Folate Deficiency in the Livers of Diethylnitrosamine-treated Rats

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SUMMARY

The effects of diethylnitrosamine on the metabolism of folate acid and related compounds in rat liver were investigated. The administration, in the drinking water, of diethylnitrosamine to rats for 3 weeks led to decreased hepatic levels of folate, S-adenosylmethionine, and 5-methyltetrahydrofolate:homocysteine methyltransferase. Liver methylentetrahydrofolate reductase levels were unaffected by administration of diethylnitrosamine. The polyglutamate fraction of hepatic folates obtained from rats treated with diethylnitrosamine for 3 weeks prior to injection with [3H]folate contained less radioactivity than did the polyglutamate fraction obtained from the livers of control rats treated with [3H]folate alone. Similarly, the polyglutamate folate fraction of rat livers that were simultaneously perfused with both diethylnitrosamine and [3H]folate contained less label than the polyglutamate fraction of rat livers perfused with [3H]folate only. Livers perfused with [2-14C]histidine and diethylnitrosamine produced more formiminoglutamate and less CO2 than livers treated with [2-14C]histidine only. The changes noted in the hepatic folate metabolism of diethylnitrosamine-treated rats resemble those seen in the livers of methyl-deficient rats.

INTRODUCTION

Previous experiments have demonstrated that the chronic administration of DENA3 to rats produces a folate deficiency that can be reversed by the administration of high dietary levels of the methyl donors, methionine and choline (29). The mechanism of this effect remains unknown. The interaction between DENA, folic acid, and methionine is important because of the role played by the lipotropes in modifying the activity of several chemical carcinogens. For example, dietary methyl donors appear to diminish the carcinogenic activity of such agents as 2-acetylaminofluorene (22), N,N-dimethyl-4-aminobenzene (7, 19), DENA (27, 28), methylcholanthrene (24, 25), and 7,12-dimethylbenz(a)anthracene (11). Furthermore, the lipotropes methionine, vitamin B12, and folic acid are all prospective targets of the electrophilic activated form of 1 or more chemical carcinogens (12, 16, 20). The metabolic interrelations among the various lipotropes are very complex, and it is impossible to alter the tissue levels or metabolism of one without simultaneously altering the metabolism of each of the others (3-5, 13, 42). We therefore decided to investigate in detail the mechanism responsible for the folate acid deficiency observed in DENA-treated rats.

MATERIALS AND METHODS

Animals, Diets, and Compounds. Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, Calif.) weighing 150 to 200 g were used in all experiments. For the chronic experiment, rats were housed singly in a wire-screen-topped cage in a constant-temperature, light-controlled room and were given a commercial stock diet (Purina rat chow; Purina Co., St. Louis, Mo.) ad libitum; experimental rats received 0.01% DENA in the drinking water for 3 weeks. In 1 experiment, 1.0% dl-methionine was added to the ground rat chow diet. Folate labeled with tritium in the 3′, 5′, and 9 positions (specific activity, ca. 40 Ci/m mole) (Amersham/Searle Corp., Arlington Heights, Ill.) and 5-14C)methyl-H4PteGlu (specific activity, 50 mCi/m mole) (Amersham/Searle) were purified over QA-E-Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.) before use (6). The [methyl-14C]AdoMet (specific activity, 55 mCi/m mole) (Amersham/Searle) and [2-14C]histidine (specific activity, 55 mCi/m mole) (Amersham/Searle) were used without further purification. In some studies, folate metabolism in perfused livers was studied. The perfusion method used was that described by Buehring et al. (5). Twenty µg of DENA were added first in the perfusion medium containing washed human red blood cells and then 10 µCi (ca. 0.25 nmole) of [3H]folate or 5 µCi (500 µmoles) of [2-14C]histidine were added next. After perfusion for 2 or 2.5 hr, livers were washed with ice-cold water and extracted for folate, AdoMet, histidine, FIGLU, or methionine.

