The Mechanism of 5-Methyltetrahydrofolate Transport by Human Erythrocytes

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ABSTRACT The mechanism involved in 5-methyltetrahydrofolate uptake by human cells is poorly understood. To more clearly elucidate this physiologically important process, transport of the vitamin was studied in human erythrocytes. 5-methyltetrahydrofolate uptake was found to increase with reticulocytosis, but measurable incorporation occurred in erythrocyte suspensions depleted of reticulocytes, leukocytes, and platelets, indicating uptake by mature erythrocytes. Incubation of erythrocytes with increasing concentrations of [^{14}C]5-methyltetrahydrofolate resulted in increasing uptake but decreasing percentage incorporation, consistent with saturation of a carrier system. Both influx and efflux phases of uptake were temperature dependent, with almost no transport at 4°C. Uptake of [^{14}C]5-methytetrahydrofolate was effectively inhibited by unlabeled 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and methotrexate, but not by pteroylglutamic acid. Prior incubation with 5-formyltetrahydrofolate increased uptake of [^{14}C]5-methyltetrahydrofolate, and extracellular 5-formyltetrahydrofolate enhanced efflux of [^{14}C]5-methyltetrahydrofolate. Nearly total depletion of ATP increased uptake of [^{14}C]5-methyltetrahydrofolate, but efflux was unchanged. Column chromatography of membrane-free hemolysate after incubation with [^{14}C]5-methyltetrahydrofolate showed 95% of radioactivity corresponded to marker radioisotope, and no other peak was noted.

Thus peripheral erythrocytes incorporate 5-methyltetrahydrofolate by a saturable, temperature-dependent, substrate-specific process which is influenced by counter-transport. This mechanism is qualitatively similar to the carrier-mediated transport of folate compounds previously described in other cell types. Therefore, human erythrocytes should be useful for detailed characterization of this membrane carrier system.

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INTRODUCTION

Mammalian cells incorporate folate compounds by specific transport mechanisms. 5-methyltetrahydrofolate (5-CH_3-H_4-folate),[1] 5-formyltetrahydrofolate (5-CHO-H_4-folate), and methotrexate (Mtx) apparently share one membrane carrier system, whereas the oxidized form of the vitamin, pteroylglutamic acid (PGA), is probably transported by a second carrier (1). Although 5-CH_3-H_4-folate transport has been well characterized in a number of experimental systems, relatively little is known about its uptake by human cells. Previous studies of human bone marrow cells (2) and of mitogen-stimulated lymphocytes (1, 3) suggest that these cells incorporate 5-CH_3-H_4-folate by processes similar to those in nonhuman cells, but such studies have been limited by difficulty in obtaining large numbers of cells for detailed characterization. Folate transport in mature human erythrocytes has not been extensively studied. We present evidence that mature erythrocytes transport-5-CH_3-H_4-folate by a carrier-mediated process similar to that found for folate and folate analogues in other normal and neoplastic cells (3–8).

METHODS

Subjects. Studies were performed with peripheral erythrocytes from normal volunteers, from patients with idiopathic aplastic anemia or chemotherapy-(Daunomycin-cytosine arabinoside) induced aplasia, and from patients with Coombs positive hemolytic anemia. Samples were obtained with the informed consent of the subjects.

Erythrocyte preparation. Heparinized (beef lung heparin, Upjohn Co., Kalamazoo, Mich.) peripheral blood was washed three times with phosphate-buffered saline (PBS) containing 5 mM phosphate, pH 7.2. In preliminary experiments, this concentration of phosphate did not decrease folate uptake compared to saline alone. The cells were resuspended in PBS

[1] *Abbreviations used in this paper*: 5-CH_3-H_4-folate, 5-methyltetrahydrofolate; 5-CHO-H_4-folate, 5-formyltetrahydrofolate; Mtx, methotrexate; PGA, pteroylglutamic acid; F/CN, sodium fluoride/sodium cyanide; PBS, phosphate-buffered saline; T_0, time zero.

