

Delivery of macromolecules into living cells: A method that exploits folate receptor endocytosis

(drug delivery/folate uptake/vitamin endocytosis)

CHRISTOPHER P. LEAMON AND PHILIP S. LOW*

Department of Chemistry, Purdue University, West Lafayette, IN 47907

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ABSTRACT Difficulties with the nondestructive delivery of macromolecules into living cells have limited the potential applications of antibodies, genes, enzymes, peptides, and antisense oligonucleotides in biology and medicine. We have found, however, that the natural endocytosis pathway for the vitamin folate can be exploited to nondestructively introduce macromolecules into cultured cells if the macromolecule is first covalently linked to folate. Thus, treatment of KB cells with folate-conjugated ribonuclease, horseradish peroxidase, serum albumin, IgG, or ferritin allowed delivery of $>10^6$ copies of the macromolecules within a 2-hr period. Cytochemical staining using 4-chloro-1-naphthol further demonstrated that the horseradish peroxidase retained activity for at least 6 hr after internalization. Since folate is an essential vitamin required in substantial quantities by virtually all cells, these observations may open the possibility of scientific and medical applications for many of the above macromolecules.

Because plasma membranes are designed to pass only those molecules required for the fitness of the cell, delivery of desired exogenous macromolecules into living cells has met with only limited success. Microinjection has permitted introduction of foreign proteins and nucleic acids into living cells, but the procedure works well only on a single-cell basis (1, 2). Electroporation, in contrast, is ideal for bulk delivery applications (3, 4); however, general cell damage is also a common consequence (4, 5). Sonication, freezing and thawing, osmotic shock, scrape or bead loading, detergent permeabilization, and other forms of cell lysis have also been employed with success in specific applications (6–9), but the inability to promote adequate resealing in some cells has limited their applicability (10–13). Erythrocyte-mediated cell fusion has also been used to deliver large amounts of macromolecules directly into the cytoplasm of cultured cells (14, 15), but the required use of fusogens, agglutinating lectins, or cytotoxic viruses, as well as the introduction of erythrocyte proteins into the target cells, may yield a system too modified for future study (16–18). Receptor-mediated endocytosis systems for natural ligands such as polypeptide hormones and viruses have been exploited to deliver desired macromolecules into certain cells (19–22). In many cases the success has been phenomenal, opening possibilities for useful medical applications. However, the absence of the desired receptors on some cell types, the ultimate deposition of most internalized molecules in the lysosomes, and the low capacities of the uptake pathways have restricted many uses of this strategy (23–25). Finally, liposome-mediated delivery has improved over the years with the conjugation of natural “recognition” ligands to the outer surfaces of the vesicles, allowing some degree of cell-type specificity (26–28). However, problems with liposome stability as well as low rates of

true cell-vesicle fusion must still be overcome before the method sees extensive application (29–31).

We have noted observations by others reporting that cells internalize vitamins such as folate by receptor-mediated endocytosis (32–34). Use of this pathway to deliver desired exogenous macromolecules into living cells seemed promising to us, since it suffered from few of the disadvantages of the aforementioned delivery protocols. Thus, unlike microinjection on scrape or bead loading, folate-conjugated proteins could conceivably contact and bind to all cells in a culture medium simultaneously. Similarly, membrane damage or alterations arising from several of the other methods would be avoided because macromolecular uptake would occur through a natural vitamin endocytosis pathway. And unlike hormone- or virus-mediated endocytosis, folate uptake is believed to occur in all dividing cells, at reasonable rates, and folate is deposited into cytosolic but not lysosomal compartments (34–36). We report here that by covalently conjugating folic acid to essentially any macromolecule, the macromolecule can be nondestructively delivered at reasonable levels into many living cells by the cellular uptake system for folate. A similar vitamin-mediated delivery system for biotin-conjugated proteins has been described in cultured plant cells (37).

MATERIALS AND METHODS

Na¹²⁵I was purchased from Amersham; Iodo-Beads were obtained from Pierce; electrophoresis chemicals were purchased from Bio-Rad; tissue culture products were purchased from GIBCO; and all cultured cells were received as a gift from the Purdue Cancer Center (West Lafayette, IN). All other chemicals were purchased from Sigma or were reagent grade chemicals from other major suppliers.

