

# Lowering the Cholesterol Content of MA104 Cells Inhibits Receptor-mediated Transport of Folate

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**Abstract.** The folate receptor is clustered on the surface of MA104 cells in association with caveolae. This relationship is thought to be essential for the proper internalization and recycling of the receptor during the delivery of 5-methyltetrahydrofolate to the cytoplasm of folate-depleted cells. Both the clustered organization of the receptor and the integrity of caveolae are disrupted when cells are deprived of cholesterol. We now

show that cholesterol depletion of MA104 cells markedly reduces the rate of 5-methyltetrahydrofolate internalization and causes a 70% decline in the number of receptors present in the internal, recycling compartment. This effect is consistent with morphologic data showing that cholesterol-depleted MA104 cells have a reduced number of caveolae as well as fewer receptors per caveolae.

**T**HE folate receptor is a glycosyl-phosphatidylinositol (GPI)<sup>1</sup> anchored membrane protein (12, 13) that mediates the high affinity uptake of 5-methyltetrahydrofolate into cells. The internalization process involves a novel endocytic pathway (2) that requires four steps (8–11): (a) binding of the vitamin to the receptor; (b) internalization of the vitamin–receptor complex into a membrane bound compartment; (c) dissociation of the vitamin from the receptor and movement across the membrane into the cytoplasm; and (d) covalent addition of multiple glutamic acid residues to the vitamin. The presence of the receptor affords cells greater than a 30-fold efficiency in 5-methyltetrahydrofolate uptake (11), which allows tissue culture cells to divide when grown in physiologic concentrations of the vitamin (14).

Biochemical (10) and immunofluorescence (19) experiments have shown that the folate receptor cyclically moves in and out of a membrane-bound compartment. Immunogold cytochemistry demonstrated that receptors are clustered in association with caveolae (19), a membrane specialization that previously has been shown to transport molecules across endothelial cells (21). Caveolae are present on the surface of most cells (23) and are the likely sites of folate receptor internalization (19).

The formation of folate receptor clusters is dependent on membrane cholesterol (18). Cholesterol binding drugs such as filipin disperse the receptors in the plane of the membrane, even if the cells are first incubated in the presence of polyclonal, anti-receptor IgG and fixed with formaldehyde. Lowering the cellular concentration of cholesterol also causes receptors to become unclustered. Receptor cluster-

ing, therefore, is dependent upon the lipid phase of the membrane.

Caveolae have a distinctive, striated coat that decorates the cytoplasmic face of the membrane (16, 20). The coat is not removed by treatment with either high salt or carbonate (20). This suggests that, unlike clathrin-coated pits, the visible portion of the coat consists of integral membrane protein(s). Recently, we identified a protein component of the coat called caveolin (20). It too behaves like an integral membrane protein. Despite the integral nature of the coat protein(s), when isolated plasma membranes are exposed to cholesterol binding drugs, the coat disassembles and caveolin scatters with the remnants of the coat material (20). Also, cholesterol depletion markedly reduces the number of caveolae in MA104 cells (18).

The profound effects of cholesterol depletion on both the organization of the folate receptor and the structure of caveolae predicts that this treatment should affect the receptor-mediated uptake of folate. The current study shows that when the cholesterol content of MA104 cells is lowered by >50%, the rate of 5-methyltetrahydrofolate delivery to the cell interior is reduced by 50% and the number of receptors in the internal, recycling compartment is reduced by 70%.

## Materials and Methods

### Materials

FBS, glutamine, trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco Laboratories (Grand Island, NY). [<sup>3</sup>H]folic acid and DL-5-methyl[<sup>3</sup>H]tetrahydrofolic acid were from Moravak Biochemicals, Inc. (City of Industry, CA). Culture flasks (T-25) and dishes (35 mm) were from Corning Glass Inc. (Corning, NY). Folic acid, DL-5-methyltetrahydrofolic acid, tris(hydroxymethyl)aminomethane, sodium taurocholic acid, Triton X-100, cholesterol, crystalline BSA, *p*-hydroxyphenylacetic acid,

1. *Abbreviations used in this paper:* GPI, glycosyl-phosphatidylinositol; LDL, low density lipoprotein.

leupeptin, aprotinin, DME (-glutamine), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride and isopropyl alcohol from Fisher Scientific Co. (Fairlawn, NJ). Cholesterol ester hydrolase, cholesterol oxidase, and HRP, from Boehringer Mannheim GmbH (Germany). Mevalonic acid lactone and paraformaldehyde were from Fluka Chemical Corp. (Ronkonkoma, NY). Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG from Zymed Laboratories Inc. (South San Francisco, CA). Dulbecco's PBS was made from a standard recipe (Gibco catalog). M199 media was prepared from a standard recipe using Sigma Chemicals. Human lipoprotein-deficient serum ( $d > 1.215$  g/ml) was prepared as previously described (6).

