Delivery of Folates to the Cytoplasm of MA104 Cells Is Mediated by a Surface Membrane Receptor That Recycles*

(Received for publication, January 20, 1988)

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MA104 cells, as well as several other rapidly dividing tissue culture cells, have a folate-binding protein associated with their cell surface. The protein has the properties of a membrane receptor: (a) 5-methyl[3H]tetrahydrofolic acid binds with high affinity (Kd ≈ 5 nM); (b) the protein is an integral membrane protein; (c) it appears to deliver physiological concentrations of 5-methyl[3H]tetrahydrofolic acid to the inside of the cell; (d) binding activity is regulated by the concentration of folate within the cell. To better understand the mechanism of action of this receptor, we have studied the pathway of folate internalization. We present evidence that during internalization: (a) folate binds to the membrane receptor; (b) the ligand-receptor complex moves into the cell; (c) the ligand is released from the receptor in an acidic intracellular compartment and moves into the cytoplasm; and (d) the unoccupied receptor returns to the cell surface.

The plasma membrane presents a formidable barrier to the movement of charged molecules, macromolecules, and particulate material from the extracellular space into the cell. Important metabolic ions and molecules that are taken up by cells depend upon specific transport systems present at the plasma membrane. These transport systems fall into two general categories: (a) membrane channels or carriers that vectorially move specific ions and low molecular weight solutes (1) and (b) endocytic vesicles that internalize macromolecules and particulate material (2). Each system utilizes a specific set of membrane proteins that bind with high affinity and specificity the molecule or ion that is being transported. For each type of transport activity, there is only a rudimentary understanding of the cellular organelles involved in the process.

One class of charged molecules that animal cells must obtain from their environment are the folates (3). Tetrahydrofolic acid is the fully reduced physiological form of folate utilized by cells for the biosynthesis of methionine, serine, deoxymethylidic acid, and purines (3). Cells obtain this folate from 5-methyltetrahydrofolic acid, which crosses the plasma membrane and enters the cell cytoplasm to donate the 5-methyl group to homocysteine during methionine synthesis, and acquires multiple glutamic acid residues. The last step is catalyzed by polyglutamate synthetase, a cytoplasmic enzyme (4).

Several studies have shown that in the presence of 1–10 μM folate tissue culture cells will rapidly accumulate folate and that eventually it becomes polyglutamated. These studies suggest that folates utilize a membrane carrier to cross the plasma membrane (5). This carrier is saturable (Kd = 1–10 μM) and internalizes folate with a Vmax of 1–12 nmol/min/g dry weight (5). The molecular basis of this carrier activity has not been determined.

Kolhouse and co-workers (6) have found that folate-depleted tissue culture cells express a folate-binding protein on their cell surface that is immunologically related to the soluble folate binder found in plasma and milk of many animals (8–12). Like the soluble protein, the membrane folate binder has a high affinity for 5-methyltetrahydrofolic acid but at least a 10-fold higher affinity for folic acid and a 5–10-fold lower affinity for methotrexate (7). In addition, folates are readily released by acid treatment (13).

Folate binding activity on the surface of MA104 cells is maximal when cells are grown in the absence of folate. Whereas at 4 °C folate-depleted cells accumulate 1 pmol/10⁶ cells, which can be released by brief acid treatment, at 37 °C cells will accumulate 5–7 pmol/10⁶ cells (7). Folate accumulation at 37 °C is linear for approximately 4 h before gradually leveling off; by 24 h of incubation, accumulation virtually ceases (7). 5-Methyltetrahydrofolic acid binding as well as uptake is inhibited by antibodies that inhibit binding to the soluble folate-binding protein (7).

The membrane-bound folate-binding protein is a likely candidate for a membrane receptor that mediates the passage of physiological concentrations of folate into the cytoplasm of the cell. In this report we present evidence that this receptor is responsible for delivering 5-methyltetrahydrofolic acid to the cytoplasm of the cell and that it does so by cyclicly moving from the cell surface into an intracellular membrane compartment and back to the cell surface.