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to a hematocrit of 50. In some experiments, leukocytes and platelets were removed by bead defibrination, Ficoll-Hypaque gradient, or sedimentation with 3% dextran for 1 h. Erythrocyte, leukocyte, and platelet counts were done with a Coulter Z; reticulocyte percentage was determined by standard procedures. The erythrocyte suspension was divided into 1-ml aliquots, and isotope added in 0.1 ml of 20 mM ascorbate. All determinations were done in duplicate or triplicate. Time zero (T_0) samples were immediately washed three times with cold PBS, lysed with 1 cm^3 of water, and stored at 4°C. Other aliquots were incubated for varying intervals at 37°C, then washed three times with PBS, and lysed with 1 cm^3 of water. Afterward, all samples were autoclaved for 15 min at 120°C, 15 lb pressure. After centrifugation at 800 *g* for 15 min, 0.5 cm^3 of clear supernate was mixed with toluene-Liquiflor-Biosolv, and radioactivity was determined in a scintillation counter. Mean counts per minute in T_0 samples were subtracted from mean counts per minute in incubated samples to adjust for nonspecific binding and trapping of radioactivity.

Chemicals. [^{14}C]5-Methyltetrahydrofolic acid, sp act 79–91 μCi/mg ([^{14}C]5-CH_3-H_4-folate), was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Radiochemical purity, assayed on an A-25 DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.) column, was 92%. Radioisotope position corresponded to 5-CH_3-H_4-folate in relation to marker compounds when monitored by fluorescence emission at 365 nm on excitation at 305 nm (9). This isotope is a racemic mixture of D and L forms. [G-^{3}H]folic acid ([^{3}H]PGA), sp act 11.3 mCi/mg, and [3′,5′,9(n)-3H]Methotrexate ([^{3}H]Mtx), sp act 13 mCi/mg, were also obtained from Amersham/Searle. Radiochemical purity was determined by column chromatography on Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) and DEAE-cellulose. Radioisotopes were dissolved in 20 mM ascorbic acid, pH 6.0, and stored frozen in the dark. 5-CHO-H_4-folate and Mtx were obtained from American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y. 5-CH_3-H_4-folate was obtained from Sigma Chemical Co., St. Louis, Mo.

Identification of accumulated material. To evaluate possible folate metabolism by peripheral erythrocytes, washed erythrocytes were incubated for 4 h at 37°C with [^{14}C]5-CH_3-H_4-folate. The cells were then washed three times with PBS and lysed by freeze thawing three times. The hemolysate was centrifuged at 9750 *g* for 15 min, the membrane-free supernate placed on a 0.9 × 50-cm column packed with Bio-Gel P-2 (Bio-Rad Laboratories), Sephadex G-25 or Sephadex G-100 (Pharmacia Fine Chemicals), and eluted with 0.1 M potassium phosphate buffer, pH 6.0. Membrane-free hemolysate, as well as clear supernate from original hemolysate after autoclaving to denature protein and conjugase, were also fractionated on a DEAE-cellulose column by a modification of the method of Corrocher et al. (10). A 0.9 × 30-cm column, packed with Whatman DE-52 (Whatman, Inc., Clifton, N. J.), was equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 0.2 M mercaptoethanol. Sample was applied to the column; 25 ml of starting buffer was followed by a linear gradient of increasing phosphate concentration from 200-ml volumes of 0.01 M and 0.7 M buffer. 2.5-ml fractions were collected. The column was calibrated with [^{14}C]5-CH_3-H_4-folate, which eluted at a buffer concentration comparable to that reported by others (11). Membrane-free hemolysate was fractionated on A-25 DEAE-Sephadex by the method previously cited (9). A 0.9 × 27-cm column was equilibrated with 0.1 M phosphate buffer, pH 6, containing 0.2 M mercaptoethanol. Sample and marker compounds (p-aminobenzoylglutamate, 5-CHO-H_4-folate, and tetrahydrofolate) were applied; 40 ml of starting buffer was followed by a gradient from 250-ml volumes of 0.1-M and 0.8-M phosphate buffers. 4.4-ml fractions were collected.

Counter transport. Erythrocytes were incubated for 1 h at 37°C in a shaker bath with either 60 μM 5-CHO-H_4-folate in 20 mM ascorbate-saline or ascorbate-saline alone. All cells were then washed three times with PBS, resuspended to 1 ml with PBS, and 2 μM [^{14}C]5-CH_3-H_4-folate added. After 90 min, control cells (those incubated with ascorbate-saline alone) were washed three times with PBS and resuspended in either fresh PBS alone or PBS containing 60 μM 5-CHO-H_4-folate.

