, with 120 ml of cold 0.9% NaCl solution; the organs were removed, blotted, put into plastic bags, and frozen at −25°. Twelve to eighteen hours later, the tissues were weighed; 5 g aliquots from each liver or two kidneys from each rat were homogenized with a Teflon pestle in 20 ml of 2% ascorbate solution (pH 6) (5). The homogenates were heated in a boiling water bath for 10 min, cooled, mixed and centrifuged at 1500*g* for 30 min at 4°. The supernatant was collected, and the debris-pellet was resuspended and recentrifuged two more times. The supernatants were pooled and stored at −25° for 6–12 weeks prior to assay. Prior experiments had shown that tissue extracts could be stored for at least 12 weeks without loss of activity. Serum samples were diluted with an equal volume of a 1% solution of ascorbic acid and frozen for 2–6 weeks prior to assay.

In a second experiment, values of liver extracts measured by radioassay were compared to values determined by microbiological assay, using *Lactobacillus casei* as the test organism. In that experiment, livers taken from rats fed O, LF, or control diets for 7 days were homogenized in a 2% ascorbate solution (pH 6) immediately after removal and incubated at 37° to allow deconjugation of folacin polyglutamates by endogenous conjugases which is necessary for the *L. casei* assay (5). After 5 hr of incubation, the samples were placed in a boiling water bath for 15 min, cooled, and centrifuged. The supernatant of the first washing only was saved

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and stored at −25° until assay.

A third experiment was performed to determine whether deconjugation of polyglutamate forms of folacin, by means of endogenous liver conjugases (5), would cause a change in the amount of folacin measured by radioassay as compared to assay by *L. casei*. The incubation was performed at pH 4.5 to maximize conjugase activity (6). In this experiment, three male rats, which had been fed a commercial chow diet, were killed by decapitation and their livers were quickly removed, weighed, minced and homogenized on ice in a 2% ascorbate solution. The homogenates were pooled and 5 ml aliquots were diluted with 2 vol of 0.1 *M* citrate buffer (pH 4.5). Two aliquots were immediately placed in a boiling water bath to inactivate endogenous conjugase activity, while the remaining samples were incubated at 37°. Two aliquots were removed and placed in a boiling water bath after 2 hr and again after 5 hr of incubation. The homogenates were centrifuged and the supernatants were frozen until the time of assay.

Assay procedures. The radioassay was a competitive protein binding procedure (1, 7) in which a demineralized cow's milk whey protein ("Hi Protal-50," Tetroid Co., Hamilton, NY) was used as the binder. Extracts of liver or kidney were diluted with freshly prepared pH 9.3 lysine buffer (0.05 *M* lysine buffer, containing 0.1% gelatin and 5 mg/ml sodium ascorbate). Aliquots of the diluted sample (100 μl) were added to 12 × 75 mm polypropylene tubes containing 800 μl of lysine buffer. Then, 100 μl of tritiated folic acid with a concentration of 2.5 ng/ml (^{3}H-PGA, 42 mCi/μ*M*, Amersham-Searle, Arlington Heights, IL), was added to the tubes, and the tubes were gently mixed. Binder solution (100 μl), diluted with buffer so that it bound 50–60% of the total radioactivity[1], was then added to the tubes. The tubes were gently mixed and stored at room temperature, in the dark, for 60 min. Ice-cold dextran-coated charcoal (0.4 ml) (2 g Darco G-60 activated charcoal: 0.2 g dextran, M.W. 40,000 in 100 ml of glass-distilled water) was added to the tubes. The tubes were then mixed, centrifuged at 1500*g* for 20 min, and the supernatants were decanted into 10 ml of liquid scintillation fluid (PCS, Amersham-Searle). The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375) for 10 min.

Serum samples, diluted with buffer, were treated the same as liver and kidney extracts, except that they were placed in a boiling water bath for 15 min to inactivate endogenous binders, then cooled prior to the addition of tritiated folic acid (7).

A set of standards containing 2.5–30.0 ng/ml folic acid was included in the procedure with each set of samples and a standard curve was constructed using a log-logit transformation (1).

Microbiological assay used *Lactobacillus casei* (ATCC 7469) as the test organism. Incubation at 37° was allowed to proceed for 42–44 hrs (8).