### Tissue Culture and Cholesterol Depletion

MA104 cells, a monkey kidney epithelial cell line, were grown as previously described (9).  $1.5 \times 10^5$  cells were seeded into a T-25 culture flask and grown for 2 d in folate-free M199 supplemented with 0.68 mM glutamine and 5% (vol/vol) FBS (medium A). Cells were then transferred to folate-free M199 supplemented with 0.68 mM glutamine and 5% lipoprotein deficient serum (medium B) containing either 200  $\mu$ M mevalonate or 200  $\mu$ M mevalonate plus 25  $\mu$ M compactin and cultured for an additional 2.5 d (60 h). The low concentration of mevalonate was required to maintain cell viability during the 60-h incubation. For indirect immunofluorescence experiments,  $5 \times 10^4$  cells were seeded onto coverslips in 35-mm dishes and grown under cholesterol depletion conditions as described above. Cells were counted using a standard hemocytometer.

### Immunofluorescence

Cells were chilled to 4°C for 20 min, rinsed with ice-cold medium C (Folate-free M199, 0.68 mM glutamine, 20 mM Hepes, pH 7.4, and 0.15% crystalline BSA) and incubated with 25  $\mu$ g/ml of monoclonal anti-folate receptor IgG (5), diluted in medium C, for 1 h at 4°C. Cells were rinsed with buffer A (PBS plus 0.15% crystalline BSA) and then fixed with 3% paraformaldehyde in PBS for 30 min. After the indicated incubation, cells were rinsed with buffer A and processed to localize mouse IgG with rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate for 1 h at 4°C as previously described (19).

### Folate Binding and Uptake

Folate binding and accumulation were measured as previously described (8, 9). On day five of cell growth, the medium was removed by aspiration and 1.5 ml of medium D (Folate-free M199, 20 mM Hepes, pH 7.4, and 0.68 mM glutamine) were added to each T-25 flask. The indicated type of radiolabeled folate was added to the dish in the presence and absence of 100-fold unlabeled folate of the same type, and the cells were incubated as indicated in the figure legends. For the measurement of folate binding using [ $^3$ H]folic acid, the medium was removed by aspiration at the end of the incubation. After rinsing with  $2 \times 5$  ml PBS, folate was released from the cells by washing rapidly for 30 s with 2 ml of acid saline (0.15 M NaCl, adjusted to pH 3 with glacial acetic acid) followed by a rinse with 1.5 ml of PBS. The radiolabeled folate in the acid saline plus the rinse equaled the acid-releasable fraction of folate. The cells were removed from the T-25 flask by addition of 1 ml 0.1 N NaOH for at least 10 min at room temperature followed by rinsing with 2 ml of PBS. To measure the membrane and cytoplasmic [ $^3$ H]folic acid, we used the following procedures at 4°C. At the end of incubation, cells were chilled on ice, and the medium was removed by aspiration. The cells were washed with  $2 \times 5$  ml ice-cold PBS and 1.5 ml of buffer B (10 mM Tris-base, pH 8.0, 0.02 mg/ml leupeptin and aprotinin, and 1  $\mu$ M DL-5-methyltetrahydrofolic acid) was added to each T-25 flask at 4°C and the flasks were placed at  $-80^\circ\text{C}$  for at least 15 min. After the freezing step, the cells were placed on an ice tray. The dissolved extract was aspirated, each flask was rinsed with 1 ml of buffer B, and the two samples were combined. The combined extracts were centrifuged for 20 min at 100,000 g in a TL-100 centrifuge using a TLA 100.3 rotor (Beckman Instruments, Inc., Palo Alto, CA) to separate the membrane fraction (pellet) from the cytoplasmic fraction (supernatant fluid). The addition of unlabeled folate to the buffer B prevented cytoplasmic [ $^3$ H]folate from binding to unoccupied receptors on the membrane. Radioactivity was measured by liquid scintillation counting using a Packard Tri-carb 1900A liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL).

### LDL Binding and Uptake

On day five of cell growth, the medium was removed by aspiration and the

cells were washed with 5 ml of PBS. Two ml of MEM supplemented with 2 mg/ml of BSA and 100 U/ml of penicillin/streptomycin (medium E) were added to each 60-mm dish. [ $^{125}$ I]-LDL (10  $\mu$ g/ml, specific activity, 350-456 cpm/ng) was added to the dish in the presence and absence of 500  $\mu$ g/ml of unlabeled low density lipoprotein (LDL), and the cells were incubated at 37°C for 4 h. The surface bound, internalized, and degraded LDL were measured by the method of Goldstein et al. (6).