Studies of energy depletion effect. Washed erythrocytes suspended in PBS were incubated for 4 h at 37°C with or without 10 mM sodium fluoride/4 mM sodium cyanide (F/CN) obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J. Subsequently the cells were again washed three times with PBS and resuspended in fresh PBS. ATP was measured by a modification of the method of Brewer and Powell (12).

RESULTS

After 90 min incubation with 10 μM [^{14}C]5-CH_3-H_4-folate, uptake by washed erythrocytes from 15 normal individuals was 6.86±1.71 pmol/10^9 cells. This uptake was by erythrocytes, rather than other formed elements, because uptake in samples from nine patients with severe aplasia (no reticulocytes seen after new methylene blue stain, white count less than 1,000/mm^3, and platelet count less than 10,000/mm^3) was not significantly different (Fig. 1). Furthermore, addition of leukocytes, or sequential depletion of leukocytes and platelets by Ficoll-Hypaque gradient and then dextran sedimentation, had no significant effect on folate uptake. Erythrocyte uptake increased with increasing reticulocytosis, but extrapolation of a line fit by the method of least squares (13) intercepted the ordinate at an uptake comparable to the measured uptake in samples with normal or few reticulocytes (Fig. 1). This intercept was statistically (14) significantly greater than zero ($P < 0.005$). Column chromatography of membrane-free or autoclaved hemolysate showed that more than 95% of the radioactivity corresponded in position to marker [^{14}C]5-CH_3-H_4-folate, and no other peak of

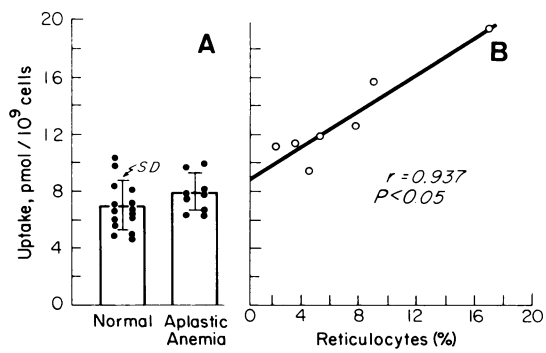

FIGURE 1 [^{14}C]5-CH_3-H_4-folate uptake in erythrocyte suspensions of varied leukocyte, platelet, and reticulocyte content. (A), uptake, in pmol/10^9 cells, by erythrocytes from normal individuals and patients with severe aplastic anemia. Bars represent the mean and brackets the SD. (B), relationship between uptake and percent reticulocytes.

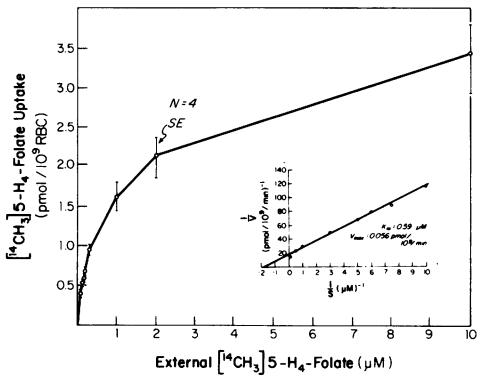

FIGURE 2 Saturable [^{14}C]5-CH_3-H_4-folate uptake by erythrocytes. Relationship between uptake, in pmol/10^9 cells, and extracellular concentration. Each point represents the mean of four experiments utilizing different erythrocyte (RBC) suspensions and brackets the SE of the mean. In the reciprocal plot (insert) the line was fitted by the method of least squares.

radioactivity was noted. Analysis of fractions from the DEAE-Sephadex column by fluorescent emission showed that radioactivity corresponded to 5-CH_3-H_4-folate in relation to marker compounds.

Uptake kinetics for 5-CH_3-H_4-folate. When erythrocytes from normal individuals, in four separate experiments, were incubated for 45 min with increasing concentrations of [^{14}C]5-CH_3-H_4-folate, the relationship between uptake and extracellular concentration was not linear (Fig. 2). In preliminary experiments, uptake was measured at 30, 45, and 60 min and found to be linear during this period for the entire range of concentrations (not shown). A Lineweaver-Burk plot of this data (15), with a straight line fit ($r = 0.99$) by the method of least squares, produced a K_m of 0.59 μM and a V_{max} of 0.056 pmol/10^9 cells per min (insert, Fig. 2).

