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Effects of pH on 5-methyltetrahydrofolic acid transport in human erythrocytes

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The effect of pH on membrane transport of folate compounds has been studied, but the results have been conflicting. For example, the optimal pH for uptake of pteroylglutamic acid by rat jejuneum was found to be 6.0 [1] to 6.3 [2], and incorporation of pteroylglutamic acid and methotrexate by brush border membrane vesicles from rat intestine was maximum at around 5.0 [3]. In contrast, the optimal pH for uptake of pteroylglutamic acid by human bone marrow was 7.4 [4], while a broad pH optimum between 4.5 and 7.5 was observed for *Lactobacillus casei* [5]. In human leukocytes, the transport of amethropterin was found to be directly proportional to pH [6], but in Sarcoma-180 cells amethopterin uptake was inversely proportional to the pH of the medium [7]. These diverse results undoubtedly reflect, at least in part, the use of different experimental conditions. In addition, it has been appreciated only recently that most anionic buffers themselves influence the transport of folate compounds, thereby necessitating the use of zwitterionic buffers for studies of pH effect [8].

The relationship between pH and folate compound permeation is of interest because it may provide insights into the mechanism of transport. Studies in a variety of experimental systems, including the human erythrocyte, have established that folate compounds are transferred across the plasma membrane by a saturable, temperature-dependent, concentrative, substrate-specific process which is influenced by heteroexchange [9]. Since folate compounds have pK values of less than 5 (the pK values of the carboxyl groups in *p*-aminobenzoylglutamic acid are 3.76 and 4.83), these compounds should be almost completely dissociated at physiologic pH [7]. Consequently, it seems possible that the folate ion, like other organic anions, may undergo co-transport with H^+ or exchange with OH^- across the membrane. If this is the case, permeation of folate compounds could be expected to be strongly pH dependent. To test this possibility, influx, efflux and steady-state levels of 5-methyltetrahydrofolic acid ($^{14}CH_3H_4PteGlu_1$) were measured in human erythrocytes suspended in media of different pH values.

Human peripheral blood was obtained from normal volunteers and depleted of white cells and platelets by dextran sedimentation as previously described [9]. The erythrocytes were washed three times with the appropriate buffer and resuspended to a hematocrit of approximately 20. The erythrocyte suspension was divided into 1 ml aliquots, and isotope was added in 0.1 ml volumes containing 20 mM ascorbate. All determinations were done in triplicate. Red cell counts were performed with a Coulter z.

Initial rates of uptake were measured by incubating red cells with the appropriate concentration of radioisotope for 45 min at 37°. In previous studies we found that uptake was linear during this period for the entire range of concentrations [9]. Steady-state levels were determined after a 4- to 6-hr incubation at 37° with 30 nM $^{14}CH_3H_4PteGlu_1$. Extracellular volume, as measured by [^{3}H]inulin concentration, does not change during this incubation period [10]. Efflux was measured by incubating washed erythrocytes in phosphate-buffered saline, pH 7.2, containing 5 mM phosphate, with 2 μM $^{14}CH_3H_4PteGlu_1$ for 90 min at 37°. After washing, the red cells were resuspended to hematocrit 20 in the appropriate buffers and divided into 1 ml aliquots. Levels of incorporated radioactivity were determined as previously described, except that protein was precipitated by the addition of 0.1 ml of 100% trichloroacetic acid to the hemolysate rather than by autoclaving [9, 11].

The pH of the erythrocyte suspension was measured before and after the incubation period and never varied by more than 0.1 unit from the indicated pH.

$^{14}CH_3H_4PteGlu_1$ (sp. act. 79–91 μCi/mg) was obtained from the Amersham/Searle Corp. (Arlington Heights, IL). Radiochemical purity was assayed by column chromatography as previously described [9].