### Cholesterol Assay

Cholesterol measurements were made using a modification of the method of Heider and Boyett (7). Cells were removed from the T-25 flask with 1 ml trypsin-EDTA and transferred to a conical graduated centrifuge tube. The cells were washed three times by suspending them in PBS, centrifuging at 800 g for 5 min, and aspirating the supernatant fluid. Isopropyl alcohol (0.5 ml) was added to the cell pellet and the sample was sonicated with  $3 \times 30$  Joules on a Vibra Cell (Sonics & Materials Inc., Danbury, CN) sonicator. After centrifugation for 15 min at 800 g the clear supernatant was decanted and an aliquot was taken for cholesterol analysis. The residue was dissolved in 0.1 N NaOH and an aliquot was taken for protein determination.

The isopropyl alcoholic solutions of standards, samples, and blank (isopropyl alcohol alone) were treated in a similar manner. An aliquot of 2 ml of reagent A (0.05 M sodium phosphate buffer, pH 7.0, 0.16 U/ml cholesterol ester hydrolase, 0.08 U/ml cholesterol oxidase, 30 U/ml HRP, 5 mM sodium taurocholate, 0.08% Triton X-100, and 0.6 mg/ml *p*-hydroxyphenyl-acetic acid) was added to a  $10 \times 75$  mm disposable glass test tube to which 200  $\mu$ l of the isopropyl alcoholic solution was added and mixed. The mixtures were incubated at 37°C for 30 min in a shaking water bath and then 350  $\mu$ l of 0.5 N NaOH was added and mixed. Samples were placed in a 1-cm light path cuvette and fluorescence was measured with a spectrofluorometer (model 8000C; SLM Instruments Inc., Urbana, IL) with an excitation wavelength of 325 nm and emission wavelength of 415 nm.

### Other Assays

All protein measurements were determined by the method of Bradford (4) using BSA as a standard.

## Results

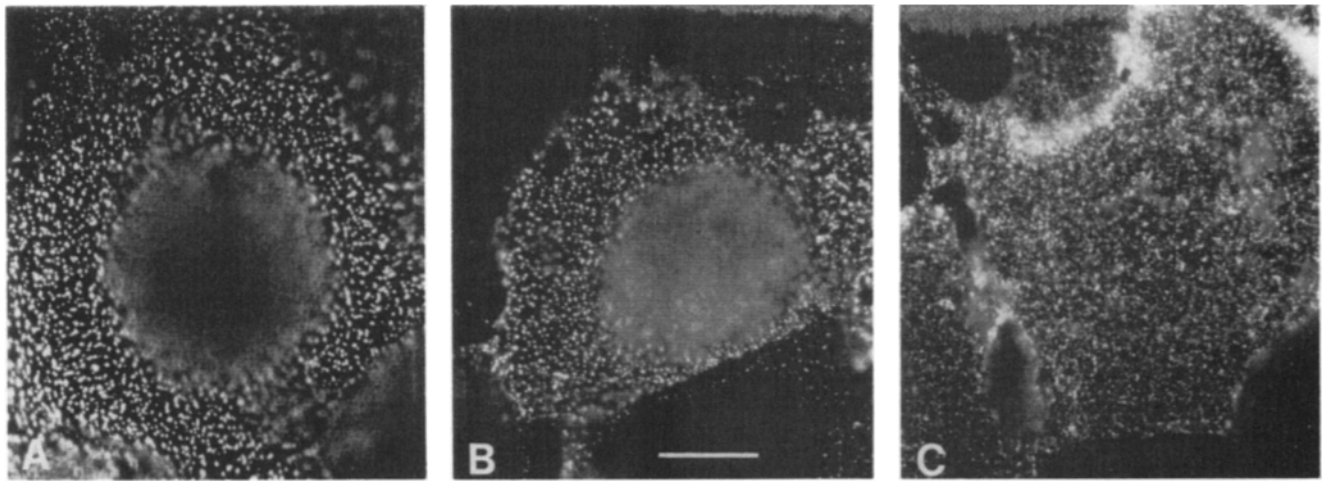
### Distribution of Folate Receptor

Cells can be depleted of cholesterol by allowing them to consume endogenous stores of sterol through repeated cell divisions. 60 h of growth without an exogenous or endogenous source of cholesterol routinely reduced total sterols in MA 104 cells from 5.86  $\mu$ g/ $10^6$  cells to 2.23  $\mu$ g/ $10^6$  cells (62% decrease). This treatment did not affect either cell viability or morphology. Further lowering the cholesterol concentration caused cell death.

Immunofluorescence showed that the degree of folate receptor unclustering among the cells was quite heterogeneous (Fig. 1). Some cells had normal-appearing receptor clusters (compare Fig. 1, A with B) but other cells (Fig. 1 C) in the same dish had smaller clusters with diffuse fluorescence across the membrane. Using these examples as extremes, we found that  $\sim 60\%$  of the cells showed some degree of receptor unclustering.