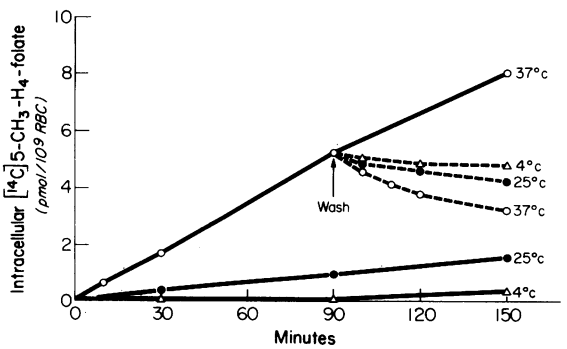

FIGURE 3 Temperature dependence of [^{14}C]5-CH_3-H_4-folate uptake and efflux in erythrocytes. Solid lines represent uptake at 4°C (△), 25°C (●), and 37°C (○). At 90 min, aliquots from the 37°C incubate were washed three times and resuspended in folate-free PBS. Broken lines represent efflux at 4°C (△), 25°C (●), and 37°C (○). Each point is the mean of three experiments.

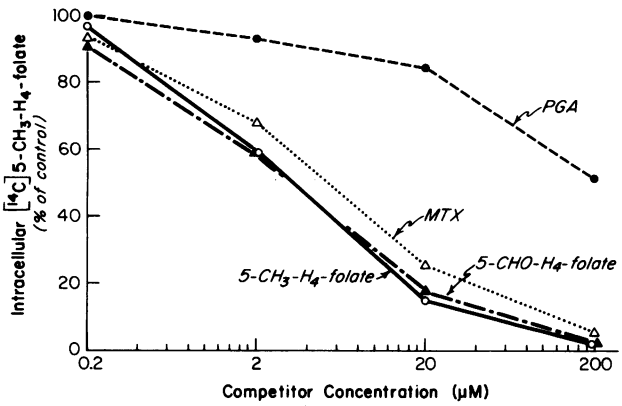

FIGURE 4 Inhibition of [^{14}C]5-CH_3-H_4-folate erythrocyte uptake by folate analogues. Erythrocytes were incubated with 2 μM [^{14}C]5-CH_3-H_4-folate and increasing concentrations of unlabeled 5-CH_3-H_4-folate (○ —— ○), 5-CHO-H_4-folate (▲ - - -▲), Mtx (△ - - - △), and PGA (● - - - ●). Each point is the mean of four experiments. Effective inhibition occurs with reduced folates (5-CH_3-H_4-folate and 5-CHO-H_4-folate) and with Mtx, but not with the oxidized folate, PGA.

Temperature sensitivity. [^{14}C]5-CH_3-H_4-folate uptake was decreased in cells incubated at 25°C and almost completely inhibited at 4°C, compared with uptake by cells at 37°C. Similarly, efflux of radioactivity was temperature dependent. Cells incubated for 90 min at 37°C with 2 μM [^{14}C]5-CH_3-H_4-folate were washed three times, resuspended in folate-free PBS, and incubated at varying temperatures. Efflux of radioactivity was greatest at 37°C, less at 25°C, and least at 4°C in three such experiments (Fig. 3).

Inhibition by other folate compounds. Erythrocytes were incubated for 90 min with 2 μM [^{14}C]5-CH_3-H_4-folate and increasing concentrations of unlabeled folate compounds. Mtx and reduced folates (5-CH_3-H_4-folate and 5-CHO-H_4-folate) effectively inhibited [^{14}C]5-CH_3-H_4-folate uptake whereas inhibition by PGA was only partial (Fig. 4).

Comparative uptake of folate compounds. Incubation of erythrocytes with equal amounts (0.1 μM) of [^{14}C]5-CH_3-H_4-folate, [^{3}H]Mtx, or [^{3}H]PGA showed that uptake of [^{14}C]5-CH_3-H_4-folate increased progressively with time, but the rate of uptake was most rapid during the first 60 min. After 150 min, uptake was ≅1 pmol/10^9 cells. Uptake of [^{3}H]Mtx increased at a lower rate for at least 240 min, when uptake was ≅0.3 pmol/10^9 cells. Uptake of [^{3}H]PGA was unchanged after 30 min at ≅0.04 pmol/10^9 cells (Fig. 5).