EXPERIMENTAL PROCEDURES

Materials—Medium 199 with Earle’s salts with (320-1150) or without (82-0002) folic acid, glutamine (320-5030), trypsin-EDTA (610-5300) were purchased from GIBCO. Fetal calf serum (12-10378) was from Hazelton Research Products, Inc. (Lenexa, KS). [3H]Folic acid (20 Ci/mmol, MT783) was from Moravek Biochemicals (City of Industry, CA). The reduced folates, L-5-methyltetrahydrofolic acid and L-5-methyl[3H]tetrahydrofolic acid (20 Ci/mmol) were synthesized and purified as previously described (7, 14). Monensin (475986) and nigericin (481990) were purchased from Behring Diagnostics. Culture flasks (T-25 and T-75) were from Costar (Cambridge, MA). Ammonium chloride (A-4514), chloroquine (C-6628), folic acid (F-7876), DL-5-methyltetrahydrofolic acid (M-0132), tetrahydrofolic acid (T3125), folinic acid (F7878), p-aminobenzoyl-1-glutamic acid (A-
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0879), and sodium Hepes (H-3375) were from Sigma. Percoll was from Pharmacia LKB Biotechnology Inc. Iodoacetic acid (16054-7) was from Aldrich. Dulbecco's phosphate-buffered saline (DPBS) was made from a standard recipe (GIBCO catalog). Folic acid polyglutamates were purchased from Dr. Nair, Department of Biochemistry, University of South Alabama. All solvents used in HPLC analysis were of HPLC grade. Salt solutions were adjusted to 3 mM MgCl₂ and 10 mM NaCl to stabilize the ionic strength. The homogenate (approximately 2 ml) was disrupted for 10 min at 25000 g at 4 °C with a Brinkmann Polytron homogenizer. Before each fraction was counted by scintillation counting, a sample was removed and the refractive index measured with a Bausch and Lomb refractometer. The refractive index was used to calculate the density of the fraction. Acid and neutral 5'-nucleotidase activity was measured by the spectrophotometric methods of Sabbadini and Okamoto (18).

Preparation of Cell Extracts and Cytoplasm for Analysis of Radioabeled Folate—To analyze radiolabeled folate in cells, cells were grown in T-75 flasks, depleted of intracellular folate, and then incubated in the presence of either 40 nm 5-methyl[14C]tetrahydrofolic acid or 5 nm [3H]folate for the time indicated. Cells were then harvested by washing the monolayers twice with ice-cold DPBS, adding 2.0 ml of buffer A (25 mM ammonium acetate, pH 4.5, 10 mM EDTA), and subjecting the sample to one round of freeze-thawing at −80 °C. The lysed cells were withdrawn, with flasks washed with 2 ml of buffer A, and the two samples pooled. The pooled samples were adjusted to 1% (v/v) 3-mercaptoethanol (3-ME) (16). The solution was adjusted to pH 3 with glacial acetic acid and then centrifuged at 20,000 g for 30 min at 4 °C in a Beckman J-21 centrifuge using a J-20 fixed angle rotor. The supernatant fraction was saved and the pellet washed by suspending in 1 ml of 0.1 M potassium phosphate, pH 7.0, containing 1% (v/v) (3-ME) and centrifuged once again. The supernatant fractions were pooled and prepared for HPLC analysis.

Preparation for HPLC Analysis—The lyophilized cell lysate was dissolved in 0.1–0.3 ml of 1% (v/v) β-mercaptoethanol and was sonicated for 5 min as described above, and 1 ml of 1% β-mercaptoethanol solution was added to pH 3 with glacial acetic acid and was added per 2.5 ml of sample. The sample was then centrifuged at 20,000 × g as described above. The supernatant fraction was saved and the pellet washed by suspending in 1 ml of 0.1 M potassium phosphate, pH 7.0, and adjusted to 1% (v/v) (3-ME). The supernatant fractions were pooled and filtered before analysis by HPLC.

Analysis of Polyglutamates—HPLC analysis of polyglutamates was performed as previously described for methotrexate polyglutamate. Analysis was performed by injecting 10,000–20,000 dpm of radiolabeled folate, which was separated by HPLC on a TSK-G2000SWXL column. The sample was analyzed for folates containing 2–20 glutamate residues. The sample was analyzed in triplicate.

Analysis of Radiolabeled Folates—to identify the type of folate present in either the cell extract or cytoplasmic fraction, the sample was treated with alkaline phosphatase. The alkaline phosphatase (Alkaline phosphatase; enzyme) was added to the supernatant fraction by incubating for 30–40 min at 4 °C under a stream of nitrogen. The sample was subjected to a second microfuge centrifugation and the supernatant fraction filtered through a 0.45-μm filter before analysis by HPLC.