Cell Culture. KB cells were grown continuously as a monolayer using minimal essential medium (MEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin (50 units/ml), streptomycin (50 µg/ml), and 2 mM L-glutamine at 37°C in a 5% CO₂/95% air humidified atmosphere. Eight days prior to each experiment, cells were transferred for two consecutive passages into folate-deficient Dulbecco's modified Eagle's medium containing the same supplements mentioned above. The reasons for these passages in folate-low medium are 3-fold: (i) high folate concentrations competitively inhibit uptake of folate-conjugated macromolecules, (ii) the folate content of MEM is 1000-fold higher than that found in normal serum *in vivo* (38), and (iii) the 10% fetal calf serum contains its normal complement of folate, which is sufficient to sustain cell growth. On the day of each experiment, monolayers were rinsed twice with warm

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Abbreviations: BSA, bovine serum albumin; ¹²⁵I-BSA, ¹²⁵I-labeled BSA; ¹²⁵I-BSA-folate, folate-labeled ¹²⁵I-BSA; HRP, horseradish peroxidase; 5MeH₄folate, 5-methyltetrahydrofolate; FITC, fluorescein isothiocyanate; RITC, rhodamine B isothiocyanate.

*To whom reprint requests should be addressed.

phosphate-buffered saline (pH 7.4, PBS) and then harvested using repeated aspiration into warm 154 mM NaCl/5.6 mM KCl/1.1 mM CaCl_2 /3.2 mM Na_2HPO_4 /0.85 mM NaH_2PO_4 /10 mM glucose, pH 7.4, containing bovine serum albumin (5 mg/ml; BSA; BSA/Locke solution). Cell viability and cell titer were determined simultaneously using a hemocytometer and the vital dye trypan blue.

Modification of BSA, RNase A, Bovine IgG, Horseradish Peroxidase (HRP), and Their Conjugates. Radioiodination. BSA was dissolved in 0.1 M NaH_2PO_4 (pH 7.0) and added to prewashed Iodo-Beads. Na^{125}I was then added and the mixture was incubated for 50 min at 23°C, after which the free unreacted ^{125}I was separated from the labeled protein using a 10 cm \times 1 cm Sephadex G-25 desalting column equilibrated in PBS (pH 7.4). The eluted protein fractions were pooled and then divided in half. One-half of the solution [^{125}I -labeled BSA (^{125}I -BSA)] was further conjugated to folic acid (39), and the other half was left unmodified as a control (*vide infra*).

Fluorescent labeling. Protein was dissolved in a minimal volume of 50 mM NaHCO_3 (pH 8.4) and incubated for 3 hr at 23°C with a 10-fold molar excess of either fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate (RITC). Unreacted FITC or RITC was inactivated by the addition of a 50-fold molar excess of ethanolamine and then separated from the labeled protein using a Sephadex G-25 column equilibrated in PBS (pH 7.4). The eluted protein fractions were pooled and then divided in half. One-half of the solution was further conjugated to folic acid, and the other half was left unmodified as a control.

Folic acid or 5-methyltetrahydrofolic acid (5MeH₄folate) labeling. Folic acid or 5MeH₄folate was dissolved in dimethyl sulfoxide and incubated with a 2- to 10-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 30 min at 23°C (39). A 10- to 30-fold molar excess of the "activated" vitamin was next added to the protein solution and incubated for 4 hr at 23°C. Unreacted material was quenched with ethanolamine and removed as described for the radio/fluorescent labeling.

Characterization of folate/5MeH₄folate-labeled proteins. The extent of folic acid or 5MeH₄folate conjugation to proteins was determined spectrophotometrically by measuring the conjugated protein's difference spectrum at 363 nm [folic acid, $\epsilon = 6197$ in PBS (pH 7.4)] or 290 nm [5MeH₄folate, $\epsilon = 20,280$ in PBS (pH 7.4)]. In general 1–10 vitamins were covalently linked per polypeptide. Alternatively, qualitative identification of folate or 5MeH₄folate conjugates was conducted using indirect immunostaining of 5- μg dot blots of the various protein conjugates. Blots were incubated first with mouse anti-folic acid monoclonal antibody or mouse anti-5MeH₄folate monoclonal antibody, followed by incubation with goat-anti-mouse horseradish peroxidase (HRP) conjugate, and then developed using the HRP substrate 4-chloro-naphthol.