The relationship between extracellular pH and initial

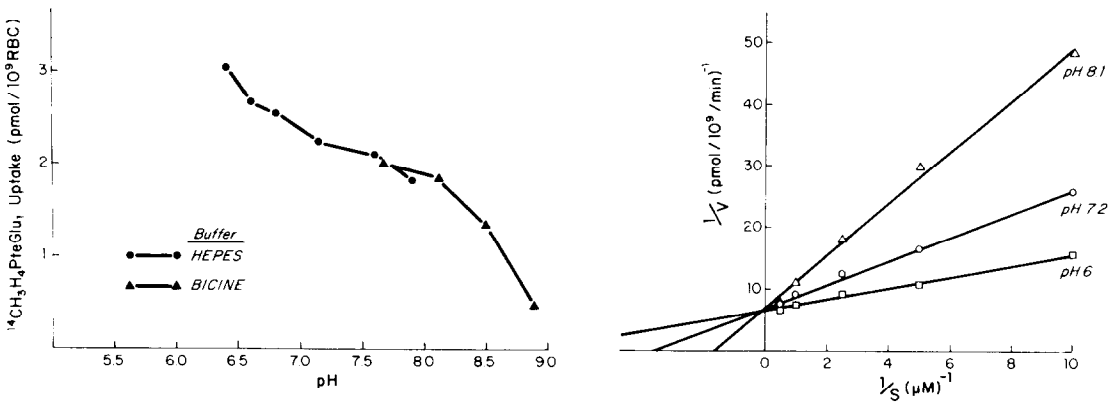

Fig. 1. Effect of extracellular pH on initial rates of $^{14}CH_3H_4PteGlu_1$ uptake. Left panel: Washed red cells were incubated for 45 min with 0.2 μM $^{14}CH_3H_4PteGlu_1$ in either 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)–100 mM NaCl or 50 mM bicine–100 mM NaCl at the indicated pH. Each point is the mean of triplicate determinations. Right panel: Double-reciprocal plot of $^{14}CH_3H_4PteGlu_1$ uptake in 50 mM HEPES–100 mM NaCl at three different external pH values. Each point is the mean of two experiments, each done in triplicate. The lines were fit by the method of least squares.

rates of $^{14}CH_3H_4PteGlu_1$ uptake by human erythrocytes is shown in Fig. 1. Using zwitterionic buffers to cover the pH range 6.4 to 8.9, we found that initial rates of uptake increased with decreasing pH. A similar inverse relationship of influx and pH has been described for other red cell organic anion transport systems (specifically lactate and β-hydroxybutyrate) and for methotrexate uptake by Sarcoma-180 cells [7, 12–14]. Uptake of $^{14}CH_3H_4PteGlu_1$ at pH 6, 7.2 and 8.1 was a saturable process exhibiting Michaelis–Menten kinetics (Fig. 1, right). The affinity for $^{14}CH_3H_4PteGlu_1$ changed with the external pH: the apparent K_m values at pH 6, 7.2 and 8.1 were 0.14, 0.25 and 0.63 μM respectively. In contrast, V_{max} (0.15 pmole/per 10^9 red cells per min) was unchanged by external pH. This influence of pH on K_m but not V_{max} is also analogous to the findings of others for lactate and β-hydroxybutyrate permeation [13, 14].

The effect of external pH on $^{14}CH_3H_4PteGlu_1$ efflux is indicated in Fig. 2. Efflux was strongly pH dependent and increased with increasing external pH. Thus, external pH had opposite effects on influx and efflux. The efflux rate constants at 6, 7.5 and 8.5 were 0.034, 0.059 and 0.067/min respectively.

From the effects of pH on $^{14}CH_3H_4PteGlu_1$ influx and efflux described above, one would deduce that steady-state levels of the vitamin should increase with decreasing pH. This prediction is confirmed by the experiments represented in Fig. 3. Net uptake of $^{14}CH_3H_4PteGlu_1$ at pH 6 is more than twice that at pH 8.5.

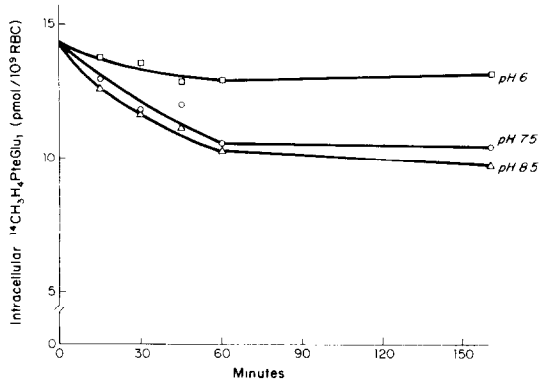

Fig. 2. Dependence of folate efflux on pH. Washed erythrocytes were incubated in phosphate-buffered saline with 2 μM $^{14}CH_3H_4PteGlu_1$ for 90 min. After washing, the red cells were resuspended in either 50 mM HEPES–100 mM NaCl (pH 6 and 7.5) or in 50 mM bicine–100 mM NaCl (pH 8.5). Each point is the mean of four experiments, each done in triplicate.

