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# Decreased Hepatic 5,10-Methylenetetrahydrofolate Reductase Activity in Mice after Chronic Phenytoin Treatment

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SUMMARY

The effects of phenytoin (DPH) on folate metabolism have been studied in female Swiss Webster mice. Doses of DPH which produce steady-state plasma levels of DPH in the therapeutic range of 10–20 μg/ml were found to decrease plasma folate levels. In addition, the *in vivo* oxidation rate of [$^{14}$C]formate and [2-$^{14}$C]histidine to $^{14}CO_2$ was increased. The increased metabolic rates were accompanied by a decrease in the hepatic activity of $N^5$,$N^{10}$-methylenetetrahydrofolate (5,10-$CH_2$-$H_4$folate) reductase. $N^5$-methyltetrahydrofolate-homocysteine transmethylase (methionine synthase); EC 2.1.1.13) activity in the liver was unchanged. The distribution of folates in the liver was determined by high-pressure liquid chromatography (HPLC) and it was found that the concentration of tetrahydrofolate ($H_4$folate) was increased in DPH-treated mice whereas the concentration of $N^5$-methyltetrahydrofolate was decreased. These effects were observed in mice treated with DPH for 4 days, but not in mice given a single DPH injection 24 hr previously. Decreased activity of hepatic 5,10-$CH_2$-$H_4$folate reductase is postulated to account for the other effects which were observed. Decreased activity presumably results in increased tissue concentrations of 5,10-$CH_2$-$H_4$folate, which is in equilibrium with its dissociation products, $H_4$folate and formaldehyde. Increased concentrations of $H_4$folate lead to increases in the oxidation rate of formate and histidine. These effects on folate metabolism may have important implications in the pharmacological and toxicological effects of DPH.

## INTRODUCTION

The majority of epileptic patients treated with anticonvulsant drugs develop a drug-related decrease in serum and red cell folate levels (1). The drug most commonly associated with folate deficiency is DPH,[1] a drug of choice for the treatment of grand mal epilepsy. DPH also reduces serum folate levels in normal subjects (2). The consequences of the altered folate status are not entirely clear. It has been suggested that decreased folate levels in the brain may contribute to the anticonvulsant action of the drugs. This hypothesis is based upon the observation that administration of folic acid to treated epileptics worsens seizure control (3) and upon the finding that 5-$CH_3$-$H_4$folate, the circulating folate form, increases brain electrical activity (3, 4). It has also been hypothesized that the high incidence of drug-induced congenital malformations in offspring of treated epileptics is a result of folate deficiency (5).

Several theories have been presented to account for DPH-induced folate deficiency. These include impaired folate absorption (6), induction of hepatic microsomal drug-metabolizing enzymes (7, 8), and increased folate catabolism (9). However, none of these mechanisms explains all of the effects on folate metabolism which have been observed following chronic DPH treatment. For example, DPH has been reported to increase the oxidation of formate and histidine in folate-deficient rats (10). In humans, DPH reduces the urinary excretion of formiminoglutamate (6, 11), an intermediate in the oxidation of histidine. The oxidation of both formate and formiminoglutamate requires $H_4$folate for the ultimate formation of 10-HCO-$H_4$folate, which is then oxidized to $CO_2$ (Fig. 1), and the urinary excretion of formiminoglutamate indicates the rate of hepatic histidine catabolism by this route (13). It has recently been reported that there is a highly significant correlation between the *in vivo* rate of formate oxidation to carbon dioxide and the hepatic concentration of $H_4$folate (14); that is, higher concentrations of $H_4$folate in the liver are associated with a faster rate of formate oxidation in rats.

Several reactions in the folate pathway generate

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[1] The abbreviations used are: DPH, phenytoin; $H_4$folate, tetrahydrofolate; 10-HCO-$H_4$folate, $N^{10}$-formyltetrahydrofolate; 5-HCO-$H_4$folate, $N^5$-formyltetrahydrofolate; 5-$CH_3$-$H_4$folate, $N^5$-methyltetrahydrofolate; 5,10-CH-$H_4$folate, $N^5$,$N^{10}$-methenyltetrahydrofolate; 5,10-$CH_2$$H_4$folate, $N^5$,$N^{10}$-methylenetetrahydrofolate; HPLC, high-pressure liquid chromatography.

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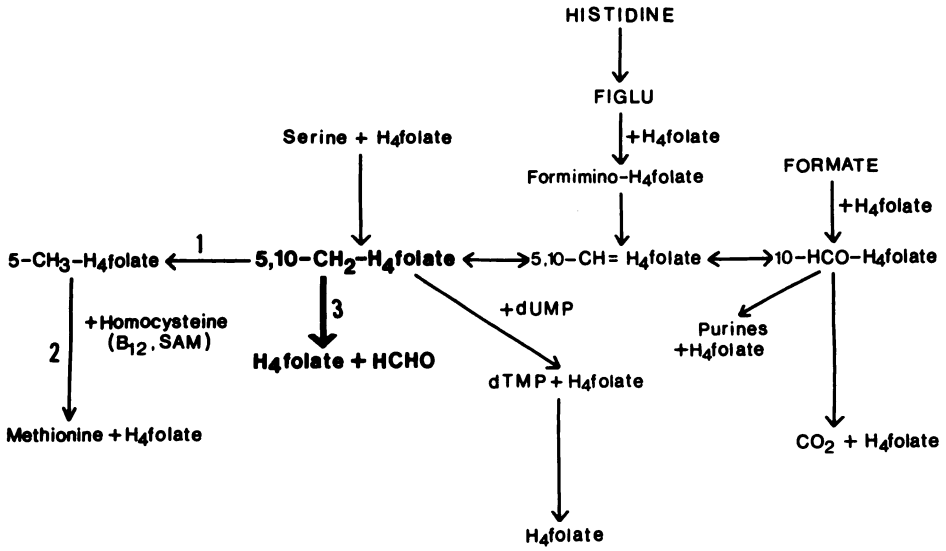