### Inhibition of [ $^3$ H]Folic Acid Internalization

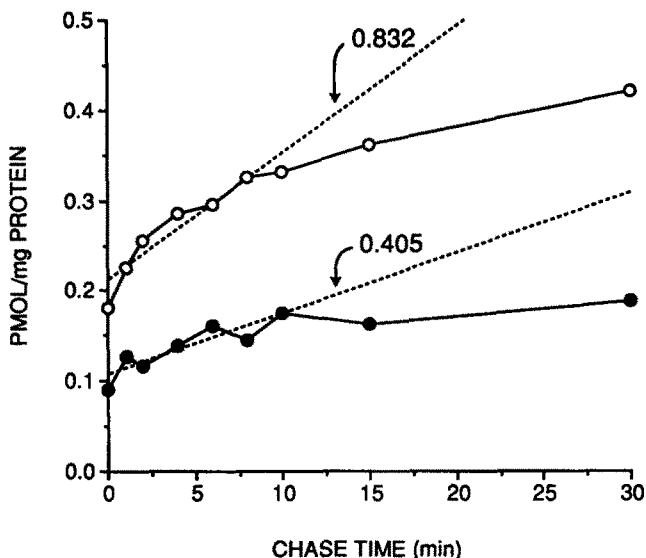
The high affinity ligand for the folate receptor, folic acid, remains bound to the receptor as it moves in and out of the cell (9). The effects of cholesterol depletion on the relative rate of [ $^3$ H]folic acid delivery to an internal compartment at 37°C is shown in Fig. 2 (dotted lines). Control cells ( $\circ$ , Fig. 2) internalized folate at 0.832 pmol/h/mg of protein. By contrast, the rate of internalization in cholesterol deplete cells ( $\bullet$ , Fig. 2) was only 0.405 pmol/h/mg of protein (a 52% de-



**Figure 1.** Effect of control incubation (A) or incubation in the presence of 25  $\mu\text{M}$  compactin (B and C) on the distribution of folate receptors. Cells were grown in medium A for 2 d and then transferred to medium B containing either 200  $\mu\text{M}$  mevalonate (A) or 200  $\mu\text{M}$  mevalonate plus 25  $\mu\text{M}$  compactin (B and C) and incubated an additional 2.5 d. Cells were then chilled to 4°C, incubated with anti-folate receptor IgG, and fixed with 3% formaldehyde. Cells were processed to localize rabbit IgG as described in Materials and Methods. Bar, 15  $\mu\text{m}$ .

crease). The uptake also plateaued earlier in the cholesterol-depleted cells ( $\bullet$ , Fig. 2). These cells only internalized  $\sim 1/3$  (0.16 versus 0.44 pmoles/mg of protein) as much [ $^3\text{H}$ ]folic acid as control cells.

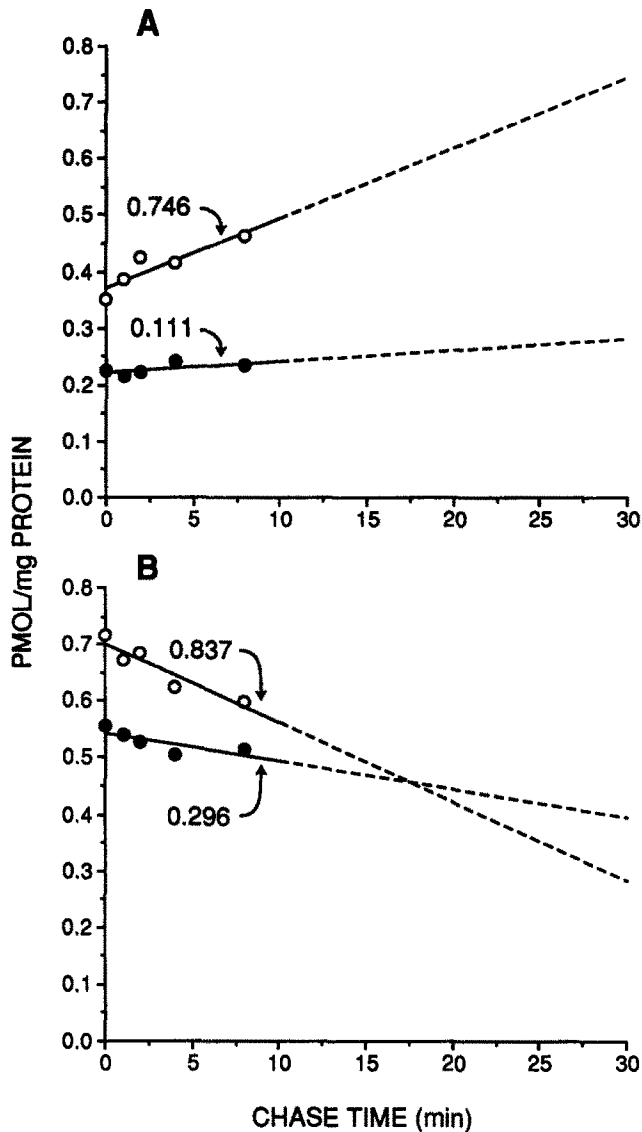
Cholesterol depletion also inhibited the rate of disappear-



