Effect of Folic Acid Supplementation on Folate Status and Formate Oxidation Rate in Mink (Mustela vision)

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ABSTRACT: We investigated the folate-dependent toxicity of formate to mink to better understand the use of formic acid in fur animal feeds. Folic acid supplementation (0, 1, 5, 10, and 20 mg/kg of DM) in the feed of weanling mink for 4 wk resulted in hepatic tetrahydrofolate (H4folate) concentrations of 3.94, 8.51, 9.15, 10.4, and 15.0 nmol/g, respectively (SE 1.03). Oxidation tests in metabolic chambers, preceding a single injection of sodium [14C]formate (500 mg/kg BW), showed that the nonsupplemented mink oxidized formate into CO2 at a rate 37% less than that of the supplemented mink. The oxidation rate increased with supplementation level and was maximal, 54.2 mEq·kg⁻¹·h⁻¹ (SE 3.0), at 10 mg of folate/kg; at the highest level of supplementation (20 mg/kg), CO2 production tended to be lower. Concentration of hepatic 14C increased with the hepatic H4folate, and its accumulation continued after the highest point of oxidation. These observations indicate that mink oxidize formate readily but at a slightly lower rate than do rats. However, if extra folate is not supplemented in the feed during the period of early intensive growth, hepatic H4folate level may decline to the levels found in humans and monkeys, which are susceptible to formate accumulation. Average daily weight gain improved with each increase in supplementation of folic acid; however, only the differences between the nonsupplemented diet and the two highest levels of the vitamin reached statistical significance (P < .05).

Key Words: Formic Acid, Oxidation, Mink, Folic Acid, Dihydrofolate Reductase


Introduction

Methanol, formaldehyde, and formic acid are related intoxicants that produce metabolic acidosis, ocular toxicity, and death in humans and monkeys. The pathological changes are caused by formate, the ultimate metabolite that accumulates in the body after administration of any of the three substances (Clay et al., 1975; Martin-Amat et al., 1978; McMartin et al., 1979).

Despite its toxic symptoms in excess, formate is also a natural metabolite, a member of the body’s folate-dependent one-carbon pool, in which a linear relationship between formate oxidation rate into CO2 and hepatic tetrahydrofolate (H4folate) concentration has been shown (Johlin et al., 1987; Tephly et al., 1992).

Activity of the CO2-cleaving enzyme 10-formyl H4folate dehydrogenase is low in animals that oxidize formate poorly, such as humans, monkeys, and pigs, whereas it is high in rats and mice, animals that oxidize formate readily (Johlin et al., 1987; Makar et al., 1990; Tephly et al., 1992).

Even though formic acid has long been used as a preservative in fur animal feeds, questions sometimes arise about whether the acid is safe to the animals. This experiment was conducted to determine the efficiency of formate oxidation in mink and its interaction with dietary folic acid.

Materials and Methods

Animals and Feeding. Thirty weanling standard dark type male mink, averaging 570 g in weight and 53 to 57 d of age, were randomly divided into five groups, weighed, and fed the following levels of supplemental folic acid: 0, 1, 5, 10, and 20 mg/kg of DM. Excluding folate level, the feed was the same for all groups (Table 1). The experimental feed contained .3 mg natural folate/kg DM, whereas the vitamin...
supplementation in the preceding lactation feed was approximately 1.3/mg DM. No formic acid was added to the diet. The mink were housed individually on a commercial mink farm in standard wire-bottom cages (310 × 400 × 700 mm) connected to nesting boxes (310 × 360 × 250 mm). Feed and water were freely available. Feeding continued for 4 wk (J July), after which the mink in groups of five (one from each group) were taken to the laboratory for final weighing and the oxidation test. Individual lengths of the feeding period ranged from 28 to 36 d. After the test, mink were killed by i.m. injection of ketamine hydrochloride (50 mg/kg, Ketalar, Parke-Davis, Barcelona, Spain). Immediately after the onset of anesthesia, cardiac puncture was used to collect blood into EDTA vacuum tubes (Becton Dickinson Vacutainer, 7 mL EDTA-K3 15%, Neylan Cedex, France). The blood samples were divided into aliquots for hematological and folate analyses. To evaluate physiological effects of the folate feeding, blood samples were analyzed for leucocyte, erythrocyte, and platelet counts, hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentration (Coulter automatic cell counter, Coulter Electronics Ltd., Luton, England). Livers were removed for 14C quantification and folate determination. The experimental procedure was approved by the experimental animal care committee of the Department of Animal Science.

