Bay may be representative of relatively low-lead coastal waters, the natural abundance data indicate that virtually all the lead is derived from Canadian gasoline lead, a conclusion that cannot be drawn on the basis of concentration determinations alone.

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Detection of a Milk Factor That Facilitates Folate Uptake by Intestinal Cells

Abstract. *Folate binding proteins in milk were tested for their effect on folate absorption. The uptake of bound folate by isolated mucosal cells from the rat small intestine was twice that of free folate and differed from it in being more effective with progression down the small intestine, in not being affected by glucose or Dilantin, in having a higher pH optimum, and in being affected by calcium concentration. This milk factor may enhance folate absorption in infants, whose risk of folate deficiency is high.*

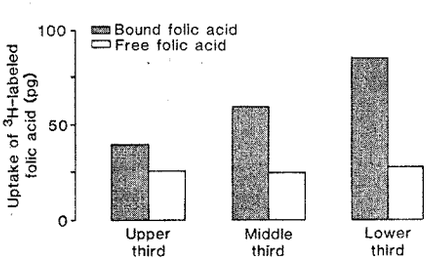
The detection of folate binding proteins in milk (*1–3*) and their subsequent characterization (*4*) led to numerous studies of their function (*5–9*). None provided a coherent explanation for their role in milk. Studies of these proteins in the milk of cows, goats, and humans (*4, 9–11*) demonstrated that they, along with the corresponding serum binders, (i) are glycoproteins with a molecular weight approximating 40,000, (ii) have isoelectric points in the neutral range, and (iii) dissociate at acid *p*H and can rebind folate when neutralized. Since the binders impair the transport of folate into both dividing cells and bacteria (*5–7*), we thought that it might be productive to study their effects on intestinal absorption.

Intestinal absorption of folate is a two-stage process. First there is obligatory hydrolysis of folate polyglutamates in food to the monoglutamate form (*12*), a process that apparently occurs in the mucosal brush border (*13*). In the second stage, the monoglutamates traverse the mucosal border at a rate modified by *p*H, fluid and electrolyte shifts, and other factors (*14*). A third stage, involving methylation and formylation of folates, may occur in mucosal cells but is not required for absorption (*15*). The absorbed folates then enter the portal circulation and are transported to the liver. In this report we show that folate binding proteins in milk mediate the folate absorption process.

The milk binder was obtained fresh from Sri Lankan goats (*16*) after the second week of lactation, centrifuged, skimmed, and freeze-dried for storage. After reconstitution, a solution of the milk (5 g per 100 ml) was incubated, without purification, with saturating quantities of ^{3}H-labeled pteroylglutamic (folic) acid for 30 minutes, dialyzed for 18 hours against three changes of buffer, and used in the various assays. To avoid possible artifacts caused by particulate matter, we centrifuged the dialyzed samples at 100,000*g* for 1 hour and used only the supernatant.

Mucosal cells were isolated from rat small intestine by vibration (*17*). The small bowel was resected from anesthetized nonfasted rats, everted over a glass spiral, and agitated at an amplitude of 1 mm for 8 minutes at 60 vibrations per second. The dislodged cells were strained through a grid with 0.4-mm^2 apertures, washed at 1000*g*, and suspended in tris-buffered Hanks solution with phosphate buffer. After incubating 10^6 cells for 1 hour at 37°C, we washed the cells three times at 1000*g*, extracted the folate by autoclaving them with 1 ml of ascorbate solution (1 g per 100 ml), and measured the radioactivity of the extracts.

Uptake of folate bound to milk binder not only exceeded that of free folate, it increased more than twofold between cells from the upper third of the intestine and cells from the lower third (Fig. 1). Free folate uptake did not change with a change in site of origin of the intestinal cells. Uptake of bound folate differed from that of free folate in being neither enhanced by 40 n*M* glucose nor inhibited by 2 m*M* Dilantin (Fig. 2). Nonradioactive free folic acid (90 m*M*) had no inhibitory effect. Calcium and EDTA barely affected free folate uptake, whereas bound folate was absorbed twice as rap-



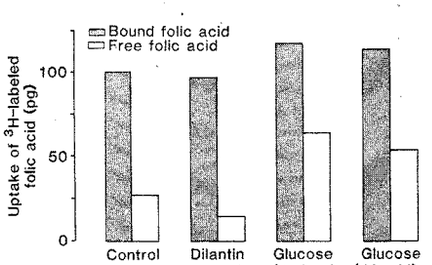


Fig. 1 (left). Effect of folate binding protein on folate uptake at *p*H 6.5 by isolated mucosal cells from the upper, middle, and lower thirds of the rat small intestine. Each incubation flask contained 10^6 cells, 1.1 ml of 0.1*M* phosphate buffer, bound or free labeled folic acid, and tris-buffered Hanks solution to a final volume of 2.5 ml (*p*H 6.5). Fig. 2 (right). Uptake of labeled folic acid by isolated cells of the rat small intestine under the influence of Dilantin and glucose.

