Effect of chronic choline deficiency in rats on liver folate content and distribution

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We have previously reported that folate and choline metabolism are interrelated. Total folate concentration was significantly diminished (31%) when rats were fed a choline-deficient diet for 2 weeks. Distribution among species of folates (differing in pteridine ring structure and/or in the number of glutamic acid residues) was not altered by this short-term dietary deficiency. In the present study we analyzed folate content and distribution in livers after 12 months on a choline-deficient diet. We found that this long-term deficiency did not significantly alter the total folate concentration in liver, however there was a significant difference between the control and deficient groups in the distribution of folate species. Whereas the major folates in the livers of control rats consisted of penta- and hexaglutamyl derivatives, those from the livers of the choline-deficient group were of longer chain lengths including hepta- and octaglutamyl derivatives. It is believed that chain elongation of the glutamate residues reflects, among other things, increased residence time of the folate molecule in the liver. A plausible interpretation of the previous and the present studies is that choline deprivation interferes with the assimilation of newly acquired folate by the hepatic tissue. Initially, during choline deprivation hepatic folate losses due to normal turnover were not promptly replenished, hence, we observed a decrease in folate level. However, when the period of choline deficiency was extended, impairment in the assimilation of folate was probably compensated for by minimizing folate losses by decreasing folate turnover rates. Hence, we observed an elongation of the glutamate ends of folate molecules.

Keywords: folate; folate polyglutamates; choline; dietary choline deficiency; affinity chromatography; HPLC

Introduction

The cellular metabolism of folate involves a variety of reactions that employ folate species differing in pteridine ring structure and/or in the number of glutamic acid residues. The capacity to discriminate between these folate species can be used to enhance our understanding of folate-dependent metabolism in the cell. We observed diminished hepatic folate concentrations and a different distribution of hepatic folate species in rats treated for 2 weeks with low doses of methotrexate as compared to controls.[1] We also observed that the effects of methotrexate on hepatic folate metabolism was exacerbated when rats were fed a choline-deficient diet.[1] Two weeks of choline deficiency alone (no methotrexate) resulted in a 31% decrease in hepatic folate concentration but did not alter the relative distribution of folate species within the liver.[1] Horne et al.[2] have reported that a diet deficient in choline and methionine, fed for 5 weeks, perturbed folate metabolism to an extent that was similar to that present in our methotrexate-choline–deficient rats. Clearly there are in-

This work was supported by Grant HD-16727 from the NIH and by the United States Department of Agriculture, Agricultural Research Service under Contract 53-3k06-5-10.
The content of this publication does not necessarily reflect the views or policies of the United States Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the United States Government.
G. Varela-Moreiras is a postdoctoral fellow who is supported by the Ministry of Education and Research of Spain.
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Received August 7, 1991; accepted November 19, 1991.

teractions between folate, methionine, and choline metabolism–these metabolic pathways intersect at the methylation of homocysteine to form methionine.[3] We undertook the present study to examine whether long-term (12 months) choline deficiency exacerbated the changes in folate metabolism that were observed in short-term deficiencies.

Methods and materials

Male Fischer 344 rats (90 g body weight, Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in suspended stainless-steel wire cages in a climate-controlled room (24° C) and were exposed to light from 06:00 to 18:00 hours daily. Animals were fed a semipurified choline-sufficient or choline-deficient diet (Lombardi Choline Diet #118753; Dyets, Bethlehem, PA, USA) that contained 0.2% methionine, 0.4% cystine/cysteine (the recommended sulfur amino acid content of the rat diet is 0.6%), 2 mg/kg folate, and 10 μg/kg B_{12}. The sufficient diet contained 0.46% choline, while the deficient diet had 0.006% choline.

Rats were divided into two groups (n = 6/group) and fed the choline-sufficient or choline-deficient diet and water ad libitum for 52 weeks. At the end of the experiment the animals were sacrificed and livers were quickly removed. Weighted aliquots were minced and placed in 10 volumes of boiling extraction solution: 10 mmol/L 2-mercaptoethanol and 0.1 mol/L sodium ascorbate in 0.1 mol/L [bis (2-hydroxyethyl) imino] tris (hydroxymethyl)-methane buffer at pH 7.85. After boiling for 15 min, the extracts were cooled in an ice bath, homogenized, and the clear supernatant fractions were collected after centrifugation at 20,000g for 15 min and kept at −70° C until analyzed.