**Figure 2.** Internalization of [ $^3\text{H}$ ]folic acid at 37°C. Control ( $\circ$ ) or cholesterol-depleted ( $\bullet$ ) MA 104 cells were washed with PBS and incubated 5 min in medium D containing 5 nM [ $^3\text{H}$ ]folic acid at 37°C. The medium was removed and the cells were washed once with PBS at 37°C and incubated for the indicated time with fresh medium D at 37°C. At the end of each incubation time, the medium was removed and 2 ml of acid saline was added at 4°C to release the external, surface bound [ $^3\text{H}$ ]folic acid. The acid-resistant [ $^3\text{H}$ ]folic acid remaining in the cell is plotted on the ordinate. The dotted lines are drawn to indicate the relative rate of internalization, where the values are calculated in units of pmol/h/mg of protein. Each value is the average of duplicate measurements. The variation in duplicate values was  $<10\%$  and the background was 0.009 and 0.005 pmol/mg protein respectively for control and cholesterol-depleted samples.

ance of [ $^3\text{H}$ ]folic acid from the cell surface (Fig. 3). Control cells ( $\circ$ , Fig. 3, A and B) and cholesterol deplete cells ( $\bullet$ , Fig. 3, A and B) were incubated in the presence of [ $^3\text{H}$ ]folic acid at 4°C. The cells were warmed to 37°C and the amount present in either an internal compartment (Fig. 3 A) or on the cell surface (Fig. 3 B) was measured at various times. Cholesterol depletion had a nearly reciprocal, inhibitory effect on the rate of [ $^3\text{H}$ ]folic acid internalization (compare  $\bullet$  with  $\circ$ , Fig. 3 A) and the rate of [ $^3\text{H}$ ]folic acid loss from the cell surface (compare  $\bullet$  with  $\circ$ , Fig. 3 B). In this experiment we observed a much greater inhibition than in other trials: the rate of internalization was inhibited  $\sim 85\%$  while the rate of loss was inhibited  $\sim 65\%$ .

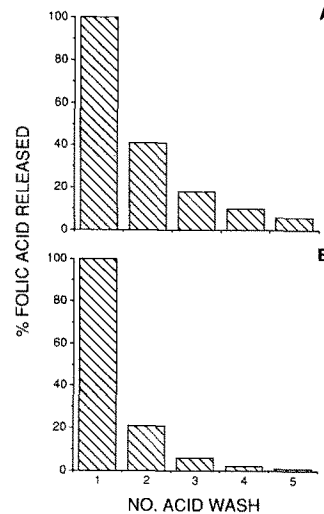
Receptor recycling is necessary for the sustained uptake of folate (9). The early plateau of [ $^3\text{H}$ ]folic acid internalization seen in Fig. 2 ( $-\bullet-$ ) prompted us to analyze the effects of cholesterol depletion on recycling (Fig. 4). Control and cholesterol-deplete cells incubated in the presence of [ $^3\text{H}$ ]folic acid for 4 h at 37°C had both internal and external receptors labeled. Treatment of these cells with an acid wash removed [ $^3\text{H}$ ]folic acid from external receptors (first wash, 100%; Fig. 4, A and B) but not internal receptors. Incubating the cells at 37°C for 30 min followed by chilling and acid stripping (second acid wash, Fig. 4, A and B) removed [ $^3\text{H}$ ]folic acid that returned from internal sites during the period. When this procedure was repeated three more times (third, fourth, and fifth acid wash, Fig. 4, A and B), any additional ligand that recycled to the cell surface was removed. A decreasing amount of ligand returned to the cell surface of both cells with each repetition, indicating that the internal pool of [ $^3\text{H}$ ]folic acid became depleted. The total amount of internal [ $^3\text{H}$ ]folic acid that came to the cell surface in control cells equaled 80% (sum of acid wash 2-5, Fig. 4 A) of the amount initially exposed at the cell surface (first wash, 100%; Fig. 4 A). This corresponds to a 1:0.8 ratio of external to internal receptors. By contrast, in cholesterol-depleted cells only 30% of what was initially bound returned (sum of acid wash 2-5, Fig. 4, B), which corresponds to a 1:0.3 ratio of external to internal receptors. Very little [ $^3\text{H}$ ]folic acid remaining associated with either set of cells at the end of the



**Figure 3.** Rate of change in the distribution of receptor-bound  $[^3\text{H}]$ folic acid at  $37^\circ\text{C}$ . MA 104 cells were prepared and labeled with  $[^3\text{H}]$ folic acid as described in Fig. 2. The internal (A) and external (B)  $[^3\text{H}]$ folic acid was measured as described. The rate of internalization (A) and loss from the cell surface (B) are calculated in units of pmol/h/mg of protein. The time scale matches Fig. 2 to compare the two experiments. Each value is the average of duplicate measurements. The variation in duplicate values was  $<10\%$  and the background in pmol/mg protein was: (A) control, 0.01 and cholesterol-depleted, 0.005; (B) control, 0.014 and cholesterol-depleted, 0.009.

last wash (data not shown), which indicates that there was not any delivery to the cytoplasm or nonspecific sticking of the label to the cell. Therefore, cholesterol depletion markedly reduced the size of the internal receptor pool but this low amount of receptor seemed to cycle to the cell surface normally.