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nitrile. The sample was centrifuged in a Beckman microfuge B at 4 °C and the supernatant fraction saved. The pellet was suspended in 0.2 ml of acetonitrile and centrifuged again. The two supernatant fractions were pooled, the acetonitrile was removed and passed through a 0.45-μm filter prior to analysis.

Folates were analyzed on HPLC by the method of Duch et al. (20), using a Waters C18 Bondapak column (3.9 mm × 15 cm) and an isocratic elution (1 ml/min) with buffer that contained 20% methanol, 5 mM Pic-A, and 10 mM sodium phosphate, pH 4.5. Prior to running each sample, the column was calibrated by running known folate standards in the same buffer system (see Fig. 3B). Each sample contained 5-10 nmol of either folic acid or 5-methyltetrahydrofololic acid as standards. We found that under the reaction conditions described, spectral quantities (5-10 nmol) of folic acid pentaglutamate were completely reduced to the monoglutamate form.

The overall recovery of 3H from the time the cells were extracted to recovery from either HPLC analysis was ≥75%.

RESULTS

Cellular Accumulation of [3H]Folate(s)—Folic acid inhibits 5-methyl[3H]tetrahydrofolic acid accumulation in MA104 cells and also competes for binding of this ligand to the folate receptor (7). To see if cells would take up folic acid, we compared the time-dependent uptake of [3H]folic acid with 5-methyl[3H]tetrahydrofolic acid. As shown in Fig. 1, 5-methyl[3H]tetrahydrofolic acid accumulated in MA104 cells with normal kinetics, reaching a maximum of 7 pmol/10⁶ cells in 24 h. [3H]Folic acid, however, was taken up more slowly, and after 24 h the cells contained only 2.3 pmol/10⁶ cells. When both sets of 24 h incubated cells were subjected to a brief acid wash at 4 °C, regardless of the ligand, 1.0 pmol/10⁶ cells of folate was released. Therefore, approximately 50% of the [3H]folic acid was acid-releasable, but only 15% of the 5-methyl[3H]tetrahydrofolic acid was released.

To be sure that the radioactivity accumulated by the cells was not a contaminant or breakdown product, we analyzed by HPLC the [3H]folic acid and 5-methyl[3H]tetrahydrofolic acid used in the incubation media and the cellular [3H] taken up by cells incubated with each ligand for 17 h at 37 °C. Although the radiolabeled folate added to the dish was >95% pure, at the end of the incubation the [3H]folic acid and 5-methyl[3H]tetrahydrofolic acid in the media were 95 and 88% chromatographically pure, respectively. On the other hand, analysis of the cell extract from either set of cells revealed that 80% of the radioactivity co-eluted with a known folate standard. (Of the remaining 20%, one-half co-eluted with p-aminobenzoyl-1-glutamic acid and the remainder did not co-elute with any standard.)

We next used a cell fractionation procedure to analyze the subcellular distribution of both [3H]folic acid and 5-methyl[3H]tetrahydrofolic acid (Fig. 2) in cells that had been incubated with the radiolabel at 37 °C for 17 h. Virtually all of the [3H]folic acid taken up by cells migrated as a single peak on a Percoll gradient at a density of 1.044 g/ml (fractions 7-10). This peak co-migrated with 5'-nucleotidase activity, a plasma membrane marker (data not shown). When we analyzed cells that had been incubated with 5-methyl[3H]tetrahydrofolic acid, the 1.044 g/ml peak contained the same amount of folate (2 pmol/10⁶ cells) but in addition, there was 4 pmol/10⁶ cells in a lighter fraction (fractions 1-4). When we measured acid 5'-nucleotidase activity, a lysosomal marker (18), peak activity was in fractions 13-16. According to the Percoll manufacturer's handbook (Pharmacia LKB Biotechnology Inc.), the lighter fraction (density of 1.00-1.040) corresponds to the cell cytoplasm.