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idly in the presence of 1 m*M* $CaCl_2$ (Fig. 3).

Bound folate uptake was not affected by varying the temperature of incubation between 4° and 37°C and the oxygen concentration between 20 and 95 percent. Temperature-independent protein-mediated uptake of vitamin B_{12} has been observed with liver slices and intrinsic factor concentrate made from hog's milk and with L1210 cells and purified *trans*-cobalamin II (*18*). It may reflect surface binding of the complex rather than internalization and subsequent release and conversion of folate, processes that are more likely to be temperature-dependent due to their enzymatic nature. The difference between surface binding and internalization of the complex may eventually be delineated by using the latex particle technique, which allows the specific location of the complex to be determined with scanning electron microscopy (*18*).

We also measured folate uptake from human milk (Mother's Milk Bank, Wilmington, Delaware) frozen immediately after expression and bovine milk from a local store. Compared with the uptake of free ^{3}H-labeled folic acid by cells of the upper and lower small bowel, respectively, each expressed as 100 percent, uptake from goat milk was 157 and 209 percent; from human milk, 109 and 156 percent; and from bovine milk, 88 and 94 percent. Failure of bovine milk to enhance folate uptake is probably attributable to pasteurization and other processing, since goat milk purchased in the United States enhanced folate uptake before it was pasteurized but not afterward.

Consonant with prior findings (*17*), the *p*H optimum for free folate uptake was 6.1 and was less than the optimum for bound folate uptake (between 6.6 and 7.1). Free folate is essentially insoluble at *p*H 7 (*19*), but is soluble at the low concentrations used in this study (5 n*M*).

The isolated mucosal cells were prepared by a technique previously used for rapid assessment of the effects of various factors on absorption of free ^{3}H-labeled folic acid (*17*). These prior studies effectively reproduced patterns of folate absorption observed with the triple lumen tube technique, namely, enhancement of uptake by glucose and inhibition of uptake by Dilantin (*20*). Our experiments were not subject to the problems of lengthened intestinal transit time or dilution with biliary folate since we used isolated cells in medium; these two factors were cited by Izak *et al.* as possible causes of artifacts in their studies of rat gut in vivo (*21*) in which milk-bound

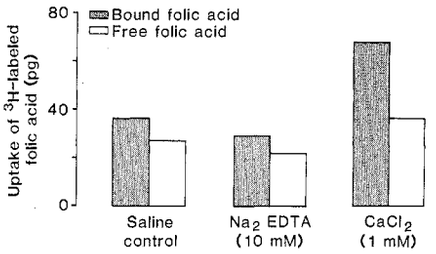

Fig. 3. Effect of calcium chloride and EDTA on the uptake of labeled bound and free folate at *p*H 6.5.

folate appeared less well absorbed than free folate in the gut as a whole. The enhancement of folate uptake demonstrated in this study therefore suggests that the prior binding of folate to milk enhances its uptake in the intestine in vivo. Uptake may be further assisted by sequestration of bound folate in a manner that prevents its utilization by bacteria (*6*).

The clinical importance of this observation is primarily related to possible effects of breast-feeding on the epidemiology of megaloblastic anemia in infants. Faber in 1928 described megaloblastic anemia in 6-week-old babies (*22*). Soon thereafter the condition was successfully treated with crude liver and yeast (*23*) and shown to be associated with nutritional deficiency, prematurity, infection, and maternal anemia (*24*). Since the calculated folate intake of week-old infants (*25*) is only a fraction of the calculated minimum daily requirement (*26*), it is not surprising that 20 to 40 percent of premature infants have changes suggestive of folate deficiency (*27, 28*).

In contrast, folate deficiency and megaloblastic anemia are extremely rare in infants of normal gestational age and birth weight who are breast-fed by nutritionally replete mothers, as reported from numerous localities including Japan (*29*). Differences in the folate status between these two groups of infants are of course attributable to multiple factors, including different folate requirements. One of the most important factors, however, may simply be whether the infant receives breast milk or cow's milk. Ek and Magnus found no folate deficiency in 35 breast-fed infants (*30*) but considerable deficiency in ten infants fed homemade cow's milk formula (*31*). Although loss of folate during preparation of cow's milk formula may have accounted for some of the differences in folate status between the two groups, it is not sufficient to explain the considerable differences observed. Identification of the factor or factors that give this advantage to breast-fed infants, such as an intrinsic factor in milk, may provide a better understanding of folate absorption during infancy and how it can be improved. A long-term objective is eliminating the need to provide folic acid supplements to infants whose birth weights are low (*32, 33*).

Although megaloblastic anemia is the predominant manifestation of folate deficiency in infancy, other effects have been suggested. These include impairment of fetal growth in utero (*34–36*) and during the first year of life (*37*) and harmful effects on central nervous system development in the perinatal period, when folate utilization in the brain is several orders of magnitude greater than that in the central nervous system of adults (*38*).