FIG. 1. *Interconversion of folate forms*

Reaction *1* is catalyzed by 5,10-$CH_2$-$H_4$folate reductase and Reaction *2* is catalyzed by methionine synthase. Reaction *3* shows the non-enzymatic dissociation of 5,10-$CH_2$-$H_4$folate to $H_4$folate and HCHO. For simplicity, only monoglutamate forms are shown. Tissue folates are predominately polyglutamates (12). $B_{12}$, vitamin $B_{12}$; *SAM*, *S*-adenosylmethionine; *FIGLU*, formiminoglutamate.

$H_4$folate (Fig. 1), but methionine synthase (EC 2.1.1.13) has been implicated as the major enzyme responsible for the regeneration of $H_4$folate (15, 16), since it is the sole known enzyme which utilizes the plasma folate form, 5-$CH_3$-$H_4$folate. Impaired methionine synthase activity in the liver reduces the hepatic concentration of $H_4$folate (17–19). This is thought to be due to the irreversibility of the 5,10-$CH_2$-$H_4$folate reductase reaction in the direction of 5-$CH_3$-$H_4$folate synthesis (20, 21). Changes in the activity of hepatic 5,10-$CH_2$-$H_4$folate reductase are also proposed to alter the liver concentration of $H_4$folate (17, 21–24).

The objective of the present study was to determine the effect of chronic DPH treatment on the hepatic activity of these enzymes, since changes in activity are known to alter tissue concentrations of $H_4$folate. Either increased methionine synthase activity or decreased 5,10-$CH_2$-$H_4$folate reductase activity would explain the increased rate of formate and histidine oxidation previously observed (10, 11) and the decreased plasma folate levels commonly seen in phenytoin-treated epileptics (1). An understanding of the interaction between DPH and folate metabolism is essential both to the treatment of the folate deficiency and to delineating the consequences of the interaction to cellular function. Initial experiments were also conducted to establish the mouse as an appropriate animal model for study of DPH/folate interactions.

## METHODS

*Materials.* [$^{14}$C]Formate was purchased from New England Nuclear Corporation (Boston, Mass.). L-[*ring*-2-$^{14}$C]Histidine and [5-$^{14}$C]$CH_3$-$H_4$folate were obtained from Amersham Corporation (Arlington Heights, Ill.). Preblend 3a70B liquid scintillation cocktail was purchased from Research Products International (Elk Grove, Ill.). DPH and 5-(4-methylphenyl)-5-phenylhydantoin were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). The Ultrasphere-ODS column (4.6 mm × 25 cm), 5-$\mu$m particle size, was purchased from Rainin Instrument Company, Inc. (Woburn, Mass.). The Micropak MCH-10 column was obtained from Varian Associates (Palo Alto, Calif.). Tetrabutylammonium phosphate was supplied by Regis Chemical Company (Morton Grove, Ill.). 5-$CH_3$-$H_4$folate, 5-HCO-$H_4$folate, and $H_4$folate were obtained from Sigma Chemical Company (St. Louis, Mo.). 5-$CH_3$-$H_4$folate and $H_4$folate were purified by DEAE-cellulose chromatography (25) prior to use as standards in the HPLC assay of liver folates. 10-HCO-$H_4$Folate was synthesized by the method of Rabinowitz (26). *Lactobacillus casei* (7469) was obtained from the American Type Culture Collection, and the growth medium (folic acid *L. casei* medium 0822 and *Lactobacillus* broth AOAC, 0901) were purchased from Difco Laboratories, Inc. (Detroit, Mich.). Polyglutamate hydrolase was prepared from pig kidneys as described by McMartin *et al.* (25). All other chemicals were obtained from commercial suppliers and were of reagent quality.

*Treatment of animals.* Female Swiss Webster mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.), and they were used for all experiments. They were maintained in temperature- and humidity-controlled rooms on a 12-hr light/dark cycle in wire-bottomed cages containing five mice per cage. DPH was administered in the diet as follows. Purina laboratory chow was finely ground, and sufficient DPH was added to give the desired concentration. Control animals received ground diet. Food was available to the mice *ad libitum*, and food consumption was monitored throughout the experiment.

*Plasma levels of DPH.* Mice were killed by decapitation, and blood was collected into heparinized tubes. The blood was centrifuged at 2000 × *g* for 10 min to obtain the plasma fraction. An internal standard of 5-(4-methylphenyl)-5-phenylhydantoin was added to give a concentration of 10 $\mu$g/ml of plasma. The plasma sample, 50–200 $\mu$l, was then extracted twice with 1 ml of ethylacetate. The ethylacetate was concentrated to about 100 $\mu$l, and 10–25 $\mu$l were injected onto an Ultrasphere ODS ($^{18}$C reverse-phase) column. The instrument used consisted of a Waters Model 6000 pump and a Waters Model U6K injector. The solvent system was acetonitrile/water/acetic acid (2:3:0.025), and the flow rate was 1.5 ml/min. DPH was eluted from the column in 4.8 min. The retention time of the internal standard was 5.5 min. The compounds were detected by measuring absorbance at 210 nm with a Waters Model 480 variable wavelength detector. The plasma concentration of DPH was calculated from a standard curve constructed by

adding known amounts of DPH and the internal standard to plasma samples and carrying these samples through the entire procedure.

