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Williamson Strum, ... , Joseph B. Bertino, Henry J. Binder

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Intestinal absorption of the monoglutamate form of the principal dietary and circulating folate compound, 5-methyltetrahydrofolic acid (5-MTHF), was studied in the rat utilizing a synthetic highly purified radiolabeled diastereoisomer. Chromatography confirmed that the compound was not altered after transfer from the mucosa to the serosa. Accumulation against a concentration gradient was not observed in duodenal, jejunal, or ileal segments at 5-MTHF concentration from 0.5 to 500 nmoles/liter. Unidirectional transmural flux determination also did not indicate a significant net flux. Mucosal to serosal transfer of 5-MTHF was similar in all segments of the intestine and increased in a linear fashion with increased initial mucosal concentrations. Further, no alteration in 5-MTHF transfer was found when studied in the presence of metabolic inhibitors or folate compounds.

These results indicate that 5-MTHF is not absorbed by the rat small intestine by a carrier-mediated system and suggest that 5-MTHF transfer most likely represents diffusion.

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Intestinal Folate Absorption

I. 5-METHYLTETRAHYDROFOLIC ACID

WILLIAMSON STRUM, PETER F. NIXON, JOSEPH B. BERTINO, and
HENRY J. BINDER

*From the Departments of Internal Medicine and Pharmacology, Yale
University, New Haven, Connecticut 06510*

ABSTRACT Intestinal absorption of the monoglutamate form of the principal dietary and circulating folate compound, 5-methyltetrahydrofolic acid (5-MTHF), was studied in the rat utilizing a synthetic highly purified radiolabeled diastereoisomer. Chromatography confirmed that the compound was not altered after transfer from the mucosa to the serosa. Accumulation against a concentration gradient was not observed in duodenal, jejunal, or ileal segments at 5-MTHF concentration from 0.5 to 500 nmoles/liter. Unidirectional transmural flux determination also did not indicate a significant net flux. Mucosal to serosal transfer of 5-MTHF was similar in all segments of the intestine and increased in a linear fashion with increased initial mucosal concentrations. Further, no alteration in 5-MTHF transfer was found when studied in the presence of metabolic inhibitors or folate compounds.

These results indicate that 5-MTHF is not absorbed by the rat small intestine by a carrier-mediated system and suggest that 5-MTHF transfer most likely represents diffusion.

INTRODUCTION

The mechanism of folate¹ absorption in man and experimental animal is not established. Both the paucity of

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Dr. Bertino is a recipient of a Career Development Award of the National Cancer Institute. Dr. Nixon was the recipient of a Merck, Sharp & Dohme International Fellowship in Clinical Pharmacology, and his present address is Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra City, Australia. Dr. Strum was a trainee of the National Cancer Institute (CA 05138), and his present address is Seymour Johnson A.F.B., N. C.

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¹Abbreviations used in this paper: DHF, dihydrofolic acid; FA, folic acid (pteroylmonoglutamate); FA poly-

experimental data and conflicting results have prevented an adequate understanding of the problem. Most previous studies have been devoted to the absorption of either folic acid (FA) or FA polyglutamates (1-10). However, the predominant circulating and storage forms of folate are neither FA nor FA polyglutamates but rather reduced forms of folate, principally 5-methyltetrahydrofolic acid (5-MTHF) (11). Moreover, the principal dietary folate compound is probably also 5-MTHF or polyglutamate forms of 5-MTHF (12). Therefore, understanding of folate absorption requires investigation of the absorption of 5-MTHF and 5-MTHF polyglutamates.

In this communication we report our studies of the absorption of 5-MTHF. The synthesis of the physiologically active diastereoisomer of radiolabeled 5-MTHF in our laboratory has facilitated these studies in the rat.