Folate Analyses. The extraction of folates from the livers of intact rats was done according to the method described by Shin et al. (36). In the perfusion studies, 40 ml of perfusate were poured into 200 ml boiling 1.0% ascorbate solution (pH 6.0); the mixture was heated in a water bath at 95°...
and cooled in ice, and the precipitate was removed by centrifugation. All extracts were stored at \(-19^\circ\)C until assayed. Total folates were determined by microbiological assay according to the method described by Waters and Mollin (43) and later modified by Tamura et al. (39). Three organisms, Lactobacillus casei (ATCC 7469), Streptococcus faecalis (ATCC 8043), and Pediococcus cerevisiae (ATCC 8081), were used. The assay procedures for S. faecalis and P. cerevisiae were the same as that for L. casei (39), except that, for S. faecalis, 10 ng of folate in 5 ml of inoculum broth and 0.1 M sodium-phosphate buffer, pH 6.7, with 0.1% ascorbate, instead of 0.05 M sodium-phosphate buffer, pH 6.1, were used. For P. cerevisiae, 5 ng of Leucovorin (N5-formyltetrahydrofolic acid) were used in the inoculum assay according to the method described by Waters and Mollin (43). DEAE-cellulose column chromatography with an exponential gradient of 0.01 to 0.5 M phosphate buffer, pH 6.0 (5), was used for the separation and identification of different forms of folic acid. Sephadex G-25 column chromatography with 0.1 M phosphate buffer (pH 7.0) (34) was used for the separation and identification of pteroylpolyglutamates. All the buffers contained 0.2 M 2-mercaptoethanol.

### Amino Acid, AdoMet, and Enzyme Determinations

For studies on the metabolism of histidine, after completion of the perfusion, the liver was homogenized with ice-cold distilled water (1:3, w/v), and protein was precipitated by the addition of 10 ml 20% sulfosalicylic acid and removed by centrifugation. To 50 ml perfusion medium, 10 ml of 20% sulfosalicylic acid were added, and precipitated protein was removed by centrifugation. Aliquots of the supernatant were used for column chromatography and radioactivity determination. Dowex 50W-X8, 200 to 400 mesh, was used for the chromatography of histidine and its metabolites (2). Five ml of the liver or perfusate extract were applied to the column (0.9 x 35 cm), and elution was carried out with 100 ml citrate buffer (pH 3.25), 100 ml citrate buffer (pH 4.25), and 300 ml citrate buffer (pH 5.28) at room temperature. The final elution was made with 100 ml NaOH to remove any radioactivity remaining on the column. Further purification of FIGLU was carried out by rechromatography on the same resin in the H+ form (5). For methionine determinations, the supernatant was adjusted to 6.0 with a saturated solution of KHCO3 at 20° in the presence of a small amount of octyl alcohol to prevent excessive frothing. The suspension was then centrifuged at 10,000 \(\times\) g for 15 min to remove precipitated KClO4. The clear yellowish solution was then chromatographed on Dowex 50W-X8 (Na+ form). Step-wise elution was done with 0.1 M NaCl and 6 \( \times\) H2SO4 or 6 M HCl. The elution was carried out in both cases until the absorbance was less than 0.03 at 256 nm. Specific radioactivities of the AdoMet peak were calculated from their UV absorption at 256 nm and from radioactivity measurements. Material in the 6 M HCl eluate and standard AdoMet migrated identically on thin-layer and paper chromatography (15).

Methyltransferase and methylene reductase activities were measured in the livers of control and DENA-treated rats. Livers were homogenized with 3 volumes of cold 0.05 M phosphate buffer, pH 7.2, and were centrifuged at 30,000 \( \times\) g for 30 min. The supernatant was frozen at \(-19^\circ\) until assayed. Just before analysis, part of each sample was dialyzed 2 hr against 2 liters of 0.05 M phosphate buffer, pH 7.2, at 4°. The methyltransferase was assayed according to the procedure of Kutzbach et al. (17). After 2 hr of incubation, the end product, \([^{14}C]\)methionine, was separated from the remaining substrate \([^{14}C]\)methyl-H4PteGlu on a 0.9 x 3 cm column of Dowex 1-Cl (100 to 200 mesh), and radioactivity in the eluate was counted (17). Methyleneetahydrofolic reductase was assayed in the reverse direction with menadione as the artificial electron acceptor (8). \([^{14}C]\)Formaldehyde formed from \([^{14}C]\)methyl-H4PteGlu was determined as the dimedone adduct that was extracted with toluene (8).