*Determination of formate and histidine oxidation.* The folate-dependent rate of formate and histidine oxidation was determined by the use of radiolabeled substrates and collection of radioactive carbon dioxide. [$^{14}$C]Formate, 15 to 20 × $10^4$ dpm/μmole, was injected i.p. at a dose of 5 mmole/kg. L-[*ring*-2-$^{14}$C]histidine, 60 to 75 × $10^4$ dpm/μmole, was given by i.p. injection at a dose of 2.5 mmole/kg. Mice were immediately placed individually into glass metabolism cages. Room air was drawn with a vacuum pump through the cages at a rate of 2 liters/min. The exit air was passed through a gas bubbler trap containing 150 ml of 2 N NaOH. A second trap in series, containing 30 ml of 2 N NaOH, was employed in order to determine that $CO_2$ absorption was complete in the first trap. Carbon dioxide was collected for 3 hr. The amount of $^{14}CO_2$ evolved was determined by counting a 0.5-ml aliquot of the 2 N NaOH in 4 ml of water and 10 ml of 3a70B liquid scintillator fluid.

*Folate assays.* Plasma folate levels were determined in mice which had been fasted overnight. Plasma samples were obtained as previously described for DPH assays. The plasma was immediately diluted with 10% ascorbate to give a final ascorbate concentration of 1%. Folate levels were determined by microbiological assay using *L. casei* (27) and a 5-HCO-$H_4$folate standard.

Hepatic folate forms were determined by HPLC. Liver samples were quickly removed from decapitated mice (within 30 sec) and immediately placed into liquid $N_2$. Within 2 hr the livers were transferred to tubes containing boiling 1.5 M 2-mercaptoethanol. The samples were heated at 100° for 5 min, then homogenized in 2 or 3 volumes of 1.5 M 2-mercaptoethanol, using a glass hand homogenizer. These extracts were prepared for HPLC analysis as described by McMartin *et al.* (25). All extracts were treated with pig kidney polyglutamate hydrolase to convert folates to monoglutamate derivatives prior to HPLC. HPLC was conducted with a Varian Micropak MCH-10 column (4 mm × 30 cm), a reverse-phase $^{18}$C column. Folates were eluted with a linear gradient of 1:5 methanol/water to 1:4 methanol/water. All solvents contained 5 mM tetrabutylammonium phosphate. The flow rate was 2 ml/min. A 100-μl aliquot of liver extract was injected onto the column, and 0.5-min fractions were collected into tubes containing 400 μl of 2-mercaptoethanol. These fractions were analyzed for folates by microbiological assay with *L. casei*. The levels of all folates were determined using 5-HCO-$H_4$folate as the standard in the *L. casei* assay. It was found that the growth of *L. casei* was equivalent with (*dl*)-L-5-HCO-$H_4$folate (calculated as the active isomer), (*dl*)-L-10-CHO-$H_4$folate, (*dl*)-L-5-$CH_3$-$H_4$folate, and (*l*)-L-$H_4$folate. However, the growth response with (*dl*)-L-$H_4$folate, calculated as (*l*)-L-$H_4$folate, was less than that with (*dl*)-L-5-HCO-$H_4$folate as reported by McMartin and Collins (28). Since tissue $H_4$folate is the (*l*)-L-$H_4$folate isomer, the levels of all folates were calculated using 5-HCO-$H_4$folate as the standard in the *L. casei* assay. *l*-L-$H_4$Folate was prepared by enzymatic reduction of $H_2$folate (29).

*Enzyme assays.* Livers were immediately removed from decapitated mice and rinsed in ice-cold 1.15% KCl. All subsequent procedures were conducted at 5°. The livers were homogenized in 3 volumes of 1.15% KCl in 10 mM $Na^+/K^+$ phosphate buffer (pH 7.4), using a glass hand homogenizer. This buffer was prepared from $NaH_2PO_4$ and $K_2HPO_4$. The homogenate was centrifuged for 15 min at 700 × *g*; the supernatant fraction was then centrifuged at 10,000 × *g* for 30 min. This supernatant fraction was then centrifuged at 100,000 × *g* for 60 min to obtain the microsomal and cytosolic fractions. Protein in these fractions was determined by the method of Lowry *et al.* (30). Microsomal cytochrome P-450 was measured by the method of Omura and Sato (31), using a Cary 219 spectrophotometer.

Methionine synthase activity and 5,10-$CH_2$-$H_4$folate reductase activity were determined in the cytosolic fraction. Methionine synthase activity was determined by the method of Weissbach *et al.* (32) as described previously (33). It was necessary to modify the reductase assay described by Kutzbach and Stokstad (22) from that previously used with rat liver cytosol (33). With the previous assay, enzymatic activity was not linear with protein concentration when mouse liver cytosol was used. It was necessary to dialyze the cytosol and to alter the concentration of several components of the reaction mixture. The following procedure was found to be optimal and to yield a reaction rate which was linear with both protein concentration and incubation time. First, the cytosolic fraction was dialyzed overnight against 0.05 M potassium phosphate buffer (pH 7.4). For assay, each incubation mixture (0.5 ml) contained 25 μM FAD, 5 mM menadione, 1.0 mM EDTA, 5 mM ascorbate, 0.5 mM [5-$^{14}$C]$CH_3$-$H_4$folate (approximately 50,000 dpm/μmole), 150 mM potassium phosphate buffer (pH 6.3), and dialyzed mouse liver 100,000 × *g* supernatant fraction containing 0.3–0.6 mg of protein. The incubation was conducted at 37° for 60 min.