The radioactivity in the cytoplasmic fraction (fractions 1-4, Fig. 2) of cells incubated with 5-methyl[3H]tetrahydrofolic acid for 17 h at 37 °C was assayed for the presence of polyglutamates by gradient HPLC (Fig. 3A). Approximately 47% of the radioactivity eluted from the column at a position between the folypentaglutamate and the folylheptaglutamate standards, indicating that the predominant form was a polyhexaglutamate. Five percent co-eluted with a polytriglutamate standard and 30% with a polymonoglutamate standard. The remaining 17% of the radioactivity (primarily fraction 3) did not co-elute with any folate standard and, therefore, most

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**Fig. 1. Uptake of 5-methyl[3H]tetrahydrofolate acid or [3H] folic acid.** Cells were grown for 4 days in the absence of folate. The medium was replaced with medium B that contained either 5 nM [3H]folic acid or 40 nM 5-methyl[3H]tetrahydrofolate acid, and the cells were further incubated for 17 h. At the end of the incubation the cells were rinsed with ice-cold DPBS, released from the dish, and homogenized as detailed under "Experimental Procedures." The homogenate was layered on a solution of Percoll (1.05 g/ml) and centrifuged at 60,000 × g for 45 min. Each fraction (0.5 ml) was analyzed for [3H] content. Fraction 8 had a density of 1.044 g/ml, corresponding to the known density of plasma membrane. In addition, it contained the peak activity for the plasma membrane marker, 5'-nucleotidase measured at neutral pH. Fractions 1-4 correspond to the cytoplasmic fraction. The specific activity for [3H]folic acid and 5-methyl[3H]tetrahydrofolate was 9,500 and 8,800 cpm/pmol, respectively. Each of the membrane fractions (fractions 7-10) contained 2 pmol/10⁶ cells of folate. In the presence of a 100-fold excess unlabeled folate during the incubation period, cellular radioactivity was reduced by 94%. Cells were grown in T-75 flasks, which at the time the experiment was done contained approximately 3.5 × 10⁶ cells.
particulate material. All of the radioactivity was removed by the radioactivity was tightly bound to a macromolecule or the radioactivity found in fraction standards (data not shown). In separate experiments, we also degradation product of folate and most likely corresponds to the total, then the radioactivity co-chromatographed with a 5-methyltetrahydrofolate standard. The latter compound is a known authentic folate. Of this, another sample of the cytoplasmic fraction was treated with plasma conjugase and analyzed on isocratic HPLC to identify the type of folate (Fig. 4B). Approximately 66% of the radioactivity co-chromatographed with a 5-methyltetrahydrofolic acid standard, 13% with a 10-formyltetrahydrofolic acid/tetrahydrofolic acid standard, and 21% with a p-amino-benzoylglutamate standard. The latter compound is a known degradation product of folate and most likely corresponds to the radioactivity found in fraction 3, Fig. 3A.

We also analyzed the cytoplasmic fraction to determine if the radioactivity was tightly bound to a macromolecule or particulate material. All of the radioactivity was removed by dialysis and migrated on Sephadex G-100 column with folate standards (data not shown). In separate experiments, we also assayed this fraction from folate-depleted cells for the presence of folate binding activity and found none to be present. Movement of Surface-bound [*H]*Folic Acid into an Acid-resistant Compartment—Even though [*H]*folic acid remained associated with the membrane fraction and did not significantly enter the cytoplasm at 37 °C (Figs. 1 and 2), only 50% could be released by a brief acid wash. The acid-resistant fraction most likely corresponds to [*H]*folic acid in a membrane compartment that was protected from the acid treatment. To determine if [*H]*folic acid could actively move from an acid-releasable to an acid-resistant compartment, we analyzed the effect of temperature on the acid releasability of [*H]*folic acid initially bound to cells at 4 °C. Cells were incubated with [*H]*folic acid at 4 °C for 0.5 h to saturate all available receptors, rinsed with folic acid-free buffer, and warmed to 37 °C for various times before assaying for amount of [*H]*folic acid that was acid-releasable (Fig. 4). Without