Phase-Contrast and Fluorescence Microscopy. After suspension in BSA/Locke solution, KB cells were deposited into the wells of a 24-well culture plate and incubated for 30 min at 37°C. Protein samples were then added to the cells to give a final protein concentration of 25 $\mu\text{g}/\text{ml}$. The cells were further incubated at 37°C for the indicated times, then collected, washed four times with 0.75 ml of ice-cold PBS, and then fixed at 4°C for 1 hr in 0.56% formalin. After one additional PBS rinse, cells were suspended in glycerin containing 5% aqueous propyl gallate and mounted on glass slides. Cells were photographed through an Olympus BHT microscope with a $\times 40$ phase-contrast/fluorescence objective.

Confocal Microscopy. KB cells were treated as described for phase-contrast and fluorescence microscopy except that the cells were fixed overnight in 0.56% formalin, washed, and then partially dehydrated in 50% (vol/vol) aqueous glycerin

overnight before suspension in the full-strength glycerin/propyl gallate solution. Cell-associated fluorescence was then imaged with a Bio-Rad MRC/500 confocal laser scanning microscope using a 514-nm excitation wavelength.

Measurement of the Time-Dependent Uptake of Folate-Conjugated ^{125}I -BSA (^{125}I -BSA-Folate). Folate-depleted KB cells were suspended in BSA/Locke solution and incubated for 30 min at 37°C as described for phase-contrast and fluorescence microscopy. ^{125}I -BSA-folate or ^{125}I -BSA was added to the cultured cells at a final concentration of 5 $\mu\text{g}/\text{ml}$. For the free folate competition studies, the water-soluble vitamin was added to the cell suspension 30 min prior to addition of the folate-conjugated BSA. The cells were further incubated at 37°C for the indicated times, collected, and washed four times with 0.75 ml of ice-cold PBS, and then cell-associated radioactivity was measured using a γ counter. The results are expressed as the number of molecules per cell based upon the known specific activities of the protein samples introduced to the cells.

Concentration Dependence of Binding and Internalization of ^{125}I -BSA-Folate. After suspension in BSA/Locke solution, KB cells were deposited into the wells of a 24-well culture plate and then incubated for 30 min at 37°C or 4°C. Labeled protein samples were then added to the cells at the indicated concentrations, and the cells were further incubated for 2 hr at either 37°C or 4°C. The ^{125}I -BSA-folate-treated cells were washed three times in either PBS (control) or 0.15 M NaCl adjusted with acetic acid to pH 3.0 (acid saline) to remove externally bound folates (40–42). Cell-associated radioactivity in final cell pellets was measured using a γ counter. Cell-surface binding capacity was estimated either from the radioactivity of the cells incubated at 4°C (where endocytosis is blocked) or from the radioactivity eluted by the acid saline wash. Internalized protein was then calculated from the difference between total cell-associated radioactivity and acid-releasable radioactivity.

Autoradiography of Internalized ^{125}I -BSA-Folate. Approximately 6.4×10^6 folate-depleted KB cells were resuspended in 4 ml of BSA/Locke solution and preincubated for 30 min at 37°C. ^{125}I -BSA-folate was added to a final concentration of 10 $\mu\text{g}/\text{ml}$ and the cells were incubated at 37°C for an additional 6 hr. The cells were washed three times with acid saline to remove externally bound ^{125}I -BSA-folate and then the entire cell sample was boiled for 3 min in 0.05 M Tris-HCl (pH 8) containing 2% (wt/vol) SDS, 20 mM EDTA, leupeptin (10 $\mu\text{g}/\text{ml}$), aprotinin (10 $\mu\text{g}/\text{ml}$), and 5% glycerin. DNA fragmentation was achieved by passage of the solution three times through a 21-gauge needle followed by three passages through a 26-gauge needle. By using an isocratic SDS/polyacrylamide gel electrophoresis system and 10% gels, 10 μg of stock ^{125}I -BSA-folate was loaded onto one lane of the gel, while 75% of the solubilized cell solution was loaded onto another. After electrophoresis, Coomassie blue staining, and gel drying, autoradiography of the two lanes was performed. The stock ^{125}I -BSA-folate autoradiogram was exposed for 30 min at -80°C , whereas the autoradiogram of the solubilized cell sample was exposed for 3 days at -80°C .