METHODS

Materials. FA,² labeled by tritium at the 3', 5', and 9' positions with a specific activity of 250 $\mu\text{Ci}/\mu\text{mole}$, L-alanine-¹⁴C³ and formaldehyde-¹⁴C³ were obtained commercially. The tritium-labeled physiologically active diastereoisomer of 5-MTHF was prepared as previously published (13). Briefly, tritium-labeled FA was reduced chemically to dihydrofolic acid (DHF) using the method of Blakley (14). The DHF was then enzymatically reduced to tetrahydrofolic acid (THF) by a highly purified dihydrofolate reductase (15). The N-5,10-methylene derivative was prepared from THF by the addition of formaldehyde, and reduced to 5-MTHF by sodium borohydride. 5-MTHF-¹⁴C was prepared in the same manner except unlabeled FA was used as the starting material, and formaldehyde-¹⁴C was added to introduce the label on the methyl group. The radiolabeled compounds were purified by column chromatography, first on A-25 DEAE-Sephadex then on Sephadex G-25. The purified ma-

glutamates, folic acid polyglutamates (usually 3- to 7-glutamates); 5-fTHF, 5-formyltetrahydrofolic acid; 10-fTHF, 10-formyltetrahydrofolic acid; 5-MTHF, 5-methyltetrahydrofolic acid; MTX, methotrexate; pABG, *p*-aminobenzoylglutamate; THF, tetrahydrofolic acid. Folate is used to refer to all folate compounds, both reduced and nonreduced.

²Amersham/Searle Corp., Des Plaines, Ill.

³New England Nuclear Corp., Boston, Mass.

terials had specific activities of either 250 $\mu\text{Ci}/\mu\text{mole}$ for ^3H or 50 $\mu\text{Ci}/\mu\text{mole}$ for ^{14}C and were greater than 99 and 95% radiochemically pure, respectively. The compounds were stored at -20°C as a solution with sodium ascorbate (3 mg/ml) at pH 6.0.

Identification of folate compounds by chromatography. Samples were desalted by chromatography (16) through a Sephadex G-15 column equilibrated with 0.1 M mercaptoethanol and eluted by the same solvent. Fractions, which contained radioactivity or folate markers, were pooled and applied to a 0.9×27 cm column of A-25 DEAE-Sephadex equilibrated with 0.1 M phosphate buffer, pH 6.0, containing 20 mM mercaptoethanol. Materials were eluted by phosphate buffer, pH 6.0, the concentration of which was increased linearly from 0.1 to 2.0 moles/liter, also containing 20 mM mercaptoethanol. The final concentration of 2.0 M phosphate buffer was obtained after passage of 500 ml of eluting buffer. Nonradioactive markers of 5-MTHF, FA, 5-formyl-tetrahydrofolic acid (5-fTHF), and *p*-aminobenzoyleglutamate (pABG) were added with the sample when applicable. Fractions of 2.5 ml were collected and monitored by radioactivity and by ultraviolet absorbance spectra.

Intestinal absorption studies in vitro. Absorption was studied by several methods: everted sacs (17), mucosal scrapings (18), and unidirectional transmural fluxes (19) in male, albino Sprague-Dawley rats weighing 250–350 g.

Everted gut sacs were prepared according to the method of Wilson and Wiseman (17), modified as previously described (20). The buffer system utilized consisted of 122 mM sodium chloride, 4.9 mM KCl, 26.5 mM sodium bicarbonate, and 1.2 mM potassium phosphate, pH 7.4. Each sac was approximately 10 cm long, and its wet weight was approximately 500 mg. The most proximal small intestinal segment was duodenum. The jejunal sacs were the next three segments; the ileal sacs were the three most distal segments. 1 ml of the buffer was placed inside the sac, which was then placed in a flask containing 5 ml of buffer solution and varying concentrations of 5-MTHF and approximately 1.5 μCi 5-MTHF- ^3H . In certain experiments the serosal fluid contained buffer only, and in other studies the serosal media had the same concentration of 5-MTHF as the mucosal solution. The flasks were gassed with $\text{O}_2\text{-CO}_2$ (95:5, v/v) and incubated at 37°C with continuous shaking for 50 min. In one series of experiments in which anaerobic incubation conditions were desired, nitrogen replaced the $\text{O}_2\text{-CO}_2$ gas mixture. At the end of the incubation period, each sac was removed, and the serosal fluid drained by gravity. The wet weight of each sac was determined. In those experiments in which 5-MTHF was not initially present in the serosal media, the serosal sample was diluted to 2.0 ml, and a portion was added to Bray's solution and counted in a Packard Tri-Carb three-channel liquid scintillation spectrometer with automatic external standardization. In these studies, 5-MTHF transfer, expressed in pg/50 min per 0.5 g wt tissue weight, is defined as the amount of 5-MTHF present in the serosal solution at the end of the incubation. In those experiments in which an equal concentration of 5-MTHF was present initially in both the mucosa and serosa solutions, portions of the final mucosal and serosal solutions were also counted. The mucosal to serosal ratio of 5-MTHF was then determined. The transport of 5 mM L-alanine was also determined in a series of experiments to demonstrate active transport in this preparation.