**Chemicals.** Tetrahydrofolic acid trihydrochloride (catalogue no. 24702, Merck, Darmstadt, Germany) and 5-methyl-tetrahydrofolic acid (calcium salt), (Merck 24708) were dissolved in 1.0% (wt/vol) sodium ascorbate. Standard solutions were flushed with nitrogen and stored at −20°C for no more than 4 mo. Aliquots of these solutions were taken to prepare a diluted standard in .01 M sodium acetate containing ascorbic acid-ascorbate (.25/.75%, wt/vol) and .1% (vol/vol) 2-mercaptoethanol. Folic acid (Merck 3984) and 5-formyl-H4folic acid (Merck 24709) were prepared accordingly and used for monitoring their presence in plasma analysis (Vahteristo et al., 1996).

Sodium [14C]formate (specific radioactivity 2.04 GBq/mmol) was purchased from Amersham International (Buckinghamshire, U.K.). Trapping agent Lumasorb II (methoxypropylamine) and a liquid scintillation counting cocktail (LSC-cocktail, isoxylene) were purchased from LUMAC*LSC B.V. (Groningen, The Netherlands). Feed-grade folic acid was purchased from Roche (Basle, Switzerland).

**Liver and Plasma Folates.** Livers were cut into 3- to 5-mm pieces, and 3 g of the cubed liver was weighed into glass tubes and stored under extraction buffer at −20°C for no more than 5 d. Extraction, incubation, and purification of the incubated extract were performed as described by Vahteristo et al. (1996). The final volume of the extract was 25 mL, of which 3 mL was used for incubation. For purification, 2.5 mL of the incubated extract was applied to a solid-phase extraction column. The retained H4folate was eluted with 2.5 mL of 10% sodium chloride in .1 M acetate containing 1% ascorbic acid, and 5 to 10 μL of eluate was injected into a reversed-phase C-18 column. Recovery of H4folate was determined by adding 3.2 μg of H4folate monoglutamate to the sample before homogenization (AOAC, 1990). Recovery of H4folate (monoglutamate) was 75 ± 10.4% (SD); CV for the liver analysis (n = 5) was less than 12%. Analysis results were not corrected according to the recovery test.

A modified method (Chapman et al., 1978) was used for plasma sample preparation. Blood samples were centrifuged for 10 min at 1,500 × g at room temperature. Plasma (2 mL) was mixed with 3.8 mL of 54.1 mM sodium ascorbate and .8 mL of 54.1 mM ascorbic acid in a capped tube, kept in a boiling water bath for 6 min, and cooled in ice. Denatured samples were centrifuged at 10,000 × g for 10 min, and the pellet was dissolved with 2 mL of 35.4 mM ascorbate solution and recentrifuged. Supernatant was collected into a 10-mL volumetric flask, which was filled with ascorbate solution (35.4 mM). Filtered supernatant (5 to 10 μL) was injected into the C-18 column. Recovery of 5-methyltetrahydrofolic acid in plasma (99 ng/mL) was 80% with relative CV of 7.7%. Reversed-phase HPLC analysis of folates was performed using 30 mM phosphate buffer at pH 2.2 and acetonitrile as described by Vahteristo et al. (1996).