Recovery of folic acid from serum was determined by adding 50 μl of a solution containing 5 ng/ml folic acid to each of 5 serum samples containing 7.9 ng/ml endogenous folacin. Recovery from liver was determined by adding a solution containing 20 μg of folic acid to each of two liver homogenates containing 70.13 μg of endogenous folacin.

Liver, serum, and kidney folacin values for each group were analyzed by one-way analysis of variance in conjunction with the Duncan Multiple Range Test. Comparison of liver folacin determined by *L. casei* and radioassay was done using a Pearson Product Moment correlation and the *t* test for correlated samples (9).

Results. There were no significant differences in food intake among any of the groups during the study. The average amount of folic acid ingested (μg/rat/day) was 1.57, 1.52, 7.13, and 47.31 for O+SST, O, LF, and Control rats, respectively.

Individual serum folacin values ranged from 32.0 to 44.0 ng/ml in the control group during the course of the study. Mean serum folacin for all time periods was 36.9 ng/ml.

Serum folacin fell rapidly in all groups of rats fed diets with decreased amounts of folic acid (Fig. 1). By the seventh day of treatment,

[1] The whey protein was initially mixed with 0.9% NaCl solution at a concentration of 100 mg/ml; this stock solution was frozen until the time of assay. Just prior to use, it was defrosted and diluted with lysine buffer; the working concentration was approximately 10 mg/ml.

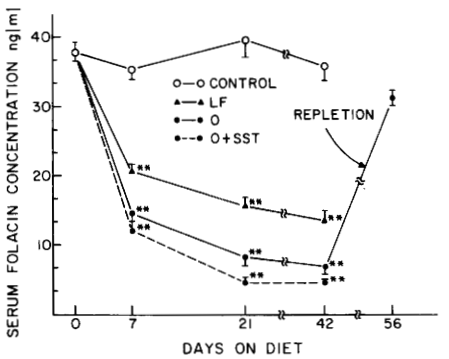

FIG. 1. Serum folacin concentration in rats given diets with varying amounts of folic acid. Abbreviations and symbols: controls = rats fed diets with 3 mg/kg folic acid; LF = rats fed diets with 0.5 mg/kg folic acid; O = rats fed diets with no added folic acid; O+SST = rats fed diets with no added folic acid plus 0.5% succinylsulfathiazole; vertical bars = ±SEM; double asterisk = $p < .01$ compared to controls.

groups LF, O and O+SST had significantly less serum folacin than the control group ($p < .01$ in each case). By the end of the study period, serum folacin of group O had fallen to 18% of the controls ($p < .01$), while that of group LF fell to 37% of control value ($p < .01$). Serum folacin fell most rapidly in the deficient group given succinylsulfathiazole; mean serum folacin of group O+SST fell to 12.5% of the control value by the end of the study ($p < .01$). Serum folacin concentration of group O+SST was lower than that of group O at each point in the study; however, the differences between these two groups were not statistically significant. Dietary repletion with folic acid was followed by a rise in serum folacin to within the control range. The correlation coefficient between folic acid intake and serum folacin (excluding group O+SST) was 0.91 ($p < .01$).

The pattern of liver folacin concentration was similar to that of the serum (Fig. 2). By the seventh day, groups O and O+SST had significantly less folacin than the control group ($p < .05$).By the end of the study, liver folacin had fallen to 58%, 28%, and 16.4% of control values in groups LF, O, and O+SST, respectively. All groups had significantly less folacin than the control group by the 42nd day of the study ($p < .01$). In addition, the group fed the diet with succinylsulfathiazole had a significantly lower liver folacin concentration than group O ($p < .05$).

Correlation between dietary folic acid intake and liver folacin was 0.86 ($p < .01$). Dietary repletion with folic acid was followed by a rise in mean liver folacin to control levels (14.8 μg/g).

Kidney folacin concentrations reflected dietary intake and were 5.14, 3.50, and 0.47 μg/g in controls, LF, and O rats, respectively. As with liver and serum, there was a significant correlation between folic acid intake and kidney folacin ($r = 0.78$, $p < .01$).