Mucosal scrapings were prepared for incubation as described by Schultz, Fuisz, and Curran (18) with 5-MTHF

concentrations of 0.5 and 50 nmoles/liter. The results were expressed as the ratio of 5-MTHF in intracellular fluid compared with its concentration in the extracellular media.

Unidirectional transmural fluxes of 5-MTHF were determined under open-circuited conditions using the methods and apparatus described by Schultz and Zalusky (19). Flat sheets of rat jejunum were mounted between Lucite chambers. 5-MTHF- ^3H was added to one bathing solution, and after a 40 min equilibration period, its rate of appearance in the opposite solution was determined. Mucosa to serosa and serosa to mucosa fluxes ($J_{m \rightarrow s}$, and $J_{s \rightarrow m}$, respectively) were determined at the same time on adjacent pieces of tissue from the same animal. Experiments to determine L-alanine unidirectional transmural fluxes were also performed (21). In all experiments both sides of the tissue was bathed with identical solutions maintained at 37°C and bubbled continuously with $\text{O}_2\text{-CO}_2$ (95:5, v/v). In this series of experiments the composition of the bathing solution in millmoles per liter was NaCl, 140; KHCO_3 , 10; K_2HPO_4 , 1.2; KH_2PO_4 , 0.6; CaCl_2 , 1.2; MgCl_2 , 1.2.

Statistics. Statistical significance was determined by the Student's *t* test (22).

RESULTS

Identification of transported folate compounds. When everted jejunal sacs were incubated in mucosal media containing 5-MTHF- ^3H , ^{14}C (500 nmoles/liter), the radiolabels were recovered unchanged from the serosal media after 60 min of incubation (Fig. 1), thus indicating no metabolic alteration of the original material occurred during the process of mucosal to serosal transfer.

Transport with no concentration gradient and mucosal scrapings. Everted sacs of duodenum, jejunum, and ileum were incubated with 50 min at 37°C with 5-MTHF on each side at equal concentration at 500, 50, 5.0, and 0.5 nmoles/liter, and at no time was the ratio of serosal to mucosal concentration greater than 1. An average of 12 experiments was performed at each concentration. Preliminary studies using identical methodology demonstrated that 5 mM L-alanine could be accumulated against a concentration gradient by jejunal mucosa. In studies using scrapings of jejunal mucosa incubated with 50 and 0.5 nM 5-MTHF, the ratio of intracellular to extracellular 5-MTHF concentration did not exceed unity.

Unidirectional transmural fluxes. At 5-MTHF concentrations of both 3000 and 3 nmoles/liter, there was no statistical difference between J_{ms} and J_{sm} (Table I), and the J_{net} present could be explained by a spontaneous transmural potential difference no more than 1.9 mv (mucosa negative).⁴ Preliminary studies demonstrated a net L-alanine flux of $0.56 \pm 0.12 \mu\text{moles/hr} \cdot \text{cm}^2$.