**Formate Oxidation Test.** A formate oxidation test was performed in acrylic metabolic chambers (7.5-L volume) for 3 h. Before the test, mink received a single intraperitoneal injection of 23% solution of buffered sodium [14C]formate (500 mg/kg BW, 74.4 Bq/mg). The injection is equal to the amount of formate mink could receive from the diet. Animals

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Table 1. Composition of the diet (as-fed basis)

<table>
<thead>
<tr>
<th>Ingredient, %</th>
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<tbody>
<tr>
<td>Beef by-products</td>
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<tr>
<td>Low-fat cod fillet offal</td>
</tr>
<tr>
<td>Baltic herring, spring</td>
</tr>
<tr>
<td>Barley-wheat mixture (50:50)</td>
</tr>
<tr>
<td>Soybean oil</td>
</tr>
<tr>
<td>Vitamin mixture</td>
</tr>
<tr>
<td>Water</td>
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</tbody>
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**Calculated content (dry matter basis)**

| ME, MJ/kg | 16.3 |
| Energy distribution, % | 
| Protein | 39 |
| Fat | 38 |
| Carbohydrates | 23 |
| Protein, % | 41 |
| Calcium, % | 2.0 |
| Phosphorus, % | 1.2 |

*Provided the following per kilogram of diet dry matter: vitamin A 12,500 IU; vitamin D 1,250 IU; vitamin E 140 mg; thiamin 54 mg; riboflavin 21 mg; vitamin B12 .07 mg; pantothenic acid 14 mg; nicotinic acid 36 mg; pyridoxine 11 mg; biotin .18 mg.
were placed in the chambers, pressurized medical-quality air (2 L/min) was circulated, and CO₂ was collected by bubbling the expired air through the trapping agent. Scrubbing flasks, containing 30 mL of trapping agent, were replaced every 30 min. For the actual ¹⁴C quantification, duplicate 8-mL aliquots were prepared.

Hepatic ¹⁴C Concentration. Triplicate liver samples were prepared to determine the concentration of labeled hepatic carbon. Samples were placed on Whatman 4 paper, weighed, and oxidized (Junitek oxidizer, Junitek Oy, Turku, Finland). The ¹⁴C quantification was performed with a Wallac 1411 LSC (Wallac Oy, Turku, Finland) liquid scintillation counter.

Statistics. Data were analyzed with least squares procedures (Neter et al., 1985) using the statistical software package Statgraphics Plus (Version 6, Manugistics Inc., Rockville, MD), with duration of the feeding period as a blocking factor and folic acid level of the diet as the main effect. In addition, initial BW was used as a covariant in weight gain analysis. After LSD analysis of the data, Tukey’s test was used to identify statistically significant differences between treatment means. Second- and third-order polynomial regression models were applied to highlight relationships among the oxidation rate, the hepatic H₄folate concentration, and the hepatic ¹⁴C concentration.

Results

Effect of Feeding. Increasing dietary supplemental folic acid clearly increased folate concentrations of plasma and liver (Figure 1). In plasma, the 5-methyl H₄folate form was the only vitamer that exceeded the detection limit (10 ng/mL). The highest plasma folate levels were three times greater than those for mink fed the nonsupplemented diet. The most pronounced relative increase (100%) occurred when supplementation was increased from 0 to 1 mg folic acid/kg DM. Hematological variables were comparable in all groups (results not shown).

Folic acid affected the growth of mink (Table 2). Average daily weight gain improved with each increase in supplementation of the vitamin; however, only the differences between the nonsupplemented diet and the two highest levels of the vitamin, 10 and 20 mg/kg, reached statistical significance (P < .05). Production of ¹⁴CO₂ started during the first 30 min of the test and from the second period onward continued almost linearly to the end of the test (Figure 2). There were no marked differences in the overall pattern of ¹⁴CO₂ production over time.