*Urinary excretion of folates.* Animals which had been treated with DPH in the diet (400 mg/kg diet) for 10 weeks were placed into metabolism cages without food. The urine was collected for 24 hr into cups containing 200 μl of 1 M mercaptoethanol. The urine sample was then filtered to remove gross debris and assayed for folates using *L. casei*.

## RESULTS

*Dose-response and time response relationships.* Initial experiments were conducted to determine the dose and time required for DPH to exert its known effects on the liver. Mice were given DPH in the diet at a level of 100, 200, and 400 mg/kg of food for 4 weeks. Food consumption was monitored three times per week, and from these data it was calculated that the DPH dose was approximately 75 mg/kg at the 400 mg/kg diet level. There was no significant difference from control animals in food consumption at any DPH dose. Figure 2 shows that the 400 mg/kg of diet dose resulted in DPH plasma levels of 16 ± 1.5 μg/ml. Considerably lower plasma levels were seen with the two lower doses, which is consistent with the dose-dependent kinetics of DPH elimination in the mouse (34). Significant differences from control mice were observed in liver weight and hepatic microsomal cytochrome P-450 content only at the highest dose (Fig. 2). None of the DPH treatments significantly affected body weight. For example, mice given DPH at the highest dose of 400 mg/kg of diet weighed 26 ± 1.0 g ($n$ = 4) after 4 weeks and control animals weighed 28 ± 1.0 g ($n$ = 4). Liver weight was increased from 1.1 ± 0.039 g to 1.5 ± 0.10 g in these animals. Thus, the liver accounted for 3.9% of body weight in control animals and 5.8% in DPH-treated animals. The oxidation rate of formate to carbon dioxide was increased in mice given 400 mg/kg of diet for 4 weeks (Fig. 2). No significant difference in the rate of formate oxidation to $CO_2$ was observed at the lower doses.

The rate of formate oxidation to $CO_2$ was also measured 2 weeks and 12 weeks following DPH administration in the diet (400 mg/kg). After 2 weeks, a 25% increase in rate was seen (1.2 ± 0.032 mmoles of $CO_2$/30 min/kg in control mice versus 1.6 ± 0.067 mmoles of $CO_2$/30 min/kg in DPH-treated mice), and this increase was not significantly different from the increase observed after 4 weeks of DPH administration. No further increase in formate oxidation was seen in mice treated for 12 weeks. A group of mice treated for 12 weeks with DPH in the diet (400 mg/kg) was subsequently given daily injections of DPH (50 mg/kg i.p.) for 15 days along with the oral dose. There was no further increase in the rate

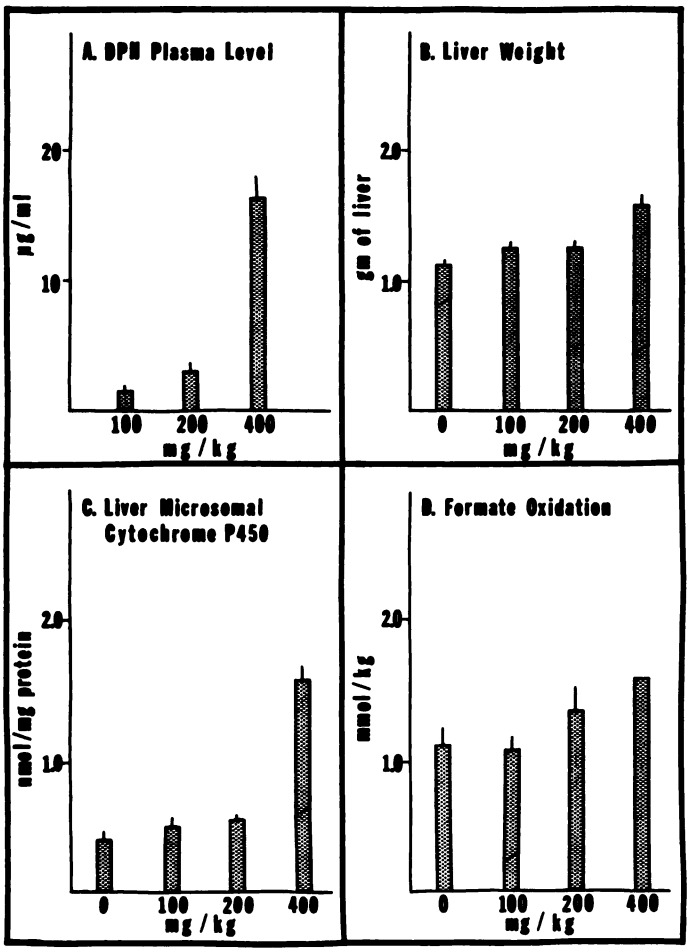


FIG. 2. *Dose-response relationship of DPH effects in mice*

Animals were treated for 4 weeks with DPH administered in the diet at a level of 100, 200, or 400 mg/kg of diet. The DPH dose was calculated from food consumption measurements to be approximately 19, 38, and 75 mg/kg, respectively. All results are expressed as means ± standard error of the mean ($n = 4$). Liver weight, liver microsomal cytochrome P-450 concentration, and the rate of [$^{14}$C]formate oxidation to $^{14}CO_2$ were significantly different from control values only at the 400 mg/kg of diet level ($p < 0.05$).

of formate oxidation in these mice given additional DPH from the increase seen with DPH administered only in the diet. Thus, in summary, the rate of formate oxidation is maximally increased to the extent of 25–30%, in mice given DPH orally in the diet at a dose of approximately 75 mg/kg. This increased rate was observed at the earliest time measured, i.e., 2 weeks. This dose of DPH was also required to cause an increase in liver weight and an increased level of liver microsomal cytochrome P-450.