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**Fig. 3. Analysis of cytoplasmic folate in MA104 cells.** Cells were grown for 4 days in folate-deficient medium and incubated with 40 nM 5-methyl[*H]*tetrahydrofolate for an additional 17 h. At the end of the incubation, cells were homogenized and fractionated on Percoll gradients as in Fig. 2. Aliquots of the cytoplasmic fraction (fractions 1–4) were either processed for polyglutamate analysis (Panel A) or first treated with plasma conjugase and then assayed for the type of folate (Panel B) as described. Approximately 10,000 cpm were loaded on each column. Panel A, the position that authentic folic acid standards eluted is indicated by the numbers: 1, folic acid; 3, folic acid triglutamate; 4, folic acid tetraglutamate; 5, folic acid pentaglutamate; and 7, folic acid heptaglutamate. 1-5-Methyltetrahydrofolyl-Glu<sub>4</sub> co-eluted with their respective folic acid analogs. Panel B, the numbers indicate the elution position of authentic folic standards: 1, paraminobenzyl-1-glutamic acid; 2, 10-formyltetrahydrofolinic acid; 3, tetrahydrofolic acid; 4, 5-formyltetrahydrofolic acid; 5, dihydrofolic acid; 6, folic acid; 7, 5-methyltetrahydrofolic acid.

**Fig. 4. Internalization of [*H]*folic acid by MA104 cells.** Cells were grown for 4 days in the absence of folate. The medium was replaced with ice-cold medium B containing 5 nM [*H]*folic acid and incubated at 4 °C for 30 min. The cells were then rinsed with DPBS at 4 °C and incubated at 37 °C in folate-free medium B for the indicated times. At the end of each incubation cells were analyzed for either acid-releasable (●) or acid-resistant (■) [*H]*folic acid. Cells were grown in T-25 flasks, as in Fig. 1. Values are means of duplicate samples, ±10%.

**Fig. 5. Appearance of the unoccupied folate receptor on the cell surface of MA104 cells.** Cells were grown in the absence of folate for 4 days. The medium was replaced with ice-cold medium B containing 5 nM unlabeled folic acid and incubated at 4 °C for 30 min. The cells were rinsed with ice-cold DPBS and warmed to 37 °C for the indicated time. At the end of each incubation, unoccupied surface receptors (●) were assayed by incubating the cells in ice-cold medium B containing 5 nM [*H]*folic acid for 30 min at 4 °C and measuring the amount of surface-bound [*H]*folic acid as described. To measure total surface receptors (○), a second set of cells at each time was acid-washed to remove any surface-bound unlabeled folic acid before incubating with [*H]*folic acid at 4 °C as above. Total surface binding (100% maximum binding) was 1 pmol/10<sup>6</sup> cells.

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**Figure captions:**

- **Panel A** shows the elution positions of authentic folic acid standards on a polyglutamate analysis column. The elution positions are indicated by the numbers: 1, folic acid; 3, folic acid triglutamate; 4, folic acid tetraglutamate; 5, folic acid pentaglutamate; and 7, folic acid heptaglutamate. The authentic folic acid standards elute at positions 1, 3, 4, 5, and 7.
- **Panel B** displays the elution positions of authentic folic acid standards on an isocratic HPLC column. The elution positions are indicated by the numbers: 1, paraminobenzyl-1-glutamic acid; 2, 10-formyltetrahydrofolinic acid; 3, tetrahydrofolic acid; 4, 5-formyltetrahydrofolic acid; 5, dihydrofolic acid; 6, folic acid; and 7, 5-methyltetrahydrofolic acid.

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**Appearance of the unoccupied folate receptor on the cell surface of MA104 cells:** Cells were grown in the absence of folate for 4 days. The medium was replaced with ice-cold medium B containing 5 nM unlabeled folic acid and incubated at 4 °C for 30 min. The cells were rinsed with ice-cold DPBS and warmed to 37 °C for the indicated time. At the end of each incubation, unoccupied surface receptors (●) were assayed by incubating the cells in ice-cold medium B containing 5 nM [*H]*folic acid for 30 min at 4 °C and measuring the amount of surface-bound [*H]*folic acid as described. To measure total surface receptors (○), a second set of cells at each time was acid-washed to remove any surface-bound unlabeled folic acid before incubating with [*H]*folic acid at 4 °C as above. Total surface binding (100% maximum binding) was 1 pmol/10<sup>6</sup> cells.
any warming, all of the radioactivity was released; however, with time there was a progressive loss of acid-releasable and a corresponding increase in acid-resistant \(^{3}H\)folic acid. Equilibrium was reached after 2-4 h when approximately 50% was in either the acid-resistant or acid-releasable form; this ratio changed very little when cells were incubated for up to 12 h at 37 °C.