Average oxidation rates were calculated as hourly means per kilogram of BW from 30 to 180 min. Results are presented in Figure 3. The mink that received nonsupplemented feed converted formate into CO₂ at a rate 37% slower than that of the folic acid-supplemented mink. Consistent with plasma and liver folate status, oxidation rate increased the most when the vitamin was increased from 0 to 1 mg. Further increases in dietary folic acid resulted in increases in oxidation rate at the 5 and 10 mg levels but decreased oxidation at the 20 mg level.

Effect of Hepatic Tetrahydrofolate Level. When individual formate oxidation rates were regressed on hepatic H₄folate levels, a quadratic relationship between these two variables was revealed (Figure 4).

Figure 1. Effect of supplemented folic acid on plasma (5-methyl tetrahydrofolate [H₄folate]) and hepatic folate (H₄folate) concentrations in mink. Each value represents the mean of six animals ± SE.
Figure 2. Formate oxidation in mink as measured by cumulative in vivo \(^{14}\text{C}\)CO\(_2\) production during the course of the test. Sodium \(^{14}\text{C}\) formate (500 mg/kg BW) was injected intraperitoneally. Each point represents the mean of six animals ± SE. Figures 0, 1, 5, 10, and 20 indicate the level of dietary folic acid supplementation (milligram/kilogram of DM).

Concentration of \(^{14}\text{C}\) in the liver first increased as hepatic H\(_4\)folate concentration increased, reached a plateau at intermediate hepatic H\(_4\)folate levels, then increased again sharply at the highest hepatic H\(_4\)folate concentrations (Figure 5).

Discussion

Folate Status of Mink. Our experiment shows that folate status of fast growing, farmed mink is strongly dependent on additional supplementation of the vitamin in the feed. Low folate status was found from feeding a nonsupplemented feed for only 4 wk. This is an important finding for mink farming, but it also makes mink a useful animal for formate metabolism research. Extremely low plasma folate values are prevented with currently recommended supplementary levels of the vitamin, .5 to 1.0 mg/kg DM (Juokslahti, 1987), but marked increases in plasma folate can be achieved only with dietary supplementation levels of 10 to 20 mg/kg DM.

It is noteworthy that the mean hepatic H\(_4\)folate level of the nonsupplemented group of mink, 3.94 nmol/g, was only one-third of the values usually analyzed from rat liver, and even less than the values measured in human and monkey livers (Johlin et al., 1987). The normal hepatic H\(_4\)folate level of young mink seems to be less than 10 nmol/g, even with dietary supplementation.

Hematology and Growth. Even though folate status in the nonsupplemented mink was low, hematological values fell within the normal range and revealed no development of megaloblastic anemia, thrombopenia, or leukopenia, which are typical of a deficiency of folate (Hawkes and Villota, 1989; Mathews and VanHolde, 1990). The actual vitamin deficiency, if it existed at all, probably did not last long due to the feed's naturally occurring vitamin and the residual effect of the preceding feeding. When the diet of young cats contained .125 mg folic acid/kg DM, it took 22 wk before the animals became folic acid deficient, and even then the cats grew normally (Thenen and Rasmussen, 1978). Therefore, absence of deficiency symptoms in our study is not unexpected. It is possible that the level of dietary folic acid required to overcome deficiency symptoms is different from that necessary for optimal growth performance.

In contrast, the fact that daily gains improved with supplementation of the vitamin was unexpected. Because the experimental period fell within a very sensitive period in the mink's life, this finding deserves a reevaluation in a more elaborate feeding trial. Similar results have been reported with pigs. Folate deficiency signs are rarely seen, but supplementation levels 10 to 20 times the usual recommendations may improve offspring survival or the total litter weight at weaning (Matte et al., 1992).