Additional experiments were then conducted to define further the time required for DPH to increase the rate of formate oxidation. In one study, four mice were given DPH (50 mg/kg, i.p.) for 4 days, and it was found on the 5th day—24 hr after the last dose—that the rate of formate oxidation to $CO_2$ was increased from 1.1 ± 0.065 mmoles/30 min/kg in control animals to 1.4 ± 0.04 mmoles/30 min/kg. This increase in the rate of formate oxidation was not significantly different from the increase observed in mice given DPH in the diet at a dose of approximately 75 mg/kg, i.e., 400 mg/kg of diet for 2 weeks. The rate of formate oxidation was not significantly different from that of control mice in animals treated with DPH (75 mg/kg i.p.) 4 hr or 24 hr previously. The DPH plasma level in these mice was 52 ± 2 μg/ml ($n = 4$) at 4 hr and 24 ± 3 μg/ml ($n = 4$) at 24 hr. These results indicate that stimulation of the rate of formate oxidation is not an acute effect of DPH mediated by a direct interaction between DPH and formate-metabolizing enzymes.

*DPH effects on folate metabolism.* Table 1 summarizes the effects of DPH in mice treated for 3 weeks with a dose of approximately 75 mg/kg (400 mg/kg diet). In addition to the effects on the liver which were previously observed, it was found that plasma folate levels were significantly decreased by DPH treatment. There was no effect of DPH on the erythrocyte folate level. The oxidation rate to $^{14}CO_2$ of both [$^{14}$C]formate and [2-$^{14}$C] histidine was increased in DPH-treated mice (Fig. 3). Both formate and carbon 2 of histidine are oxidized to $CO_2$ by reactions dependent upon $H_4$folate (Fig. 1).

Figure 4 shows the distribution of hepatic folates in untreated mice. The chromatographic method used in these studies provides essentially baseline separation of the various folates. It is apparent that the predominant folate in mouse liver is $H_4$folate. The effect of DPH on this distribution is shown in Fig. 5. Livers of DPH-treated mice had an increased concentration of $H_4$folate and a decreased concentration of 5-$CH_3$-$H_4$folate. There was no change in the concentration of HCO-$H_4$folate forms. The total hepatic folate level was not significantly affected by DPH treatment.

Concomitant with the increased hepatic concentration of $H_4$folate and decreased level of 5-$CH_3$-$H_4$folate, decreased activity of 5,10-$CH_2$-$H_4$folate reductase was observed (Fig. 6). There was no significant effect of DPH treatment on hepatic methionine synthase activity. These effect are observed whether the activities are expressed as specific activity or as total activity in the liver in order to account for the increase in liver weight in DPH-treated mice. Enzymatic activity was measured with combinations of homogenates from control and DPH-treated mice in order to determine whether homogenates of treated mice contained an inhibitor of 5,10-$CH_2$-$H_4$folate reductase activity. This was found not to be the case. Activity with combined homogenates was

TABLE 1

*Effects of DPH in mice*

DPH was administered in the diet at a dose of 400 mg/kg of diet for 3 weeks. The DPH dose was calculated to be about 75 mg/kg. Results are expressed as means ± standard error of the mean ($n = 5$).

| | Control | DPH |
|---|---|---|
| Body wt. (g) | 23 ± 0.9 | 23 ± 1.3 |
| Liver wt. (g) | 1.0 ± 0.03 | 1.4 ± 0.13[a] |
| Liver microsomal protein (mg/g of liver) | 16 ± 1.2 | 21 ± 1.3[a] |
| Liver microsomal cytochrome P-450 (nmoles/mg of protein) | 1.1 ± 0.02 | 2.3 ± 0.11[a] |
| Plasma folate (ng/ml) | 52 ± 3 | 40 ± 2[a] |
| Erythrocyte folate (μg/ml) | 755 ± 97 | 728 ± 58 |
| Plasma DPH (μg/ml) | — | 12 ± 4 |

[a] Significantly different from control ($p < 0.05$).