We next measured the effects of temperature on receptor internalization in cholesterol-depleted cells (Fig. 5). Incubation of control and cholesterol-depleted cells in the presence of 5 nM  $[^3\text{H}]$ folic acid for one h at  $4^\circ\text{C}$  followed by washing and warming to either 37 (Fig. 5,  $37^\circ\text{C}$ ) or  $8^\circ\text{C}$  (Fig. 5,  $8^\circ\text{C}$ )

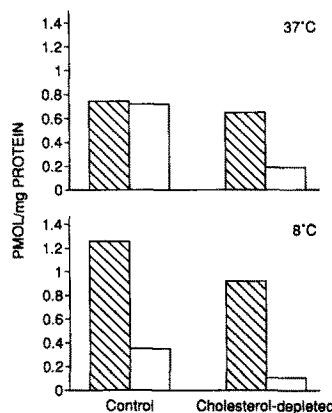


**Figure 4.** Effects of cholesterol depletion on folate receptor recycling. Cells were prepared as described in Fig. 1. The medium was replaced with medium D containing 5 nM  $[^3\text{H}]$ folic acid, and the cells were incubated for 4 h at  $37^\circ\text{C}$ . The same set of cells was subjected to the following protocol five times (*No. Acid Wash*): chilled to  $4^\circ\text{C}$ , washed with acid saline to release  $[^3\text{H}]$ folic acid, and warmed to  $37^\circ\text{C}$  for 30 min in medium B. The 100% values for control (A) and cholesterol-depleted (B) cells were 1.02 and 0.68 pmol/mg protein, respectively. Each value is the average of duplicate measurements. The variation in duplicate values was  $<10\%$  and the background was 0.023 pmol/mg protein in A and 0.006 pmol/mg of protein in B.

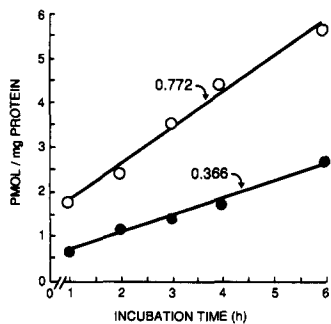
for 1 h allowed cells to internalize prebound ligand. Chilling the cells followed by an acid wash at  $4^\circ\text{C}$  removed only the folate bound to external receptors. The ratio of external to internal radioactivity in cells incubated at  $37^\circ\text{C}$  was 1:1 for control cells and 1:0.3 for cholesterol-depleted cells. The cells warmed to  $8^\circ\text{C}$ , on the other hand, had an external to internal ratio of 1:0.3 for control cells and 1:0.1 for cholesterol-depleted cells. Extending the warm-up period to 4 h did not change these ratios. Therefore, regardless of the incubation temperature cholesterol-depleted cells internalized  $\sim 70\%$  less  $[^3\text{H}]$ folic acid (the ratio of the two internal receptor pools was 1:0.3 at  $37^\circ\text{C}$  and 0.3:0.1 at  $8^\circ\text{C}$ ).

#### Inhibition of $[^3\text{H}]$ -5-Methyltetrahydrofolate Internalization

The previous experiments used  $[^3\text{H}]$ folic acid to monitor the effects of cholesterol depletion on folate receptor behavior. This folate remains tightly bound as the receptor moves in and out of the internal, membrane-bound compart-



**Figure 5.** Effect of temperature on the internalization of  $[^3\text{H}]$ folic acid. Cells were prepared as described in Fig. 1. Cells were chilled on ice for 20 min. The medium was replaced with medium D containing 5 nM  $[^3\text{H}]$ folic acid and then incubated for 1 h at  $4^\circ\text{C}$ . Medium D, either at  $37^\circ\text{C}$  ( $37^\circ\text{C}$ ) or  $8^\circ\text{C}$  ( $8^\circ\text{C}$ ), was added and then the cells were incubated for 1 h at the same temperature. The cells were chilled on ice to  $4^\circ\text{C}$  and external ( $\square$ ) and internal ( $\square$ )  $[^3\text{H}]$ folic acid was measured as described. Each value is the average of duplicate measurements. The variation in duplicate values was  $<10\%$  and the background ranged from 0.004 to 0.017 pmol/mg of protein.