⁴ The unidirectional fluxes under open-circuited conditions were analyzed by the Ussing flux ratio equation (23):

$$\frac{J_{ms}}{J_{sm}} = \frac{a^m}{a^s} e^{ZF\Delta\psi/RT},$$

where J_{ms} and J_{sm} are the mucosal to serosal flux and serosal to mucosal flux, respectively; a^m and a^s are the

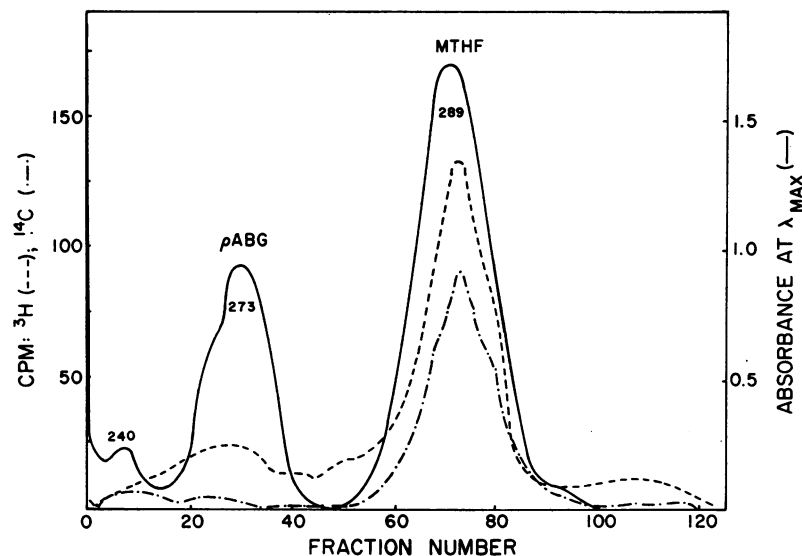


FIGURE 1 Chromatographic identification on DEAE-Sephadex (16) of folate compounds found in serosal media of everted jejunal sac incubated in mucosal media containing 5-MTHF- ^3H , ^{14}C . Essentially all radioactivity was identified in the 289 μm peak which cochromatographs with unlabeled 5-MTHF. 5-MTHF and pABG were added to the column as markers (see Methods).

All of the following everted sac experiments were performed with 5-MTHF present initially only in the mucosal media.

Transfer of 5-MTHF vs. time. Everted jejunal sacs containing 50 nM 5-MTHF in the mucosal media were incubated for 5, 15, 30, and 50 min, and an increase transfer of 5-MTHF was noted with increasing incubation time (Fig. 2).

5-MTHF transfer: location and variation with concentration. Transfer of 5-MTHF from mucosa to serosa at 0.05–50.0 $\mu\text{moles/liter}$ was quantitatively simi-

lar in everted sacs of duodenum, jejunum, and ileum (Fig. 3). The increase in transfer at 0.05 $\mu\text{mole/liter}$ in jejunal segments as compared with duodenal and ileal segments was not statistically significant. The effect of a 1000-fold increment in substrate concentration (0.05–50 $\mu\text{moles/liter}$) on transfer of 5-MTHF from mucosa to serosa of the duodenum, jejunum, and ileum was to produce a linear increase in the total amount transferred (Fig. 3).

Effect of modified incubation conditions on 5-MTHF transfer. Everted jejunal sacs were incubated in a mucosal media containing 50 nM 5-MTHF under anaerobic conditions, in a sodium-free medium, and in the presence of 10^{-4}M dinitrophenol. No significant decrease in mucosal to serosal transfer was observed as compared with paired controls. 500 nM 5-MTHF transfer in the presence of 10 mM glucose was similar to controls (Fig. 4).

Effect of folate analogues on 5-MTHF transfer. Everted jejunal sacs were incubated in a mucosal media containing 50 nM 5-MTHF and with 500 nM FA, 500 nM 5-fTHF, or 500 nM methotrexate (MTX). No significant decrease in mucosal to serosal transfer was noted in those tissues incubated with FA and MTX. A significant increase in transfer of 5-MTHF was noted during incubation with 5-fTHF ($P < 0.05$) (Fig. 5).

TABLE I
Transmural Fluxes of 5-MTHF*

Concentration nmoles/liter	J_{ms}	J_{sm}	J_{net}
3000	2944 \pm 170 (30)	2559 \pm 268 (21)	385 \pm 317
3	4.89 \pm 0.32 (40)	4.55 \pm 0.18 (29)	0.34 \pm 0.37

* Unidirectional transmural fluxes determined utilizing the method and apparatus of Schultz and Zalusky (19) as described in the text. 5-MTHF flux is expressed as 10^{-2} pmoles/hr \cdot cm 2 . The number of flux determinations is in parenthesis.

chemical activity of the ion in the mucosal and serosal media, respectively; z, F, R, and T are, respectively, the change of the ion, the Faraday, the gas constant, and absolute temperature; and $\Delta\psi$ is the electrical potential difference across the jejunum.