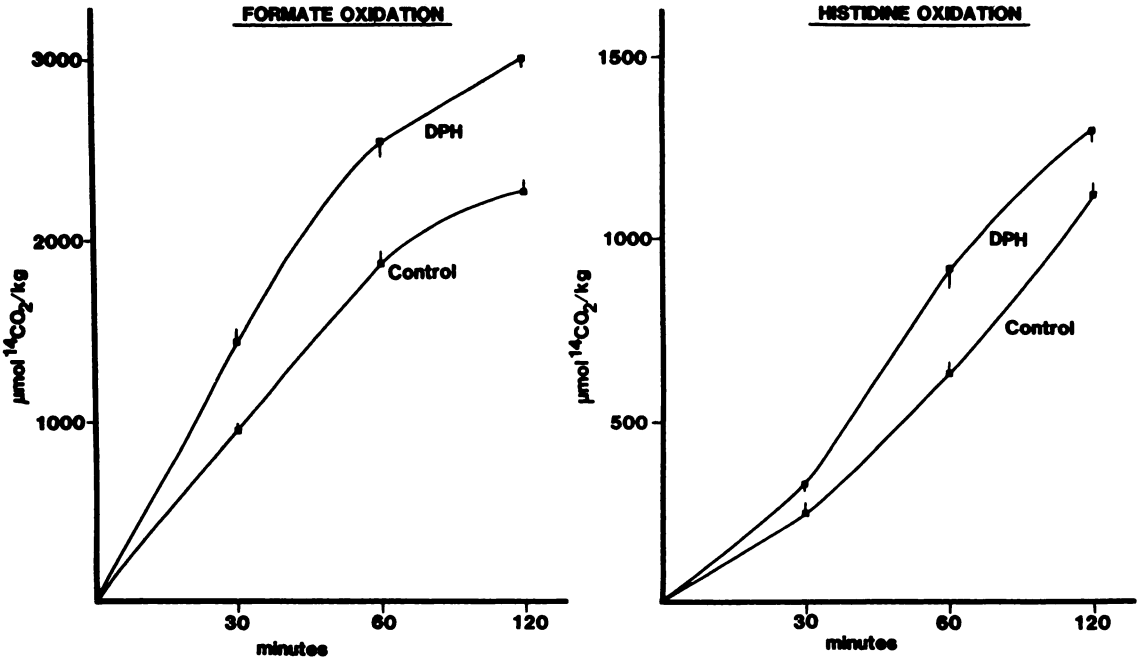


FIG. 3. *Effect of DPH on the oxidation of [$^{14}$C]formate and L-[ring-2-$^{14}$C]histidine to $^{14}CO_2$*

DPH was administered for 3 weeks in the diet at a dose of approximately 75 mg/kg. Results are expressed as the cumulative excretion of $^{14}CO_2$ per kilogram of mouse (mean ± standard error of the mean, $n = 4$). The excretion of $^{14}CO_2$ was significantly different ($p < 0.05$) at all time points with both compounds.

additive when compared with activity determined with homogenate from control or treated mice alone. DPH, added at concentrations of 10 μM–1 mM directly to the reductase assay incubation, did not affect the activity measured.

5,10-$CH_2$-$H_4$Folate reductase activity was maximally decreased within 1 week of DPH treatment (Table 2). However, a single dose of DPH administered 24 hr previously did not affect the enzymatic activity. DPH was given by i.p. injection when the time of administration was less than 1 week because it was observed that food consumption was erratic for the initial 2–3 days of dietary administration. On the other hand, long-term (>5 days) i.p. injections of DPH were found to cause severe abdominal adhesions, and therefore this route is unsuitable for chronic experiments. The time course of DPH effects on 5,10-$CH_2$-$H_4$folate reductase activity paralleled the effects on formate metabolism which were already described. Thus, there is an inverse relationship between the rate of formate oxidation to $CO_2$ and the hepatic activity of 5,10-$CH_2$-$H_4$folate reductase. Identical experiments were conducted with male mice, and it was found that 5,10-$CH_2$-$H_4$folate reductase activity is decreased by DPH treatment to an extent similar to that in female mice.

Urinary excretion of folates was found to be unaffected

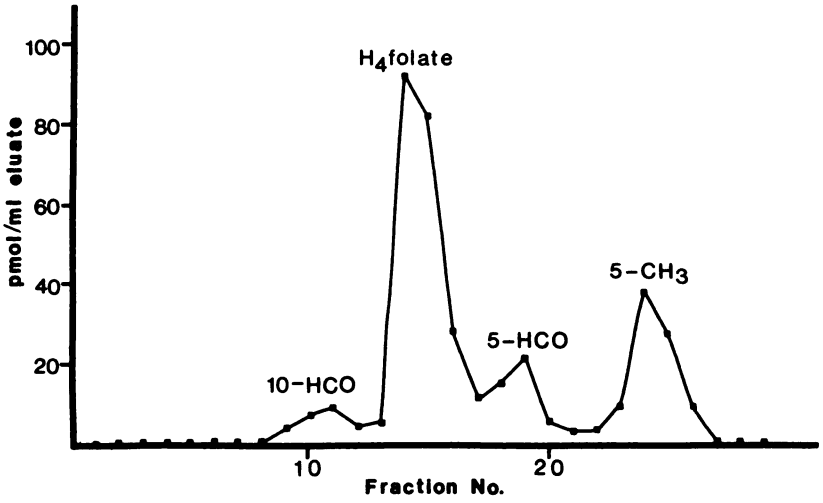


FIG. 4. *Typical HPLC chromatogram of the folate forms in mouse liver*

The HPLC analysis was conducted as described under Methods, using liver extracts which had been treated with pig kidney conjugase to hydrolyze folylpolyglutamates to monoglutamates. Folates were measured by microbiological assay with *Lactobacillus casei* and are expressed as picomoles per milliliter of eluate from the HPLC column.

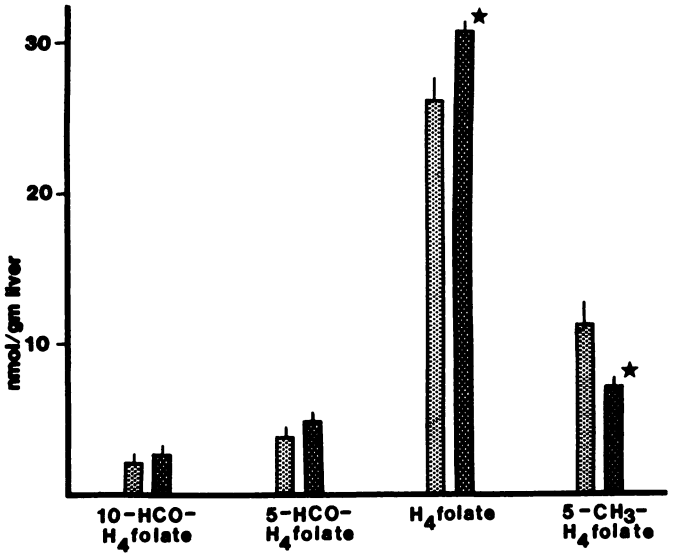


FIG. 5. *Effect of DPH on the distribution of folate forms in mouse liver*

DPH was administered for 3 weeks in the diet at a dose of approximately 75 mg/kg. Results are expressed as means ± standard error of the mean ($n = 4$). The concentrations of $H_4$folate and 5-$CH_3$-$H_4$folate were significantly different ($p < 0.05$) from control values.

by DPH treatment of mice for 10 weeks. The excretion was 7.7 ± 1.0 nmoles/24 hr in control mice ($n = 4$) and 6.9 + 0.80 nmoles/24 hr in DPH-treated mice ($n = 5$).