Counter-transport. Uptake of [^{14}C]5-CH_3-H_4-folate was enhanced in cells previously incubated with 5-CHO-H_4-folate, and efflux of radioactivity was greatly increased by extracellular 5-CHO-H_4-folate (Fig. 6).

Effect of energy depletion on uptake. After 4 h incubation, ATP levels in control cells slightly dropped to 3.27±1.21 μM/g hemoglobin (ATP in normal fresh

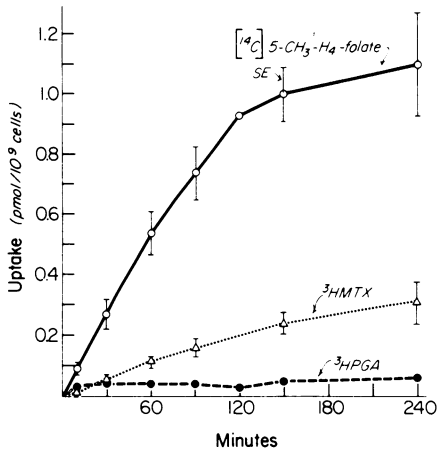

FIGURE 5 Comparative uptake of folate analogues by erythrocytes. Erythrocytes were incubated with 0.1 μM of [^{14}C]5-CH_3-H_4-folate (○ —— ○), [^{3}H]Mtx (△ - - - △), or [^{3}H]PGA (● - - - ●). Each point is the mean of duplicate assays in three separate experiments utilizing different erythrocyte (RBC) suspensions; brackets represent SE of the mean.

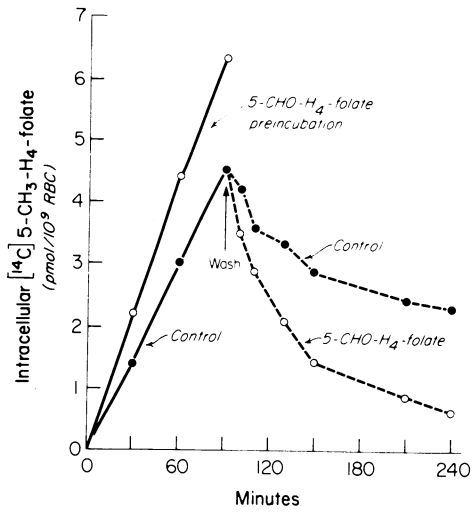

FIGURE 6 Stimulation of [^{14}C]5-CH_3-H_4-folate uptake and efflux by counter-transport. Uptake of [^{14}C]5-CH_3-H_4-folate is increased in erythrocytes incubated with 5-CHO-H_4-folate (○ —— ○) compared to control cells (● —— ●). When cells containing [^{14}C]5-CH_3-H_4-folate were washed (↑) and resuspended in folate-free PBS (● - - - ●) or PBS containing 5-CHO-H_4-folate (○ - - - ○), efflux of radioactivity was enhanced by the presence of 5-CHO-H_4-folate.

erythrocytes by this method is 3.79±0.70 μM/g hemoglobin) whereas incubation with F/CN almost totally depleted ATP (0.28±0.14 μM/g hemoglobin).

When energy-replete and -depleted cells were incubated for 90 min at 37°C with increasing concentrations of [^{14}C]5-CH_3-H_4-folate, uptake was consistently higher in the ATP-depleted cells at all concentrations tested. Statistical analysis with the paired t test (16) showed this difference was significant for all concentrations tested ($P < 0.01$ for 0.2 μM [^{14}C]5-CH_3-H_4-folate or higher; $P < 0.05$ for lower concentrations). The kinetic curve resembled that of control cells in that both deviated from linearity, consistent with saturation (Fig. 7). A double-reciprocal plot of this data (15) produced a K_m of 0.75 μM and a V_{max} of 0.069 pmol/10^9 cells per min for control cells, and a K_m of 0.80 μM and a V_{max} of 0.094 pmol/10^9 cells per min for F/CN-treated cells. These differences were statistically significant for V_{max} ($P < 0.001$) but not for K_m ($P > 0.3$) (16).