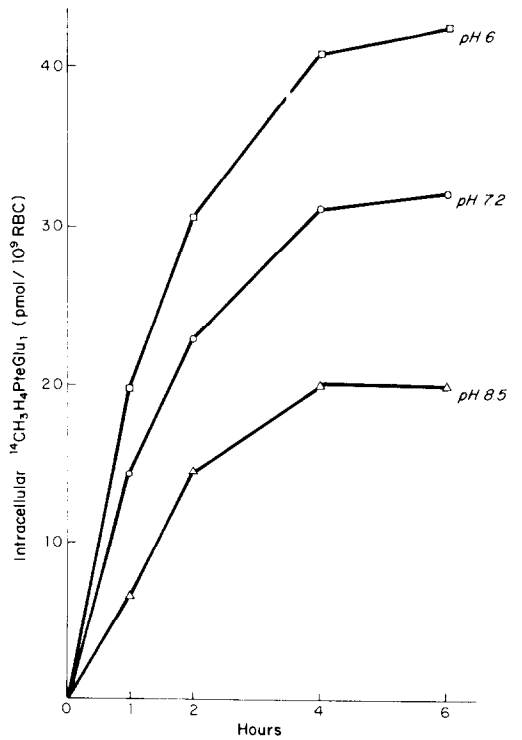

Fig. 3. Effect of external pH on net $^{14}CH_3H_4PteGlu_1$ uptake. Red cells were suspended in either 50 mM HEPES–100 mM NaCl (pH 6 and 7.2) or in 50 mM bicine–100 mM NaCl (pH 8.5) and incubated with 30 nM $^{14}CH_3H_4PteGlu_1$. Each point is the mean of triplicate determinations.

These findings suggest that the direction of a pH gradient across the membrane is an important factor influencing the direction of net folate flux. It seems likely, therefore, that $^{14}CH_3H_4PteGlu_1$ permeates through the membrane by a process that does not translocate electric charge. This postulated mechanism of coupled H^+ copermeation or OH^- exchange is analogous to lactate and β-hydroxybutyrate transfer [13–15] and indicates further similarities between these erythrocyte membrane transport systems. Previous studies in our laboratory showed that $^{14}CH_3H_4PteGlu_1$ transfer, like lactate transport, is sensitive to sulfhydryl reagents and amino reactive agents but is resistant to proteolytic enzymes [10]. We also found that several amphipathic drugs known to inhibit anion transport, such as ethacrynic acid, sulfinpyrazone and dipyridamole, also impair folate transport [16]. These findings, taken together with the present studies, indicate that the red cell membrane carrier systems for folate compounds and for other, smaller organic anions are remarkably similar.

In summary, we measured the effects of external pH on influx, efflux and net steady-state levels of $^{14}CH_3H_4PteGlu_1$. Initial rates of uptake were inversely proportional to external pH. Lowering the pH of the suspending medium increased influx by enhancing the affinity of the carrier: the apparent K_m values at pH 6, 7.2 and 8.1 were 0.14, 0.25 and 0.63 μM respectively. In contrast, V_{max} was independent of external pH. Efflux rate constants at pH 6, 7.5 and 8.5 were 0.034, 0.059 and 0.067/min respectively. Consequently, lowering the external pH increased steady-state levels of $^{14}CH_3H_4PteGlu_1$.

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Distribution of pepstatin and statine following oral and intravenous administration in rats. Tissue localisation by whole body autoradiography

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Pepstatin A is a potent inhibitor of pepsin activity with a K_i of the order of 10^{-11} M, and is one of several low molecular weight protease inhibitors isolated by Umezawa *et al.* [1] from the culture filtrates of various strains of *Streptomyces* (Fig. 1). In addition to pepsin, pepstatin A also inhibits many other acid proteinases, in particular renin (K_i 10^{-7} M) and cathepsin D (K_i 5×10^{-10} M). It has been advocated as a possible chemotherapeutic agent for the treatment of duodenal ulceration [2], hypertension [3] and inflammation [4]. We have used whole body autoradiography to compare the distribution *in vivo* of the relatively insoluble radiolabelled analogue, pepstatinyl-[^{14}C]glycine, which has a similar K_i value to pepstatin A [5], with that of the soluble derivative [^{3}H]acetylstatine which also inhibits pepsin but has a K_i value 10^6-fold less than pepstatin A [6].

Materials and methods

Pepstatin A was a kind gift from Dr. Jan Muller (H. Lundbeck & Co., Copenhagen-Valby, Denmark). [1-^{14}C]Glycine (40 mCi/mmole) and [^{3}H]acetic anhydride (10 Ci/mmole) were from Amersham International (Amersham, U.K.). Cremophor EL (polyoxyethylated castor oil) was from Blagdon, Campbell Chemicals Ltd. (Croydon, U.K.).

Pepstatinyl-[^{14}C]glycine was prepared by the method of Knight and Barrett [5]. The specific radioactivity of pepstatinyl-[^{14}C]glycine (15.5 mg) was estimated to be 5 mCi/mmole. An R_f value of 0.8 was obtained for pepstatinyl-[^{14}C]glycine by thin-layer chromatography [1] and this agreed closely with a value of 0.76 for unmodified pepstatin A [1]. The radiolabelled analogue was homogeneous since [1-^{14}C]glycine did not move from the origin