## DISCUSSION

The mouse has been established as a good animal model for studying the effect of DPH on folate metabolism. Thus, plasma folate levels are decreased and stimulation of the rate of formate and histidine oxidation to $CO_2$ is observed in mice treated chronically with DPH. Decreased plasma folate levels (1, 2) and an increased rate of histidine metabolism (6, 11) in DPH-treated humans have been reported. In addition, DPH treatment of mice was found to increase liver weight, hepatic microsomal protein, and the liver content of microsomal cytochrome P-450. Induction of microsomal enzymes is known to occur in DPH-treated epileptics (1). These effects of DPH in the mouse were found at doses which produce steady-state plasma levels in the "therapeutic" range, i.e., 10–20 μg/ml. This dose was approximately 75 mg/kg when DPH was given in the diet at a level of 400 mg/kg. It is unlikely that DPH induction of microsomal cytochrome P-450 accounts directly for the stimulation of formate and histidine metabolism. Preliminary studies with rats have shown that phenobarbital or 3-methylcholanthrene increases the liver microsomal cytochrome P-450 (P-448) concentration but does not affect the rate of formate or histidine metabolism.

DPH was found to reduce the hepatic activity of 5,10-$CH_2$-$H_4$folate reductase. This decreased activity is postulated to account for the other effects on folate biochemistry which were observed. Increases in the activity of this enzyme have been previously associated with increases in tissue concentrations of $H_4$folate, which are accompanied by reciprocal decreases in the concentration of 5-$CH_3$-$H_4$folate (17, 21–24). For example, 5,10-$CH_2$-$H_4$folate reductase activity is decreased in thyroidectomized rats. Elevated concentrations of $H_4$folate and decreased concentrations of 5-$CH_3$-$H_4$folate are observed (24, 35). Increased concentrations of $H_4$folate and enhanced rates of formate and histidine metabolism also occur following methionine administration to vitamin $B_{12}$-deficient rats (17, 36, 37) and nitrous oxide-treated

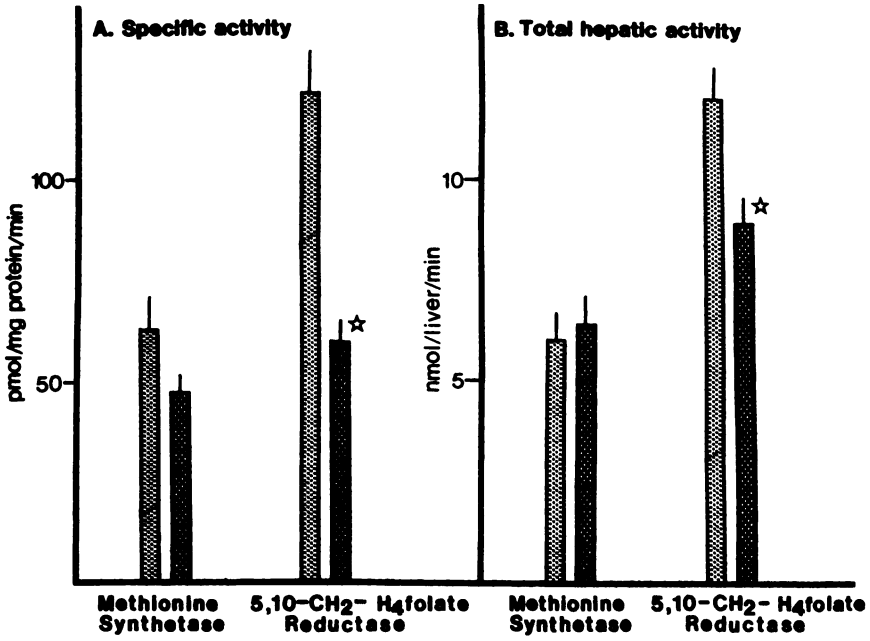


FIG. 6. *Effect of DPH on the hepatic activity of methionine synthase and 5,10-$CH_2$-$H_4$folate reductase*

DPH was administered for 3 weeks in the diet at a dose of approximately 75 mg/kg. Results are expressed as means ± standard error of the mean. 5,10-$CH_2$-$H_4$folate reductase activity was significantly different ($p < 0.05$) from control values whether it was expressed on a protein basis or per total liver.

TABLE 2

*Effect of DPH on 5,10-methylenetetrahydrofolate reductase activity*

| Time[a] | Dose | Route of administration | Activity[b] |
|---|---|---|---|
| *days* | *mg/kg* | | *pmoles/min/mg protein* |
| 0 | — | — | 110 ± 9.3 (12) |
| 1 | 75 | i.p. | 100 ± 11 (4) |
| 4 | 50 | i.p. | 67 ± 7.4 (4)[c] |
| 7 | 75 | Diet | 68 ± 5.3 (3)[c] |
| 21 | 75 | Diet | 70 ± 7.0 (8)[c] |

[a] Number of days DPH was administered.