DISCUSSION

Previous investigations of folate absorption utilizing FA have yielded conflicting results: evidence of active

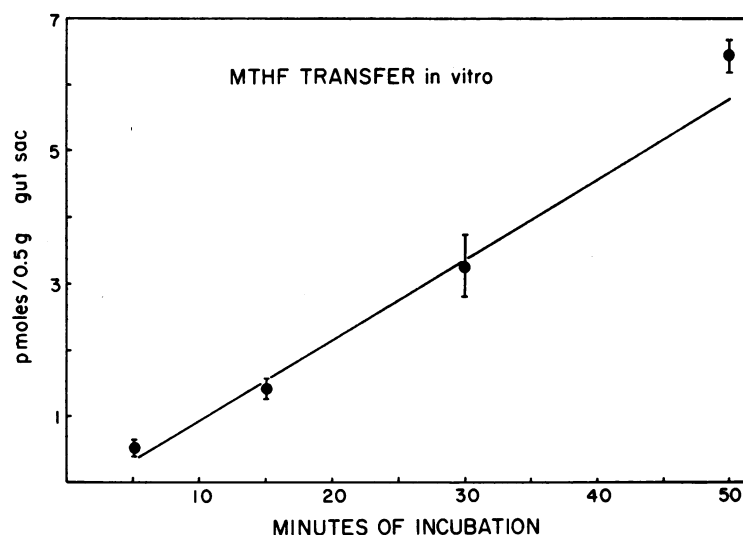


FIGURE 2 5-MTHF transfer from mucosa to serosa across an everted jejunal sac as a function of time. Three sacs from different animals were incubated at each time. See text for details.

transport or movement against the concentration gradient has (1, 4-7) and has not (2, 3, 8, 10) been obtained. Our evaluation of two of these studies (1, 7) suggest that the original conclusions indicating the presence of saturation kinetics may need modification. Cohen (5) originally suggested that the apparent accumulation of FA against a concentration gradient could be accounted for by the conversion of FA to reduced folate by hamster jejunum. Additional evidence of the reduction and methylation by FA by the intestinal mucosa has been observed in rat (24), and in man (9, 25), although reduction and methylation of FA by the human intestine has been disputed (26). Whether these differences are explained by species and concentration differences is not known, but the question requires further study. FA, however, is not the major dietary form of folate. Analysis of liver, pea seedlings, yeast, and milk, indicates that significant amounts of reduced folate, primarily 5-MTHF, are found (11, 27-29). Further, analytical methods would tend to underestimate the reduced folate forms, and some of these reduced forms may exist as polyglutamates (12).

Synthesis of highly purified, radiolabeled 5-MTHF in our laboratory has facilitated the study of the absorption of this physiologic folate compound, 5-MTHF (13). Intestinal absorption of 5-MTHF was studied in the rat by several different *in vitro* methods: everted intestinal sacs, mucosal scrapings, and unidirectional transmural fluxes. These investigations indicate that 5-MTHF is transferred unchanged across the rat small intestine and suggest that the mechanism of 5-MTHF absorption is a nonenergy dependent process. These

conclusions are supported by (a) the failure to demonstrate movement of 5-MTHF against a concentration gradient in the everted sac experiments, lack of accumulation of 5-MTHF within the intestinal mucosa in mucosal scraping, and no net flux of 5-MTHF in the unidirectional transmural flux studies; (b) the absence of saturation kinetics in that an increase in 5-MTHF mucosal to serosal transfer was linearly related to increased mucosal concentration of 5-MTHF; (c) the inability to inhibit the rate of 5-MTHF transfer with either nitrogen replacement or the addition of a metabolic inhibitor, dinitrophenol; and (d) the failure to inhibit 5-MTHF transfer significantly with folate analogues, FA and MTX. A preferential site of 5-MTHF absorption in the small intestine was not observed. Absorption was quantitatively similar in the duodenum, jejunum, and ileum.