The effect of energy depletion on [^{14}C]5-CH_3-H_4-folate efflux was measured in control and energy-depleted cells containing approximately equal concentrations of [^{14}C]5-CH_3-H_4-folate. Control cells were incubated for 90 min with 2 μM of radioisotope, and F/CN-treated cells were incubated for 80 min with 1 μM [^{14}C]5-CH_3-H_4-folate. The cells were then washed; some samples from each group were hemolysed, and the remaining cells were resuspended in fresh PBS (control) or PBS and F/CN (energy-depleted). Subsequently, cells were harvested after 10, 20, 30, 60, 120, and 160 min of incubation at 37°C. Neither initial concentration ($P > 0.4$) nor rate constants ($P > 0.1$) differed significantly in the two groups when tested by the paired t test (16). The observed efflux rate constants±1 SD for control cells was 0.036±0.003 pmol/10^9 cells per min; for F/CN-treated cells was 0.031 ±0.008 pmol/10^9 cells per min (Fig. 8).

DISCUSSION

These studies indicate that human peripheral erythrocytes transport, but do not metabolize, 5-CH_3-H_4-folate

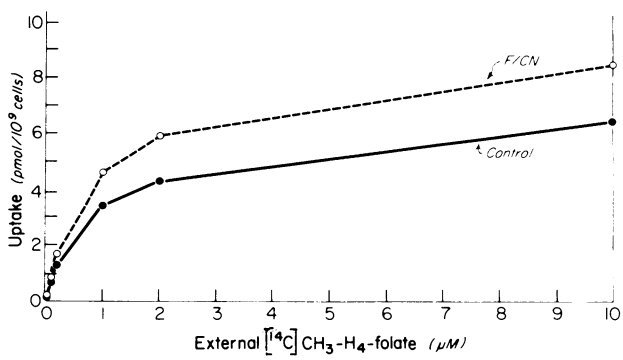

FIGURE 7 Effect of erythrocyte ATP depletion on [^{14}C]5-CH_3-H_4-folate uptake. Erythrocytes after 4 h in PBS (● —— ●) or in PBS plus 10 mM sodium fluoride/4 nM sodium cyanide (○ - - - ○) were washed, then incubated for 90 min with increasing concentrations of [^{14}C]5-CH_3-H_4-folate. Each point represents the mean of six experiments. The difference between uptake by control and F/CN-treated cells is statistically significant at all concentrations tested.

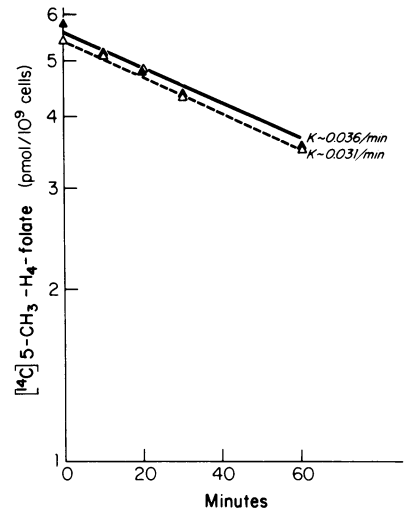

FIGURE 8 Release of [^{14}C]5-CH_3-H_4-folate from control and energy-depleted cells. When loaded with approximately equal amounts of radioisotope, as described in the text, rate constants of efflux (K) from control (▲ —— ▲) and F/CN-treated (△ - - - △) cells are nearly identical. Each triangle represents the mean of six experiments, done in triplicate. Lines were fit by the method of least squares.

under the conditions of these experiments, and that uptake represents membrane transport function independent of intracellular folate metabolism. Uptake of 5-CH_3-H_4-folate was by erythrocytes, rather than other formed elements, because uptake did not change after addition or depletion of leukocytes and platelets, and was nearly identical in erythrocytes from normal individuals and in cells from patients with profound leukopenia, reticulocytopenia, and thrombocytopenia. Although the possibility must be considered that the observed uptake is the result of the few remaining reticulocytes (4, 17), extrapolation of the linear relationship between uptake and reticulocyte count to zero reticulocytes predicts an uptake by mature erythrocytes comparable to our measured values. Thus, uptake in these studies is probably not significantly influenced by the small number of reticulocytes present.