[b] Hepatic 5,10-$CH_2$-$H_4$folate reductase activity values are means ± standard error of the mean. The number of animals in each group is given in parentheses. These data were compiled from several separate experiments. Activity in untreated mice ranged from 92 to 121 pmoles/min/mg of protein in separate studies.

[c] Significantly different from control ($p < 0.05$).

rats (14), and the effect of methionine has been postulated to be due to its conversion to *S*-adenosylmethionine, which inhibits 5,10-$CH_2$-$H_4$folate reductase activity (21, 22). In addition, cultured fibroblasts from humans with a genetic deficiency of 5,10-$CH_2$-$H_4$folate reductase have increased concentrations of non-methylfolate forms, including $H_4$folate, and decreased concentrations of 5-$CH_3$-$H_4$folate (23).

Based largely on the data from these various studies, the concept has evolved that the activity of both 5,10-$CH_2$-$H_4$folate reductase and methionine synthase control the cellular concentration of 5-$CH_3$-$H_4$folate and $H_4$folate. DPH was found in the present study to decrease the hepatic concentration of 5-$CH_3$-$H_4$folate and increase the concentration of $H_4$folate, as well as to decrease the activity of 5,10-$CH_2$-$H_4$folate reductase. Methionine synthase activity was not altered by DPH treatment. The working hypothesis suggested by these data is that the decreased 5,10-$CH_2$-$H_4$folate reductase activity is responsible for the reciprocal changes in folate forms which were observed. An effect of DPH on enzymes which utilize $H_4$folate, such as serine hydroxymethyl transferase, would be expected to alter the concentration of $H_4$folate, but a concomitant change in 5-$CH_3$-$H_4$folate concentration is unlikely to occur by this mechanism.

Decreased 5,10-$CH_2$-$H_4$folate reductase activity leads directly to increased concentrations of 5,10-$CH_2$-$H_4$folate rather than elevated concentrations of $H_4$folate (Fig. 1). However, 5,10-$CH_2$-$H_4$folate reversibly dissociates to $H_4$folate and formaldehyde. The dissociation of 5,10-$CH_2$-$H_4$folate to $H_4$folate might occur both in intact cells and in the preparation of the liver for folate analysis. The observation in the present study of increased rates of formate and histidine oxidation *in vivo*, reactions which depend upon the hepatic $H_4$folate concentration, suggests that increased concentrations of $H_4$folate did indeed occur *in vivo* as a result of decreased 5,10-$CH_2$-$H_4$folate reductase activity. An alternate explanation for the stimulation of formate and histidine oxidation to $CO_2$ is that decreased 5,10-$CH_2$-$H_4$folate reductase activity leads to increased conversion of 10-HCO-$H_4$folate to $CO_2$ (38). This is unlikely, however, since $H_4$folate is both utilized and generated in the metabolism of formate to $CO_2$ (Fig. 1). Therefore, no change in hepatic concentrations of either $H_4$folate or 5,10-$CH_2$-$H_4$folate would accompany the increased rate of formate metabolism to $CO_2$. However, increased concentrations of apparent $H_4$folate were observed. Whether or not this increase is attributable in intact cells to both $H_4$folate and 5,10-$CH_2$-$H_4$folate has yet to be determined by methods which can definitively prevent the *in vitro* interconversion of the two forms. The molecular mechanism responsible for the decreased 5,10-$CH_2$-$H_4$folate reductase activity is not apparent from these studies. However, the time required, i.e., more than 24 hr, suggests that neither DPH nor its metabolites have a direct effect on the enzyme.

The decrease in plasma folate levels caused by DPH can be accounted for by the effects which were observed on the distribution of hepatic folate forms. This could be the direct result of decreased hepatic concentration of 5-$CH_3$-$H_4$folate, the circulating folate form, or the indirect result of the increased concentration of $H_4$folate. Non-methyl folate forms are the favored substrates for polyglutamate synthesis, and polyglutamate folate derivatives are preferentially retained within cells. Interestingly, drugs such as nitrous oxide, which have the opposite effect of DPH on 5-$CH_3$-$H_4$folate and $H_4$folate concentrations in the liver, have the opposite effect on plasma folate levels (18, 19); that is, nitrous oxide increases plasma folate levels. An alternate mechanism for the decreased plasma folate levels caused by DPH, i.e., increased urinary excretion of folates, was not demonstrable in the present study.

The present study clearly shows that DPH decreases the activity of a key enzyme in the folate pathway, namely 5,10-$CH_2$-$H_4$folate reductase, and alters the hepatic distribution of folate forms. These effects can account for the effects of DPH previously observed and also shown in the current experiments, namely increased rates of formate and histidine oxidation to $CO_2$ and decreased plasma folate levels. However, effects of DPH on other enzymes involving folate interconversions have not been ruled out, and such effects may also contribute to the over-all effect of DPH on folate biochemistry. Additional experiments are necessary to determine the consequence of these effects on folate metabolism to the pharmacological and toxicological effects of DPH. Whether DPH affects brain folate metabolism in a manner similar to its effects on liver folate metabolism has not yet been studied. The effects of DPH which have been observed could disrupt embryonic folate metabolism and lead to malformed offspring. This could occur as a result of either decreased maternal plasma folate levels or effects on embryonic 5,10-$CH_2$-$H_4$folate reductase activity. Experiments are in progress to examine whether disrupted embryonic folate metabolism contributes to the mechanism of DPH teratogenicity.

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