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Aspirin Prevention of Cholesterol Gallstone Formation in Prairie Dogs

Abstract. *When prairie dogs (*Cynomys ludovicianus*) are fed a diet containing cholesterol, a marked increase in gallbladder mucin secretion parallels the evolution of cholesterol supersaturated bile. Gelation of mucin precedes the precipitation of cholesterol liquid and solid crystals and the development of gallstones. Aspirin given to prairie dogs inhibited mucin hypersecretion and gel accumulation and prevented gallstone formation without influencing the cholesterol content of supersaturated bile. This suggests that gallbladder mucin is a nucleation matrix for cholesterol gallstones.*

Cholesterol gallstones afflict 30 percent or more of the adult population in certain Western countries and ethnic groups (*1*). Their prevalence is increasing in the Orient where many Western dietary habits have been adopted (*2*). A critical step in the formation of cholesterol gallstones is the nucleation of cholesterol from supersaturated bile (*3*). Depending on the degree of supersaturation, the initial cholesterol microprecipitates may be either liquid-crystalline (*4*) or amorphous (*5*); both types contain substantial amounts of the biliary phospholipid, lecithin. These microprecipitates give rise to crystals of cholesterol monohydrate which in turn aggregate to form macroscopic gallstones (*6*). The importance of gallbladder mucin in the formation of gallstones has long been recognized. In 1856 Meckel von Hemsbach (*6a*), who had studied the formation of pearls, attributed gallstones to an accumulation of gallbladder mucin that served as a nucleus for the stones that formed. Womack (*7*) obtained evidence that hypersecretion of gallbladder mucin glycoprotein occurred in lithogenic hamsters and suggested that this high-molecular-weight polymer acted as an organic binder in the formation of cholesterol gallstones. Increased gallbladder mucin has been observed in other animal models (*8*) as well as in human gallbladders containing stones (*9*).

On the basis of morphologic studies, Hultén (*10*) suggested that cholesterol crystals grew predominantly in mucin-packed niches of the human gallbladder wall. More recent observations (*11*) suggest that a nucleation factor is present in supersaturated lithogenic human bile, and that this factor accelerates cholesterol crystal formation; the factor is not found in supersaturated nonlithogenic bile from control patients (*11*). Studies at our laboratory demonstrated (*12*) that marked gallbladder mucin hypersecretion occurred in prairie dogs fed a cholesterol diet for several days; this paralleled the enrichment of bile with cholesterol and occurred prior to nucleation and the formation of liquid crystals, crystals, or gallstones. Cholesterol crystals were particularly prominent in a visible mucin gel that accumulated in the gallbladder of the cholesterol-fed animals.

We designed the present study to test the hypothesis that gallbladder mucin glycoproteins are important in the nucleation of cholesterol microprecipitates from lithogenic bile. We inhibited gallbladder mucin glycoprotein secretion with aspirin, since this compound, as well as other nonsteroidal anti-inflammatory drugs, are known to inhibit mucin secretion in other organs (*13*) but are without effect on biliary lipid saturation (*14*). We found that aspirin administered to cholesterol-fed prairie dogs inhibited the nucleation of cholesterol-supersaturated bile without altering biliary lipid composition and prevented the appearance of cholesterol microprecipitates and gallstone formation. The prevention of crystals and stones in the aspirin-treated animals was correlated with the absence of a mucin gel and a marked diminution in gallbladder mucin concentration.

We first studied the effect of aspirin on gallbladder mucin secretion in vitro, using an organ culture technique (*15*). Gallbladder explants from cholesterol-fed and control prairie dogs were incubated with [^{3}H]glucosamine and various concentrations (1 to 25 m*M*) of aspirin (*16*). Aspirin at all doses inhibited gallbladder glycoprotein secretion from normal gallbladder explants as well as those from prairie dogs fed the cholesterol diet for 5 days. The addition of aspirin caused a dose-dependent inhibition of mucin secretion reaching 80 to 85 percent inhibition at 25 m*M*.

To confirm the inhibitory effect of aspirin on gallbladder mucin secretion, we compared mucin glycoprotein synthesis and secretion in gallbladder explants from animals fed cholesterol and animals fed cholesterol plus aspirin. Aspirin doses of 25, 50, 75, and 100 mg per kilogram per day were well tolerated. The largest dose of aspirin caused mild gastritis but did not cause significant hepatotoxicity, weight loss, or hemorrhage. Prairie dogs ($N = 6$) were then pair-fed with either the 1.2 percent cholesterol diet or the 1.2 percent cholesterol diet plus 100 mg of aspirin per kilogram of body weight per day for 6 days. As shown in Fig. 1, aspirin in vivo significantly ($P < .001$) inhibited the incorporation of [^{3}H]glucosamine into gallbladder tissue and secreted mucin glycoproteins after 6 and 24 hours of incubation.

We then tested the effects of aspirin on total and relative biliary lipid composition and the formation of cholesterol crystals and gallstones (*17*) in prairie dogs fed the cholesterol diet for 14 days. Three groups of prairie dogs were studied (Table 1). Control animals were given

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