During the warm up period, there was not any \(^{3}H\)folic acid released into the media; therefore, under these conditions all of the ligand remained membrane-bound. This allowed us to examine cells for the presence of any unoccupied receptors that might appear during the temperature-dependent transfer of folic acid to an acid-resistant compartment. Cells were chilled to 4 °C and incubated in the presence of 5 nM unlabeled folic acid to saturate all available surface binding sites. The cells were then washed and incubated at 37 °C for various times before measuring \(^{3}H\)folic acid binding to the cell surface (at 4 °C). As seen in Fig. 5, before warming to 37 °C, there was very little \(^{3}H\)folic acid binding detected, indicating that all external receptors were occupied with unlabeled folic acid. With time at 37 °C, however, there was a progressive increase in surface binding, which reached ~50% of maximal binding by 4 h. The kinetics of appearance of unoccupied receptor on the cell surface was similar to the kinetics of appearance of acid-resistant \(^{3}H\)folic acid (compare Fig. 5 with Fig. 4). During the course of this experiment, the total number of \(^{3}H\)folic acid binding sites did not change (Fig. 5).

The appearance of an unoccupied receptor on the cell surface at the same time as folic acid moved into an acid-resistant compartment suggested that both occupied and unoccupied receptors could shuttle between the acid-resistant and acid-releasable compartments at 37 °C. Additional evidence was provided by a third experiment (Fig. 6). A set of cells was incubated with \(^{3}H\)folic acid at 37 °C for 4 h, a time when binding to the membrane fraction is maximal. The same set of cells was then subjected to repeated cycles of acid washing at 4 °C followed by warming to 37 °C for 0.5 h in
Folate Uptake Via a Membrane Receptor That Recycles

The Folate Receptor Mediates 5-Methyltetrahydrofolic Acid Accumulation—If the folate receptor is responsible for the uptake of 5-methyl[H]tetrahydrofolic acid into folate-depleted cells, then inactivation of the receptor should prevent cellular accumulation. One way to inactivate the receptor is with antibodies. This was done by Antony et al. (25), who showed that antibodies capable of inhibiting folate binding to the soluble folate binder completely block the uptake of 5-methyl[H]tetrahydrofolic acid at 37 °C in KB cells. Another way is with a high affinity, competitive ligand such as folic acid. Previously we showed that folic acid blocks accumulation of 5-methyl[H]tetrahydrofolic acid, even when present in the medium at one-eighth the concentration of the radiolabeled folate. The current results show that folic acid has this effect by inactivating the surface receptor. [H]Folic acid was only found tightly associated with the plasma membrane fraction and the amount bound was equal to the number of 5-methyl[H]tetrahydrofolic acid binding sites. Therefore, under conditions where folic acid blocks 5-methyl[H]tetrahydrofolic acid accumulation, we detected only one population of folate binding sites on the membrane.

Recycling of the Folate Receptor—Even though at 37 °C [H]folic acid never appeared in the cytoplasmic fraction nor was it polyglutamated (data not shown), the membrane fraction contained twice as much ligand as could bind to cells at 4 °C. Therefore, to establish a function, we must be able to distinguish between these two steps: (a) binding to the cell surface receptor followed by (b) delivery to an intracellular site. Therefore, to establish a function, we must be able to distinguish between these two steps. We used acid releasability at 4 °C to identify folates on the cell surface, and cell fractionation together with measuring polyglutamation to identify folate that reached the cytoplasm.

Many ligand-receptor interactions are sensitive to pH (2). This property has been exploited by investigators to identify ligands that are bound to receptors at the surface of cells (2, 24). Since acid treatment of the soluble folate binder is a standard method for releasing folates (13), we reasoned that low pH should release folate bound at the cell surface of MA104 cells and leave behind any folate that had entered an intracellular compartment. To verify this method, we measured the amount of folate that was acid-releasable from cells incubated with the ligand at 4 °C, conditions that prevent intracellular accumulation (7). We found that virtually all of the folate could be released. Moreover, when cells were incubated with 5-methyl[H]tetrahydrofolic acid at 37 °C for up to 24 h, chilled to 4 °C, and treated with acid saline, the same amount of ligand was released. However, in this case there was always a substantial amount that remained associated with the cell. As judged from cell fractionation experiments, much of this 5-methyl[H]tetrahydrofolic acid had been delivered to the cytoplasm of the cell (Fig. 24).