### Measurement of Radioactivity

Radioactivity in liver extracts, perfusate extracts, and column fractions was measured using scintillation liquid, 48.48 g PPO and 3.25 g POPOP dissolved in 11.4 liters toluene for 3 to 4 hr and mixed with 6.6 liters Triton X-100 overnight.

### RESULTS

#### In Vivo

The folate contents of the livers of rats receiving DENA for 3 weeks were determined microbiologically and compared with the corresponding values in the livers of control rats (Table 1). Ten to 33% decreases in the responses of the 3 organisms to hepatic folate were observed when the rats were given 0.01% DENA in the drinking water, but these differences obtained by microbiological assays were not statistically significant. The effect of DENA on the distribution of folate derivatives was also studied radiochemically. Rats were treated with 25 \( \mu\)Ci of \([^{3}H]\)folate i.p. and were sacrificed 24 hr later. Liver extracts containing folate derivatives were chromatographed on Sephadex G-25 columns to separate the mono- and polyglutamate folic acid derivatives. The data, given in Table 1, show that \([^{3}H]\)folate uptake by the liver was lower in the animals receiving DENA in the drinking water, but the differences obtained by microbiological assays were not statistically significant. The effect of DENA on the distribution of folate derivatives was also studied radiochemically. The rats were treated with 25 \( \mu\)Ci of \([^{3}H]\)folate i.p. and were sacrificed 24 hr later. Liver extracts containing folate derivatives were chromatographed on Sephadex G-25 columns to separate the mono- and polyglutamate folic acid derivatives. The data, given in Table 1, showed that the liver uptake of \([^{3}H]\)folate was lower in the animals receiving DENA in the drinking water, but the difference was of marginal statistical significance (\(p < 0.03\)). The levels of methyltransferase and of methylene reductase were determined in the livers of control and DENA-treated rats. Carcinogen administration decreased the hepatic content of methyltransferase but exerted no significant effect on hepatic levels of methylene reductase (Table 1). Furthermore, as shown in Table 1, the chronic administration of DENA to rats led to a 27% decrease in the hepatic level of AdoMet. The addition of DENA to the drinking water reduced the hepatic level of AdoMet by 27%.
The effects of chronic DENA administration to rats on the hepatic levels of folate cofactors and enzymes, and of AdoMet

DENA (0.01% in the drinking water) was administered to male Sprague-Dawley rats (150 to 200 g) for 3 weeks prior to sacrifice. Group 1-carbon metabolite Control DENA p" Folate activitya L casei 18.4 ± 1.1c (3) 15.0 ± 1.8 (3) >0.2 S. faecalis 9.6 ± 0.4 (3) 8.6 ± 0.6 (3) >0.2 P. cerevisiae 10.0 ± 2.1 (3) 6.7 ± 0.9 (3) >0.2 Folate uptakeb by liver Monoglutamates 0.34 ± 0.05 (3) 0.35 ± 0.05 (4) >0.2 Polyglutamates 4.09 ± 0.52 (3) 2.85 ± 0.25 (4) <0.07 Totalb 5.59 ± 0.61 (3) 3.75 ± 0.33 (4) <0.03 Folate enzymesa Methyltransferase 0.14 ± 0.007 (9) 0.11 ± 0.003 (11) <0.005 Methylene reductase 0.31 ± 0.02 (9) 0.35 ± 0.02 (11) >0.2 AdoMetc 66.6 ± 4.8 (12) 48.6 ± 2.0 (16) <0.005 a Statistical significance, as determined by Student's t test. b µg folate per g liver. c Mean ± S.E. d Numbers in parentheses, number of livers assayed. e Twenty-four hr prior to sacrifice, each rat received an i.p. injection of 25 µCi of [3H]folate. Values are expressed as percentage of dose injected. f The difference between the sum of mono- and polyglutamates and total radioactivity in liver represents unidentified breakdown products. g µmol of folic acid per mg protein per hr. h µmol/g liver.

Effect of DENA on the metabolism of [2-14C]histidine in the perfused rat livers

Experiment details given in "Materials and Methods." DENA in perfusate Total CO2/2 hr FIGLU in liver Diet µmoles µmoles µmoles Chow + 31 145 Chow + 30 129 Chow - 45 96 Chow - 41 105 30.5 ± 0.7n 137 ± 11.3 43 ± 2.8 100.5 ± 6.4 a Mean ± S.D.