Table I shows the folacin concentration of liver samples which had been autolyzed for 5 hr then assayed by either radioassay or by *L. casei*. Mean folacin values determined by the two methods did not differ significantly ($t = 1.68$, $p > .10$). Moreover, correlation analysis between the two sets of values was

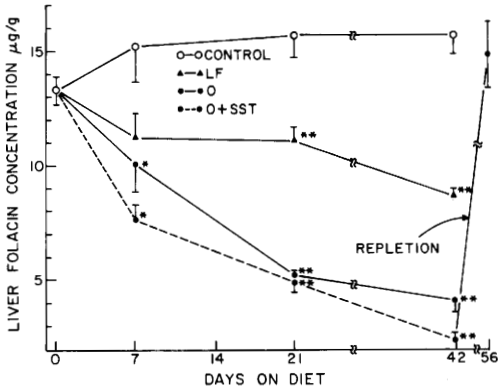

FIG. 2. Liver folacin concentration in rats given diets containing varying amounts of folic acid. Abbreviations and symbols: asterisk = $p < .05$ compared to controls. For other symbols see Fig. 1.

TABLE I. COMPARISON OF LIVER FOLACIN CONCENTRATIONS MEASURED BY *Lactobacillus casei* OR RADIOASSAY.

	Folacin μg/g[a]	
	L. casei	Radioassay
	5.8	5.3
	4.6	4.2
	5.6	7.9
	4.6	4.8
	7.1	7.2
	7.9	9.0
	7.9	8.9
	7.8	7.5
	7.4	8.7
(mean)	6.52	7.05
(SEM)	±0.46	±0.62

[a] Each value represents the mean of duplicate or triplicate determinations for one liver sample.

statistically significant ($r = 0.87$, $p < .01$).

Table II shows the effects of incubation at 37° on liver folacin concentration as measured by radioassay and *L. casei.* Folacin values determined by *L. casei* increased approximately three-fold after 3–5 hr of autolysis, while the values determined by radioassay remained essentially unchanged.

Recovery of folic acid added to serum was 101.9 ± 2.12% ($\bar{X}$ ± SEM). Recovery from liver extracts was 104.7 ± 4.9%. Intra-assay coefficients of variation (% CV) ranged from 1.0 to 7.5% for liver, kidney and serum; inter-assay % CV ranged from 2.7 to 10.4%.

Discussion. Folacin values and the pattern of tissue depletion in liver, kidney, and serum were similar to values found by others using *L. casei* to measure tissue folacin, in rats fed levels of folic acid which were comparable to those used in the present study (4, 10, 11). Moreover, comparison of liver samples concurrently measured by radioassay and *L. casei* gave excellent agreement. Similar data have been found by others comparing radioassay and microbial folacin values of human serum or whole blood (1, 12). The present study extends those findings to include rat liver.

Radioassay, using a milk binder, appears to measure folacin regardless of glutamate chain length. This was suggested by the incubation experiment, in which there was a threefold increase in folacin measured by *L. casei* after incubation at 37°, but no change in folacin measured by radioassay. Moreover, folacin concentration of unincubated liver samples measured by radioassay was virtually identical with that of incubated liver measured by *L. casei.* This result supports previously published data showing tht pteroylmono-, pteroyltri- and pteroylheptaglutamates had the same capacity to displace ^{3}H-PGA from milk binder (13) or hog-kidney binder (2).

TABLE II. EFFECTS OF INCUBATION AT 37° ON FOLACIN CONCENTRATION OF LIVER EXTRACTS DETERMINED BY *L. casei* OR RADIOASSAY.

	Hours incubated		
	0	3	5
μg/g folacin by *L. casei*[a]	6.61	19.51	21.23
	6.70	20.23	17.74
μg/g folacin by radioassay	20.47	19.75	20.59
	20.43	21.69	16.47

[a] Each number represents the mean of duplicate or triplicate determinations for the time period.

Taken as a whole, the present findings suggest that radioassay offers a feasible method for measuring total tissue folacin in experimental laboratory animals.

Summary. Serum and liver folacin levels determined by competitive protein binding assay fell rapidly after the administration of folacin-depleting diets to female rats and returned to normal after dietary repletion with folic acid. Values of liver folacin measured by radioassays were comparable to values concurrently-determined by assay with *Lactobacillus casei.* Liver, serum, and kidney folacin levels were similar to those previously determined in this laboratory and to published values for total folacin determined by *L. casei.* Incubation of liver homogenates for 3–5 hr at 37° led to a threefold increase in folacin concentration when measured by *L. casei,* but did not alter values determined by radioassay.

The results suggest that competitive protein binding offers a feasible and conventional technique for measuring total folacin content of rat tissues.

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