Polyglutamate synthetase, the enzyme that adds glutamic acid residues to folate by a γ-carboxyl linkage, is a cytoplasmic enzyme (4). Therefore, polyglutamation of folate should serve as a measure of the ligand that reached the cytoplasm of the cell. HPLC analysis of the cytoplasmic fraction showed that 63% of the folate radioactivity was in the form of polyglutamate.

| Treatment                  | 5-Methyl[H]tetrahydrofolic acid pmol/10^6 cells |
|----------------------------|----------------------------------------------|
| Control                    | 6.0                                          |
| NH₄Cl (20 mM)              | 3.6                                          |
| Chloroquine (50 μM)        | 3.8                                          |
| Chloroquine (500 μM)       | 2.2                                          |
| Nigericin (5 μM)           | 2.0                                          |
| Monensin (25 μM)           | 2.0                                          |

**TABLE 1**

Effect of nigericin and weak bases on folate accumulation

MA104 cells were grown in the absence of folate for 4 days. The medium was replaced with medium B containing the indicated concentration of the drug and incubated for 30 min at 37 °C. Total cellular 5-methyl[H]tetrahydrofolic acid was measured as described. Value is the mean of two experiments (±10%) where each experiment had duplicate flasks per treatment.

**DISCUSSION**

The most fundamental question about the folate receptor is whether or not it mediates the cellular accumulation of physiological folate. Cellular accumulation can be divided into two steps: (a) binding to the cell surface receptor followed by (b) delivery to an intracellular site. Therefore, to establish a function, we must be able to distinguish between these two steps. We used acid releasability at 4 °C to identify folates on the cell surface, and cell fractionation together with measuring polyglutamation to identify folate that reached the cytoplasm.

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**The Folate Receptor Mediates 5-Methyltetrahydrofolic Acid Accumulation**

If the folate receptor is responsible for the uptake of 5-methyl[H]tetrahydrofolic acid into folate-depleted cells, then inactivation of the receptor should prevent cellular accumulation. One way to inactivate the receptor is with antibodies. This was done by Antony et al. (25), who showed that antibodies capable of inhibiting folate binding to the soluble folate binder completely block the uptake of 5-methyl[H]tetrahydrofolic acid at 37 °C in KB cells. Another way is with a high affinity, competitive ligand such as folic acid. Previously we showed that folic acid blocks accumulation of 5-methyl[H]tetrahydrofolic acid, even when present in the medium at one-eighth the concentration of the radiolabeled folate (7). The current results show that folic acid has this effect by inactivating the surface receptor. [H]Folic acid was only found tightly associated with the plasma membrane fraction and the amount bound was equal to the number of 5-methyl[H]tetrahydrofolic acid binding sites. Therefore, under conditions where folic acid blocks 5-methyl[H]tetrahydrofolic acid accumulation, we detected only one population of folate binding sites on the membrane.

**Recycling of the Folate Receptor**

Even though at 37 °C [H]folic acid never appeared in the cytoplasmic fraction nor was it polyglutamated (data not shown), the membrane fraction contained twice as much ligand as could bind to cells at 4 °C. In addition, 50% of [H]folic acid taken up at 37 °C was released by acid treatment, indicating that only half of the [H]folic acid was exposed at the cell surface. When cells incubated with [H]folic acid at 4 °C were washed and shifted to 37 °C, there was a reciprocal disappearance of surface-bound folic acid and an increase in the number of unoccupied receptors at the cell surface. These results suggest that the membrane fraction contains two populations of receptors:
50% are at the cell surface exposed to the extracellular space and the other 50% are in an internal membrane compartment. Moreover, these two receptor pools can exchange with each other at 37 °C.

Folic acid, then, is a high affinity ligand for the folate receptor that does not enter the cytoplasm. Instead, it remains bound to the receptor as the receptor cyclically moves in and out of the cell. Therefore, this ligand serves as a convenient way to tag the receptor to monitor receptor recycling. The finding that a maximum of 50% of the [3H]folic acid bound at 4 °C was internalized at 37 °C indicates that ordinarily both occupied and unoccupied receptors can shuttle in and out of the cell at 37 °C.