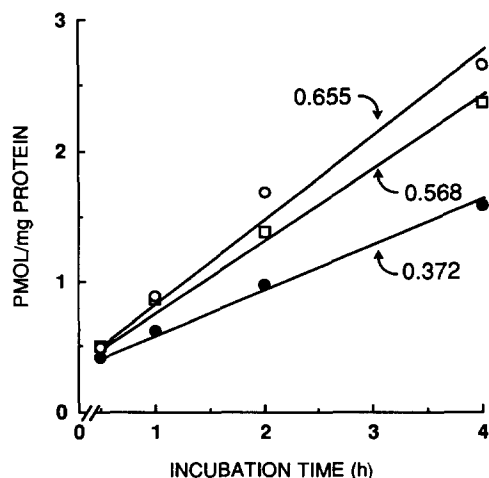


**Figure 6.** Effect of cholesterol depletion on the appearance of 5-methyl[<sup>3</sup>H]tetrahydrofolic acid in the cytoplasm. Control (○) and cholesterol deplete (●) cells were prepared as described in Fig. 1. The medium was replaced with medium D containing 20 nM 5-methyl[<sup>3</sup>H]tetrahydrofolic acid and then incubated at 37°C for the indicated time.

At the end of incubation, the cells were chilled to 4°C and the membrane and cytosol fraction were prepared as described. The rate of 5-methyl[<sup>3</sup>H]tetrahydrofolic acid delivery to the cytoplasm was measured in units of pmol/h/mg of protein. Each value is the average of duplicate measurements. The variation in duplicate values was <10% and the background was 0.122 and 0.073 pmol/mg of protein respectively for control and cholesterol-depleted cells.

ment, which allows the detection of receptor movement. Fig. 6 shows that cholesterol deprivation also inhibited the rate of [<sup>3</sup>H]-5-methyltetrahydrofolate delivery to the cytoplasm. The rate of delivery in cholesterol deplete cells (●, Fig. 6) was 0.366 pmole/h/mg of protein, a decline of 53% compared with control cells (0.772 pmole/h/mg of protein). Thus, 5-methyltetrahydrofolate delivery was inhibited to the same extent as folate receptor internalization.

The observed inhibition of internalization could be because of the side effects of the compactin treatment rather than cholesterol depletion. Therefore, we measured the ability of exogenously added cholesterol to reverse the inhibition of [<sup>3</sup>H]-5-methyltetrahydrofolate uptake (Fig. 7) in chole-



**Figure 7.** Internalization of 5-methyl[<sup>3</sup>H]tetrahydrofolic acid in cells grown in the absence of compactin (○), in the presence of compactin (●), or in the presence of compactin plus LDL (□). Cells were prepared as described in Fig. 1. 24 h before the beginning of the experiment, 25 μg/ml of LDL was added to one set of compactin-treated cells (□). The rate of 5-methyl[<sup>3</sup>H]tetrahydrofolic acid delivery to the cytoplasm was measured in units of pmol/h/mg of protein. Each value is the average of duplicate measurements. The variation in duplicate values was <10% and the background was 0.074, 0.062, and 0.053 pmol/mg of protein respectively for control, plus cholesterol and cholesterol-depleted cells.

**Table I.** Effects of Cholesterol Depletion on Internalization Index

Trial	Treatment	Surface Bound (a)	Internalized (b)	Index (b/a)
		LDL, ng/10 <sup>6</sup> cells		
1	+ Cholesterol	4.86	203.40	41.80
	- Cholesterol	43.20	1621.20	37.50
2	+ Cholesterol	8.23	278.40	33.80
	- Cholesterol	52.40	1484.80	28.30
5-methyltetrahydrofolate, pmol/10 <sup>6</sup> cells				
1	+ Cholesterol	3.00	6.15	2.05
	- Cholesterol	2.58	3.23	1.25
2	+ Cholesterol	4.55	8.50	1.87
	- Cholesterol	3.75	3.98	1.06

Cells were grown in the presence (- Cholesterol) or absence (+ Cholesterol) of 25 μM compactin as described in Fig. 1. Cells were washed with PBS and incubated in the presence of either medium D containing 20 nM 5-methyl[<sup>3</sup>H]tetrahydrofolic acid or medium E containing 10 μg/ml <sup>125</sup>I-LDL for 4 h at 37°C. At the end of the incubation, the surface-bound (acid releasable) and internalized 5-methyl[<sup>3</sup>H]tetrahydrofolic (acid resistant membrane fraction plus the cytoplasmic fraction) were measured as described. The surface-bound (heparin releasable) and internalized (heparin resistant plus degraded) <sup>125</sup>I-LDL were measured by the method of Goldstein et al. (6).

sterol-deprived cells. Incubating the cells in the presence of compactin plus serum lacking LDL inhibited uptake by 45% (compare control ○, with cholesterol deplete ●, Fig. 7). The uptake rate was restored to near normal when the cells received LDL as a source of cholesterol 24 h before the measurements (□, Fig. 7).