Affinity chromatography using immobilized milk folate binding protein, followed by ion pair high pressure liquid chromatography was used to determine folate distribution.[4-6] Briefly, an aliquot of the supernatant fraction was mixed with a trace amount of [^{3}H]folic acid (1.83×10^5 Bq, 0.66 TBq/mmol; Amersham, Arlington Heights, IL, USA) and applied to a folate binding protein-Sepharose affinity 1 mL bed volume column. The column was then washed and eluted with 0.02 mol/L of trifluoroacetic acid containing 10 mmol/L dithioerythritol. The acid fraction was promptly neutralized and a 1 mL aliquot was used for the analysis of folate distribution using a combination of ion pair high performance liquid chromatography and diode array detection system as described.[4-6] Folates elute in the order of increasing number of glutamate residues. Therefore, those with the same number of glutamate residues elute as a separate cluster arranged in three groups: group 1 consists of 10-formyltetrahydrofolates, tetrahydrofolates, and dihydrofolates; group 2 consists of 5-formyltetrahydrofolates; and group 3 consists of 5-methyltetrahydrofolates. An identification of the pteridine ring structure was made on the basis of the UV absorption values at 280, 350 and 258 nm. The individual chromatograms were analyzed to determine total folates and distribution of the various derivatives. Individual peak areas were used for quantitative estimation of folate activities and the sum of these activities was used for estimation of total folate concentrations. Folate distribution was expressed on the basis of glutamic acid chain lengths and pteridine ring structure. Choline and phosphocholine were determined using separation by HPLC and quantitation using gas chromatography/mass spectrometry.[7] Data were analyzed for statistical significance by using Student's t test. The Bonferroni adjustment inequality[8] was used for comparison of glutamate chain length and ring distribution within the same tissue in choline sufficient and choline deficient rats.

Results

During the 52 weeks of this study, rats on a choline-deficient diet gained 351 ± 13 (standard error of mean) g compared with those on the choline-sufficient diet (control), which gained 380 ± 9 g (no significant difference). Hepatic choline concentration was 124 ± 6 nmoles/g liver in control rats and was 94 ± 7 nmoles/g liver in deficients ($P < 0.05$). Hepatic phosphocholine concentrations, which previously have been shown to be a much more sensitive indicator of choline status,[3] was 2,293 ± 176 nmoles/g liver in controls and was 182 ± 28 nmoles/g liver in deficients ($P < 0.01$).

Figure 1 shows representative chromatograms of folates from liver extracts from the two groups of rats. Hepatic folates from control animals *(Figure 1A)* exhibited a fractionation pattern consisting of two major clusters representing penta- and hexaglutamyl derivatives in addition to some minor clusters representing mono-, tetra-, and heptaglutamyl folates. The distribution pattern of folates from livers of animals on the choline-deficient diet *(Figure 1B)* was similar except for a decrease in the magnitude of the pentaglutamyl folate cluster, an increase in the heptaglutamate cluster, and the appearance of octaglutamyl folates. These differences are depicted in *Figure 2*, which shows the means of the relative distribution of the various clusters as determined for each group of animals. As indicated, livers from animals on the choline-deficient diet had significantly (Bonferroni P value = 0.05) lower pentaglutamyl and higher hepta- and octaglutamyl folates than did the livers from animals on the choline-sufficient diet.

Hepatic folates from rats receiving the choline-

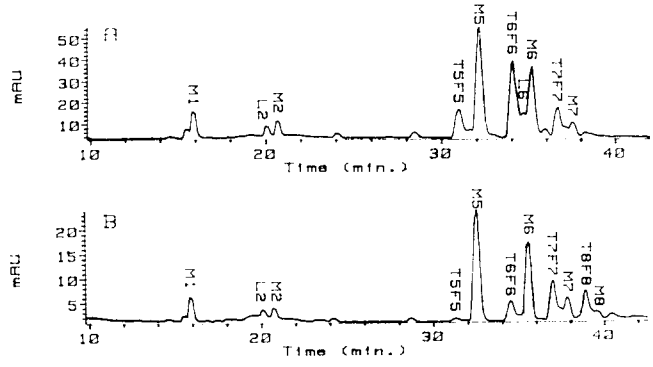

Figure 1 Chromatographic separation of rat liver folates. Each chromatogram was obtained from the purification of folates from 1.0 g liver (chromatogram A, control) and 0.5 g liver (chromatogram B, choline deficient) and are representative of six liver samples from equally treated animals: A, from choline sufficient rats; B, from choline deficient rats. Identification of the folate derivatives in the various peaks is denoted as: F, 10-formyltetrahydrofolates; L, 5-formyltetrahydrofolates; T, unsubstituted tetrahydrofolates, and M, 5-methyltetrahydrofolates. Numbers following the letters represent the total number of glutamate residues.