These studies in the rat were performed *in vitro* and must be confirmed in man before our conclusions can be extended. Although it is conceivable that at lower 5-MTHF concentrations a carrier-mediated system might be identified, it is doubtful that folate concentrations below those used in these studies would have physiologic significance.

The stimulation of 5-MTHF transfer by 5-fTHF is unexplained. However, Goldman (30) recently reported that in L 1210 cells, the presence of 5-fTHF intracellularly will stimulate both 5-MTHF and MTX influx. He has suggested that this observation could be explained by counter flow or counter transport. It would be intriguing to speculate that a similar mechanism exists in the gut. However, our studies do not indicate the presence of a carrier-mediated system in the small intestine.

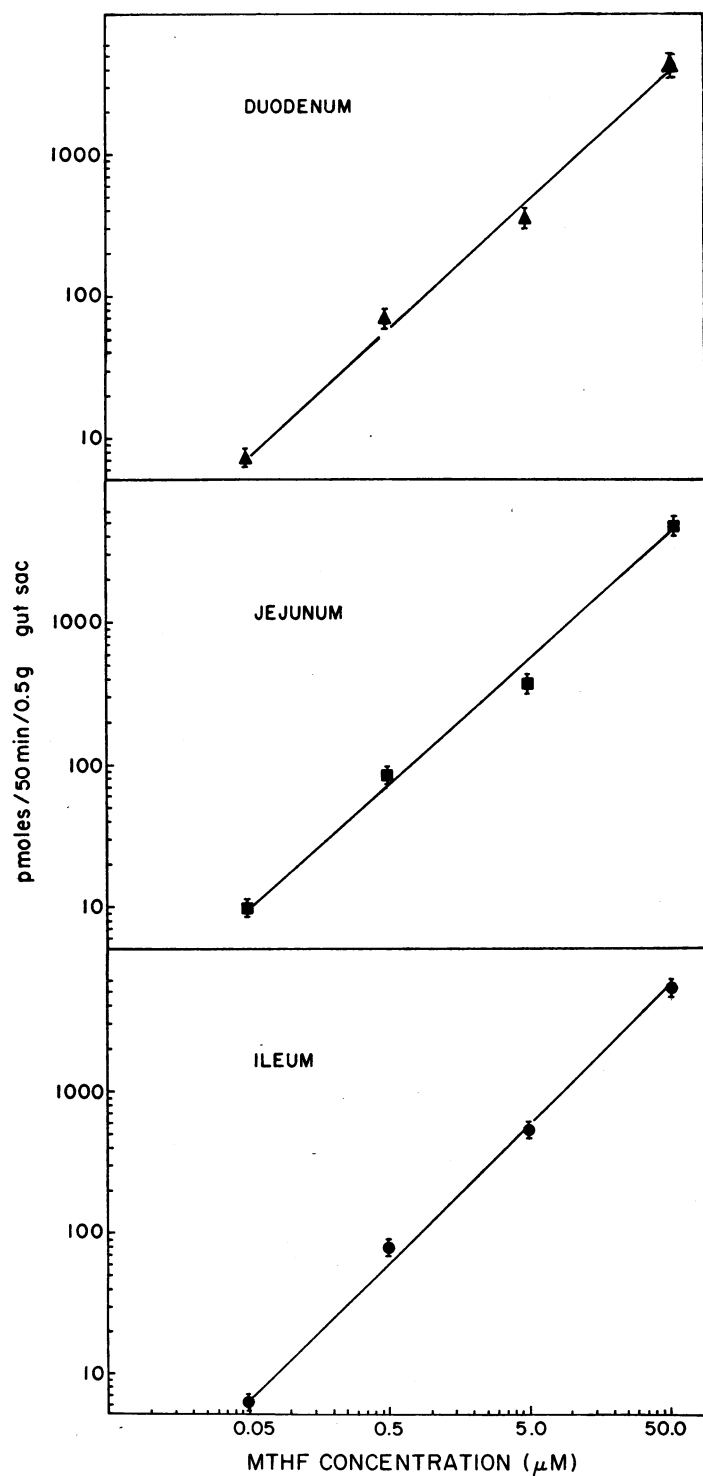


FIGURE 3 5-MTHF transfer across duodenal, jejunal, and ileal everted sacs as a function of increasing 5-MTHF concentration in the mucosal media. An average of nine sacs from each segment was studied at each concentration.