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DISCUSSION

The present results confirm and partially explain the previous results (29) on the methionine-reversible folate deficiency produced in rats by the chronic administration of DENA. DENA administration in the drinking water produced a significant drop in the polyglutamate derivatives of liver folate (29), the major form of hepatic folate (12, 14, 29, 36). In perfusion studies, the addition of DENA to the perfusate decreased the formation of polyglutamate derivatives of folic acid from [3H]folate. The addition of DENA during perfusion also increased FIGLU levels in the liver and decreased the formation of carbon dioxide from [2-14C]histidine.

Direct evidence indicates that the altered folate distribution in the livers of DENA-treated animals is a consequence of methionine deficiency which, in turn, reduces the level of AdoMet. The livers of rats treated with DENA contain signifi-
The influence of DENA on folate uptake, folate distribution, and the levels of methionine and AdoMet in rat liver perfused with 10 μCi [3H]folate. The DENA lowering of hepatic AdoMet can be reversed by methionine. Physiological methyl donors also appear to decrease the carcinogenic activity of DENA (31) and other carcinogens (10, 21, 28, 30, 31).

The effects of DENA may be explained on the basis of the methyl trap theory (13). This postulates that an increase in the proportion of 5-methyl-H₄PteGlu results in a decrease in the physiologically active H₄PteGlu forms which are involved in most folate-dependent reactions (23, 40, 42). This would result in a decreased catabolism of FIGLU (3). This increased proportion of 5-methyl-H₄PteGlu occurs in vitamin B₁₂ deficiency where there is a decreased activity of methyltransferase. Methionine can be converted to AdoMet which in turn inhibits methylenetetrahydrofolate reductase (18), thereby reducing the formation of 5-methyl-H₄PteGlu and increasing the proportion of H₄PteGlu (5). This is consistent with the observed effect of methionine in decreasing FIGLU excretion in the whole animal (4), facilitating FIGLU catabolism in the perfused liver (5), and reducing the elevated FIGLU excretion occurring in animals treated with DENA (29). Decreased levels of polyglutamate may be the result of decreased levels of AdoMet with a corresponding increased activity of methylenetetrahydrofolate reductase. This would decrease the level of H₄PteGlu. This, rather than 5-methyl-H₄PteGlu, is the preferred substrate for polyglutamate synthetase (32, 38). It is also consistent with the effect of methionine in increasing the level of polyglutamate formation in the perfused liver (5). Thus the effects of DENA in decreasing polyglutamate levels may be explained by its role in reducing levels of AdoMet. Polyglutamates have a lower rate of membrane transport than folic acid monoglutamates, and the formation of polyglutamates represents a mechanism whereby the organism can concentrate higher levels of folic acid within the cell. The effect of DENA on the hepatic distribution of vitamin B₁₂ has not yet been reported.

It is tempting to speculate that the effects of DENA on folate metabolism are a specific biochemical consequence of its carcinogenic activity. As yet we have not accumulated enough evidence to demonstrate this point. Possible causes of the DENA-induced folate deficiency unrelated to the carcinogenic process include partial inanition, altered hepatic cell population, and nonspecific toxicity.

In previous studies, chronic DENA administration to rats...
restricted both their growth rates and food consumption (26, 29). However, no folate deficiency, measured by elevated FIGLU excretion following a loading dose of histidine, could be observed in rats fasted for 3 days (L. Poirier, unpublished observations) or pair-fed with FIGLU-excreting, tumor-bearing rats (26). The histological changes produced by the chronic administration of DENA have been well described (41).

A folic acid deficiency has been observed in rats receiving 0.01% DENA in the drinking water for as little as 1 week (29); the alterations seen in the liver cell populations at this time are minimal (41). A possible role for nonspecific toxicity in the production of a folate deficiency by DENA cannot be excluded without examining the mechanism producing the methyl deficiency or by screening hepatocarcinogens, related noncarcinogens, and other hepatotoxic agents for their ability to induce a methyl-dependent folate deficiency. Further experimentation will be required to demonstrate which of the possible factors is responsible for the altered folate distribution.

REFERENCES

23. Poirier, L. A. Liver Cell Populations at this Time are minimal (41). Further experimentation will be required to demonstrate which of the possible factors is responsible for the altered folate distribution.

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