Formate Oxidation. The relationship between formate oxidation rate and hepatic H\(_4\)folate concentration in mink (Figure 4) agrees with reports on other species (Johlin et al., 1987). Due to the quadratic response in our study, it follows that supplemental folic acid can increase CO\(_2\) production only to a certain point, in mink up to 50 to 55 mEq/(kg·h), after which the rate can be reduced. The experiment also suggests the existence of another rate-limiting factor in formate oxidation, most probably the enzyme 10-formyl H\(_4\)folate dehydrogenase (Johlin et al., 1987; Makar et
al., 1990; Tephly et al., 1992). The oxidation curve will obviously reach a plateau and may even decline as a result of a relative deficiency of this enzyme. The fact that the concentration of hepatic $^{14}$C increases sharply with a concomitant decrease in oxidation rate, reveals a dramatic change of metabolism. This observation may be a practical implication of what Choe (1989) suggested after folate studies with rats; because of tight binding of product to the dehydrogenase enzyme, oxidation of one-carbon moieties may be regulated by the ratio of formyl $H_4$folate to $H_4$folate in liver. The results of our experiment are different from those of Palese and Tephly (1975), who found that formate oxidation also increased due to injected formate, but oxidation rates remained within levels characteristic of the species.

Even though the main causes for susceptibility to formate accumulation are known, differences in accumulation among individual animals of the same strain and fed the same diet are still difficult to explain (Dorman et al., 1993). Some strains of pigs are very susceptible to formate toxicity (Makar et al., 1990; Tephly et al., 1992). Formic acid toxicity has been of minor importance in nutritional studies. When pigs were fed supplemental formic acid, toxic signs did not occur until the acid content reached 1.8% (Grassmann et al., 1992). Differences among individuals may be due to differences in activity of the dehydrogenase enzyme or the rates at which formate carbon is incorporated into other one-carbon pool metabolites, such as serine, purines, and proteins (Mathews and VanHolde, 1990). Even though formate metabolism by alternate metabolic pathways has been shown to be only 15% in incubated rat hepatocytes (Billings and Tephly, 1979), incorporation of formate into livers of folic acid-treated rats was 10 times greater than incorporation rates of folic acid-deficient rats (Plaut et al., 1950). Our study shows that folic acid supplementation per se does not improve oxidation of formate into CO$_2$, but it still leaves open the possibility that a high level of dietary folic acid supplementation may increase incorporation of formate carbon into body proteins. Because only radioactivity in liver was determined, distribution of $^{14}$C into different metabolites remains unknown.

The oxidation test gives us basic knowledge about the levels of formic acid that mink can tolerate in the feed. According to the test, mink's formate oxidation capacity is approximately 1,000 mg of formic acid (85%)/kg of BW in 24 h. Thus, safe formic acid percentage in feed DM could be 1% during periods of fast growth (feed intake of 100 g DM$^{-1}$·kg BW$^{-1}$), but could increase to 2% in the maintenance feed of an adult mink (feed intake 50 g DM$^{-1}$·d$^{-1}$·kg BW$^{-1}$). In the latter situation, it is also possible that reduced palatability would limit feed intake so formate consumption would be below the level required to cause toxicity. Oxidation capacity of young mink fed 1% formic acid in the diet DM exceeded the oxidation rates found in the present study (our unpublished observations). It is obvious that mink can fairly well avoid formate accumulation. Even though enzyme
activities are to a large extent genetically determined, adaptation to formate feeding cannot be totally excluded, especially if folate status of an animal is adequate. Our experimental mink did not receive formic acid before the test.

We conclude that mink oxidize formate readily, but with a slightly lower rate than rats. In an animal with adequate folate status, formate will not accumulate in the body; formate is activated into the body's one-carbon pool and, to a large extent, is oxidized to CO₂. However, if the diet fed to mink during the early intensive growth period contains markedly less than 1 mg of folate/kg DM, hepatic H₄folate level may drop below the levels analyzed in humans and monkeys, species that are very susceptible to formate accumulation.

**Implications**

The capacity of mink to oxidize formate removes most of the concerns about safety of formate as a feed preservative. This research suggests 1% formic acid in diet DM is safe for mink and emphasizes the importance of supplemental folic acid during the early postweaning period in a mink's life. During that period, supplemental folic acid is important for formate metabolism, and it may improve growth rate.

**Literature Cited**