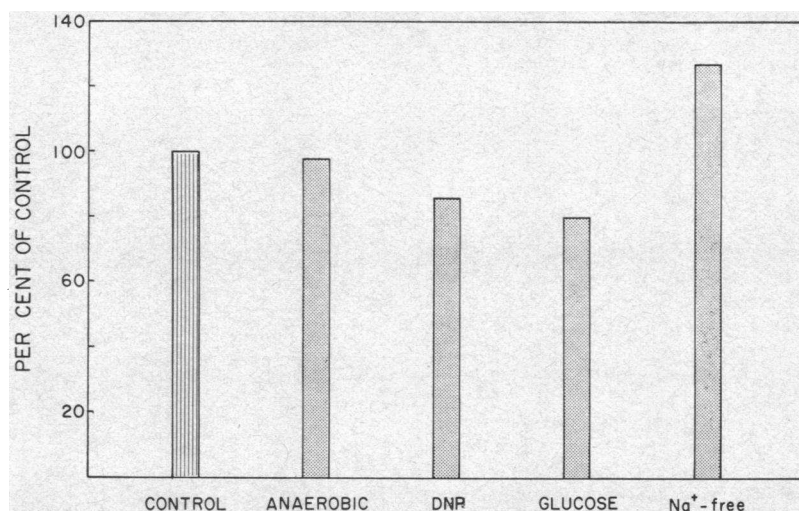


FIGURE 4 5-MTHF transfer was determined with several variations of the incubation conditions as described in the text. The differences observed between experiments and paired control studies were not significant.

Evidence to suggest carrier-mediated transfer of folate has been reported in L 5178Y leukemia cells (31), L 1210 leukemia cells (32, 33), lymphocytes (34), and across the blood brain barrier in dogs (35). FA transfer in platelets (36) and *Lactobacillus casei* (37) does not appear to be carrier mediated and, as previously noted,

conflicting evidence exists whether FA is actively transported in the small intestine.

Our studies do not indicate the existence of a carrier-mediated system but rather suggest that 5-MTHF mucosal transfer occurs by a nonenergy dependent non-saturable system.

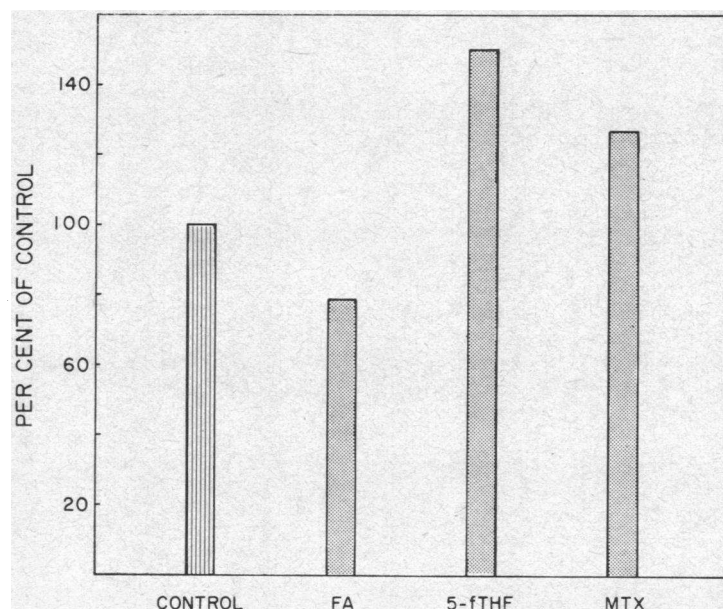


FIGURE 5 MTHF transfer was determined in the presence of various folate analogues as described in the text. In the presence of FA and MTX, the differences in 5-MTHF transfer were not significant from those of paired control studies. 5-MTHF transfer was significantly increased in the presence of 5-fTHF.

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