We consistently found that at 37 °C [3H]folic acid was taken up more slowly than 5-methyl[3H]tetrahydrofolic acid (Fig. 1). In this experiment there was a 4-fold difference in the initial rate of uptake. Therefore, the normal rate of receptor recycling may be much faster than was detected by following [3H]folic acid movement. Attempts to follow the internalization of 5-methyl[3H]tetrahydrofolic acid prebound at 4 °C were unsuccessful because when the cells were warmed to 37 °C, >90% of the ligand was released into the surrounding medium.

Release of Internalized 5-Methyltetrahydrofolic Acid—All of the drugs that are known to dissipate proton gradients within intracellular membrane-bound compartments (22, 23) interfered with the uptake of 5-methyltetrahydrofolic acid. Iono- phosphes such as monensin and nigericin had the most profound effect. Monensin most likely had its effect by inhibiting the release of 5-methyl[3H]tetrahydrofolic acid from internalized receptors because it did not measurably affect receptor recycling. Thus at equilibrium monensin had no effect on the amount of membrane-associated 5-methyl[3H]tetrahydrofolic acid but caused a 6-fold inhibition of accumulation in the cytoplasm. These results suggest that the release of physiological folate takes place in an acidic intracellular compartment.

The inhibitory effect of monensin on 5-methyl[3H]tetrahydrofolic acid accumulation is strong evidence that the receptor enters a membrane-bound compartment, e.g. an endocytic vesicle, during recycling. However, we cannot rule out the possibility that ionophores and weak bases are affecting some other cell process that is required for delivery of 5-methyltetrahydrofolic acid to the cytoplasm.

Mechanism of Receptor-mediated Internalization—Collectively the data we have gathered suggest a novel mechanism for folate internalization by the folate receptor. Extracellular 5-methyltetrahydrofolic acid binds to a population of externally oriented membrane receptors. The receptor-linked complex moves into the cell, most likely by becoming entrapped in a vesicular compartment that cannot be readily distinguished from the plasma membrane on Percoll gradients. The interior of the compartment becomes acidic, causing the ligand to dissociate from the receptor. The acidic environment of this compartment may facilitate the movement of 5-methyltetrahydrofolic acid into the cytoplasm. The unoccupied receptor then moves back to the cell surface to participate in another round of internalization.

The relationship between receptor-mediated internalization and carrier-mediated internalization (5) remains to be clarified. Most likely these are two separate internalization processes that operate in different tissues to serve the special metabolic demands of individual cells. There is an intriguing possibility, however, that the two processes are coupled in some fashion. For example, the transmembrane movement of folate that is internalized by the folate receptor might be mediated by a carrier mechanism. The receptor, which is maximally expressed in folate-deficient cells, seems adapted to concentrate folates at the surface membrane, which may be required for the carrier to transport in a cellular environment that is low in folate.

Physiological Significance—We think that the folate-depleted tissue culture cell model used in our studies (7) and those of Kolhouse and co-workers (6, 25) as well as McHugh and Cheng (26) has revealed a physiologically important folate transport pathway that cannot be detected in cells grown in standard folic acid-containing media (10−6−10−8 M), because receptor activity is regulated by intracellular folate concentration (7). We found that, in the presence of only 0.04 μM 5-methyl[3H]tetrahydrofolic acid, within 4 h the cellular folate concentration increases from 0.5–1 μM to 5–6 μM, which is a 100-fold higher concentration of [3H]folate in the cell than is in the media. During this time, the cells accumulate 10−15% of the total 5-methyl[3H]tetrahydrofolic acid in the media, resulting in 70–80% of the cellular folate being radiolabeled (50–60 × 103 cpm/106 cells in a typical experiment). This high proportion of labeled folate facilitates the chemical characterization of the intracellular folate (Fig. 5), which is important for detecting any metabolic modifications that have occurred within the cell. Most importantly, this uptake process occurs at near physiological concentrations of folate.

Whether the folate receptor functions the same way in vivo remains to be established. The recent evidence that the soluble folate binder found in plasma may be derived from the membrane folate receptor (6) together with the finding that human placenta (27) and rat kidney (28) are rich sources of the membrane receptor suggests that the receptor is expressed on the surface of tissue cells.

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