The internalization index is the amount of a ligand internalized in a period divided by the amount bound to receptors at the cell surface. The index measures the efficiency of uptake by a receptor-mediated process (1). The folate receptor is not internalized by clathrin-coated pits in MA104 cells even though LDL receptors do use this route (19). For this reason, we compared the effects of cholesterol depletion on the internalization index for LDL and folate (Table I). This treatment decreased the internalization index for LDL by ~13%, even though a lack of cholesterol caused a five- to sixfold increase in the number of LDL receptors. By contrast, cholesterol depletion reduced the internalization index for 5-methyltetrahydrofolate an average of 41%. These results also show that folate internalization in MA104 cells is far less efficient than LDL internalization.

## Discussion

The results of this study functionally connect together two previous studies showing that both folate receptor clustering (18) and the structure of caveolae (20) are dependent on membrane cholesterol. The impaired folate uptake observed in cholesterol-deprived cells can be directly attributed to the disruption of these two organizational features of the plasma membrane.

Cholesterol depletion had several effects on the behavior of the folate receptor: as judged by ligand binding, a 40% reduction in the total number of receptors; a shift in the steady distribution of surface membrane receptors so a greater proportion were external; and a markedly slowed internalization rate. An alteration in receptor traffic from the

Golgi apparatus to the cell surface can account for the first effect, although we do not have any direct evidence concerning the fate of newly synthesized receptors. The second effect can be attributed to the smaller size of each receptor cluster. This would result in fewer receptors over each caveolae, thus reducing the number of receptors available for internalization. Fewer receptors to internalize would reduce the size of the internal receptor pool at steady state. Finally, cholesterol depletion has been shown to reduce the number of caveolae (18). Fewer caveolae available for taking up receptors would cause a slower rate of folate internalization.

Other evidence also indicates that cholesterol-depleted cells have fewer functional caveolae. First, receptor recycling was unaffected by cholesterol depletion even though there were fewer internal receptors (Fig. 3 B). This is consistent with there being a residual population of functional caveolae capable of both internalizing receptors and returning them to the cell surface. Second, in normal cells only a subpopulation of caveolae are able to function at low temperature (9). We observed that at both 37 and 8°C there was a 70% decline in the number of internalized receptors in cholesterol-depleted cells (Fig. 5). Therefore, in both control and cholesterol-depleted cells low temperature appears to inactivate a constant percentage of those caveolae that are functional at 37°C.

Indirect immunofluorescence staining for folate receptor clusters showed that not all cells were equally affected by the cholesterol depletion protocol. We attribute this to an incomplete removal of cholesterol from the cell population. The functional caveolae as well as the normally clustered receptors most likely were enriched on the surface of cells that had not responded to the cholesterol lowering conditions. This heterogeneity accounts for why this protocol does not completely inhibit folate receptor internalization.

Other studies have shown that a variation in the cholesterol content of the cell membrane can alter the rate of transmembrane ion transport (3). Therefore, the altered transport of 5-methyltetrahydrofolate observed in cholesterol-depleted cells could have been due to changes in the function of the anion carrier that allows 5-methyltetrahydrofolate to reach the cytoplasm. The parallel reduction in the rates of both [<sup>3</sup>H]folic acid internalization and [<sup>3</sup>H]-5-methyltetrahydrofolate delivery to the cytoplasm argues that the primary effect of cholesterol depletion is on the function of receptors in caveolae.

Cholesterol depletion reduced the internalization index of 5-methyltetrahydrofolate (Table I), which is expected if receptor function is impaired. The LDL receptor internalization index, by contrast, was much less inhibited by this treatment. A requirement for cholesterol is expected for coated pit function because it is abundant in endosomal membranes (15) and the infectivity of Semliki Forest virus, which enter cells through coated pits, is inhibited in cholesterol-depleted cells (17). Nevertheless, these results indicate that a clear difference exists in the cholesterol requirement of these two endocytic pathways.

We do not know how cholesterol controls folate receptor clustering. On the other hand, the sterol may have its effect on caveolae function by altering membrane fluidity or reducing the ability of the caveolae to seal off from the extracellular space. Previous studies have found evidence for the presence of high concentrations of cholesterol around the rim of

each caveolae (22). Conceivably this cholesterol is essential for membrane-membrane interactions that are necessary for caveolae internalization.

The receptor-mediated uptake of folate involves a novel endocytic pathway called potocytosis (2). A model for this pathway proposes that many different GPI-anchored membrane proteins are able to generate high concentrations of physiologically important, low molecular weight molecules within closed caveolae. These molecules then move across the caveolae membrane by a carrier-mediated process. The effect of cholesterol depletion on folate uptake directly tests the model by showing that when sequestration in caveolae is impaired, the delivery of 5-methyltetrahydrofolate to the cytoplasm of the cell is inhibited. Cholesterol depletion may be a useful method for identifying other examples of GPI-anchored membrane proteins that deliver molecules to cells by potocytosis.

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