The mechanism of erythrocyte folate uptake appears to involve a membrane-carrier system, because uptake is saturable, temperature dependent, substrate specific, and influenced by counter transport (18). Like the carrier systems previously described in L-1210 leukemia cells (8, 19) and in stimulated lymphocytes (3), this transport system has markedly different relative affinities for folate compounds, consistent with the presence of two carrier systems in erythrocytes as well, one specific for reduced folates and Mtx, and a second for PGA (1). These differences in carrier affinity for folate analogues are also consistent with prior observations that there is no measurable increase of erythrocyte folate after injection of PGA or [^{3}H]PGA into animals or man (20, 21), and that incubation of erythrocytes with [^{3}H]-PGA in vitro results in only low levels of uptake (17), whereas there is significant erythrocyte incorporation of [^{14}C]5-CH_3-H_4-folate in vitro (22). The affinity of the erythrocyte transport system for 5-CH_3-H_4-folate approximates that reported in other experimental systems; our observed K_m of 0.6 μM is comparable to K_t's of 1 μM in L-1210 leukemia cells (19), 0.9 μM in hog choroid plexus (6), and 0.9 μM in *Lactobacillus casei* (23). This K_m for erythrocytes must be considered at present to be only an approximation, however, because the stereospecificity of the transport mechanism is unknown. The radioisotope used in these experiments was a mixture of D and L stereoisomers. If only the biologically active form (+) L-CH_3-H_4-folate is transported, the true K_m may be lower than our measured value.

Although these characteristics of the folate uptake system in erythrocytes, consistent with a carrier system, are similar to those in other experimental systems (3, 19, 24), human erythrocytes appear to incorporate less 5-CH_3-H_4-folate during incubations with the vitamin than do reticulocytes, bone marrow cells (2), or stimulated lymphocytes (1, 3). This quantitative difference may result from loss, with maturation, of transport systems (25), or may reflect the absence of folate utilization by mature erythrocytes. We favor the latter possibility because folate uptake in other cell types is clearly related to metabolic activity (3, 23, 26).

Uptake of 5-CH_3-H_4-folate by human erythrocytes increases after ATP depletion, thereby resembling Mtx uptake by rabbit reticulocytes (4) and L-1210 leukemia cells (19, 27). However, in contrast to these experimental systems, wherein metabolic poisons apparently inhibit an energy-dependent efflux mechanism without altering influx (resulting in a net increase of uptake) (4, 19), we found efflux rate constants in control and F/CN-treated human erythrocytes were nearly identical. Other possible explanations for the consistent increase of uptake after energy depletion were also considered and excluded. For example, passive diffusion was not increased after F/CN treatment because the slope of the relationship between 5-CH_3-H_4-folate uptake and extracellular 5-CH_3-H_4-folate concentration at high levels was the same in control and energy-depleted cells. Swelling of the cells after energy depletion, with a concomitant increase in surface area of membrane available for diffusion, also is not a likely cause of the observed increase in uptake; if uptake were standardized by hematocrit rather than by cell number, the increment after energy depletion remained, and mean corpuscular volume actually decreased slightly rather than increased. Therefore these studies, while showing that 5-CH_3-H_4-folate uptake can occur in the absence of ATP, do not provide an explanation for the enhanced uptake by energy-depleted erythrocytes.

The physiologic significance of erythrocyte folate uptake is not clear. Erythrocyte folate is largely polyglutamates of 5-CH_3-H_4-folate (28), and its function, if any, is unknown. Under the conditions of these experiments, incorporated folate was not conjugated and no transfer of label to other compounds was detected. In cells actively utilizing folate, 5-CH_3-H_4-folate is rapidly and almost completely demethylated to tetrahydrofolate with production of methionine (29). Significant labeling of this low molecular weight compound should have been detected in our system, but was not, suggesting little or none was formed. Therefore, although peripheral erythrocytes retain the capacity to transport folate, this newly incorporated vitamin probably does not have a major role in erythrocyte metabolism. Further investigations of erythrocyte folate uptake seem warranted, however, because membrane transport function can be studied directly, and quantities of these cells are readily available for detailed characterization of the membrane-carrier system.

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