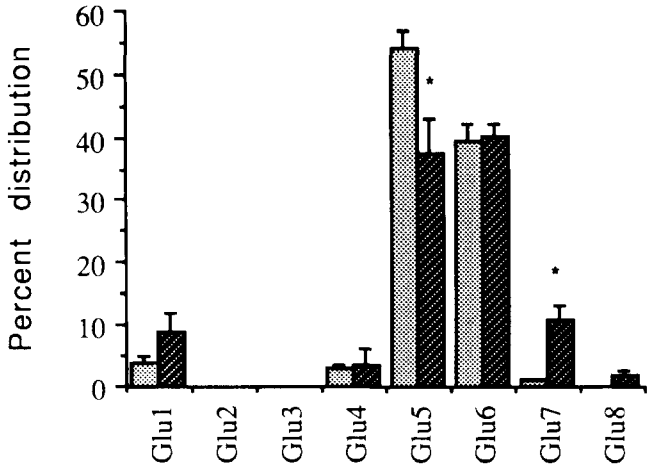

Figure 2 Effect of chronic choline deficiency on hepatic folate distribution in rats. The "choline-sufficient" group is represented by the light bars and the "choline-deficient" is represented by the dark ones. Data are presented as mean ± SE for six animals per group, percentage of total folate. * denotes values that are significantly different (Bonferroni P value < 0.05) than the respective values from control animals. Abbreviations used: Glu_n, number of glutamate residues.

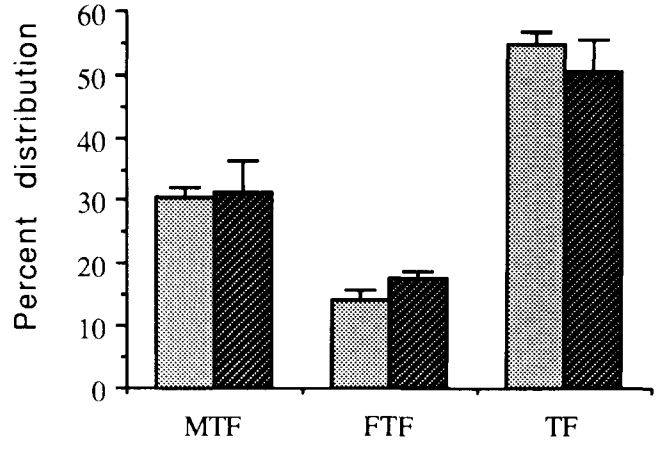

Figure 3 Effect of chronic choline deficiency on the pteridine ring distribution. Data represent means ± SE for six animals per group, given as percentage of total folates, irrespective of the glutamic acid chain length. The "choline-sufficient" group is represented by the light bars, whereas the darker bars represent the "choline-deficient" group. Abbreviations used: MTF, 5-methyltetrahydrofolates; FTF, formyltetrahydrofolates; and TF, unsubstituted tetrahydrofolates.

deficient diet exhibited similar pteridine ring structure distribution as those from livers from rats receiving the choline-supplemented diet *(Figure 3)*. Total hepatic folate concentration was not significantly different in rats on the choline-deficient diet as compared with the rats on the choline-sufficient diet (14.2 ± 1.9 nmol/g liver and 17.3 ± 1.5 nmol/g liver; $P > 0.2$).

Discussion

Short-term (2 weeks) feeding of rats with a choline-deficient diet caused the hepatic folate level to be 31% lower, with conservation of the pteridine ring distribution and minimal elongation of the glutamate chains, as compared with hepatic folates from control animals.[1] In the present study, a long-term (12 months) feeding of rats with the same choline-deficient diet caused minimal changes in hepatic total folate content and in the pteridine ring structure distribution of folates. However, the glutamate chains of hepatic folates were significantly elongated after 12 months of choline deficiency. The extent of this elongation (estimated on the basis of the total number of glutamate residues in γ-peptide linkage versus total number of polyglutamyl folate[9]), amounted to an extra 1.3 glutamate residues per folate polyglutamate.

As was pointed out by Foo and Shane,[10] and recently discussed by us,[1] elongation of the glutamic acid chain of folates beyond the five to six residues that normally are found in liver is, at least in part, the result of a higher residence time of these folates within the liver tissue. The synthesis of folate polyglutamate occurs by a stepwise mechanism that is governed by the affinity of the folate substrate for polyglutamate synthase. Affinity is highest when the substrate (tetrahydrofolate) contains five or fewer residues and much lower when the number of residues is greater. Accordingly, the addition of glutamate residues to the folate substrate proceeds rapidly until the total attached residues equals five to six. Thereafter, the reaction proceeds very slowly. The presence of hepta- and octaglutamyl folates therefore may reflect slower folate turnover. We did not measure folate turnover rates to confirm this. Under conditions of normal supply of folate to the liver, an increase in residence time should result in a higher concentration of folate in the tissue. We suggest that an increase in residence time (decrease in folate turnover) was a compensatory mechanism that resulted in the maintenance of normal hepatic folate stores in spite of 12 months on a choline-deficient diet.

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