Cytochemical Staining of Internalized HRP-5MeH₄folate. After suspension in BSA/Locke solution, KB cells were deposited into the wells of a 24-well culture plate and then incubated for 30 min at 37°C. The cells were treated with either HRP-5MeH₄folate or unmodified HRP at 10 $\mu\text{g}/\text{ml}$. After a 4-hr incubation at 37°C, cells were collected and washed four times with 0.75 ml of ice-cold PBS, and then stained for HRP using the substrate 4-chloro-1-naphthol as described (43), except 0.06% H_2O_2 was employed.

RESULTS

Uptake of Fluorescent Folate-Labeled Proteins. Bovine IgG and bovine RNase were first labeled with FITC or RITC and

then further conjugated to folic acid or left unmodified as controls. In this manner the same specific fluorescence was present on both folate-conjugated and control samples in all experiments. When folate-conjugated FITC-labeled IgG was added to KB cells (a human nasopharyngeal cell line), a rapid avid association of the protein with the membrane was observed that was sustained through five washes. As seen by a comparison of the phase-contrast and fluorescence images of the same cell clusters in Fig. 1 *A–H*, folate-conjugated IgG was found to bind avidly to the cell surface within 5 min of mixing and then to gradually enter the cell's interior over a period of several hours. In contrast, when the same cells were treated with FITC-labeled IgG lacking the attached folate, no binding or internalization was observed (Fig. 1, *I–L*). Because the only difference between the two samples was the covalent attachment of folate, these data suggest that covalently attached folate can promote the uptake of fluorescent IgG into KB cells. To ensure that the internalization process was neither protein nor cell type specific, the same study was conducted with RNase and BSA in HeLa cells with virtually identical results (data not shown).

Because the fluorescence images of the later time points in Fig. 1 (*F* and *H*) revealed the possible presence of IgG-containing vesicles within the cytoplasm, a more thorough examination of the distribution of fluorescence in the cells was conducted using the horizontal plane selectivity of the confocal microscope. One representative plane of focus in KB cells treated with folate-labeled RNase for 30 min is

shown in Fig. 1*M*. Similar to other planes of focus, circular spots of intense fluorescence $\approx 0.5 \mu\text{m}$ in diameter were clearly identified.

Time-Dependence of the Association of ^{125}I -BSA-Folate with KB Cells. To more accurately evaluate the time course of folate-mediated protein uptake, the binding and internalization kinetics were quantitatively monitored using folate conjugated to ^{125}I -labeled BSA. Fig. 2 shows that 3.5×10^6 molecules of folate-conjugated BSA became KB cell-associated within the first half-hour of incubation compared with only 0.18×10^6 molecules of the unconjugated BSA. That this cell association was folate-receptor-mediated could be demonstrated by the ability of a 100-fold molar excess of free folate to competitively block the interaction (see Fig. 2). Thus, in the presence of the free folate, association of BSA-folate was no more apparent than binding of the unconjugated BSA. Importantly, the kinetics of the interaction of BSA-folate with the KB cells are similar to those published for the association of free methotrexate (a folate analogue), with the same cells, both exhibiting half-maximal binding 3 min after mixing (44).

Concentration Dependence of the Association of ^{125}I -BSA-Folate with KB Cells. To further confirm the involvement of a cell surface folate receptor in mediating the internalization of folate-conjugated proteins, the binding and internalization of folate-conjugated BSA were evaluated as a function of the concentration of the BSA. As shown in Fig. 3, incubation of the cells with BSA-folate for 2 hr at 4°C led to a maximal

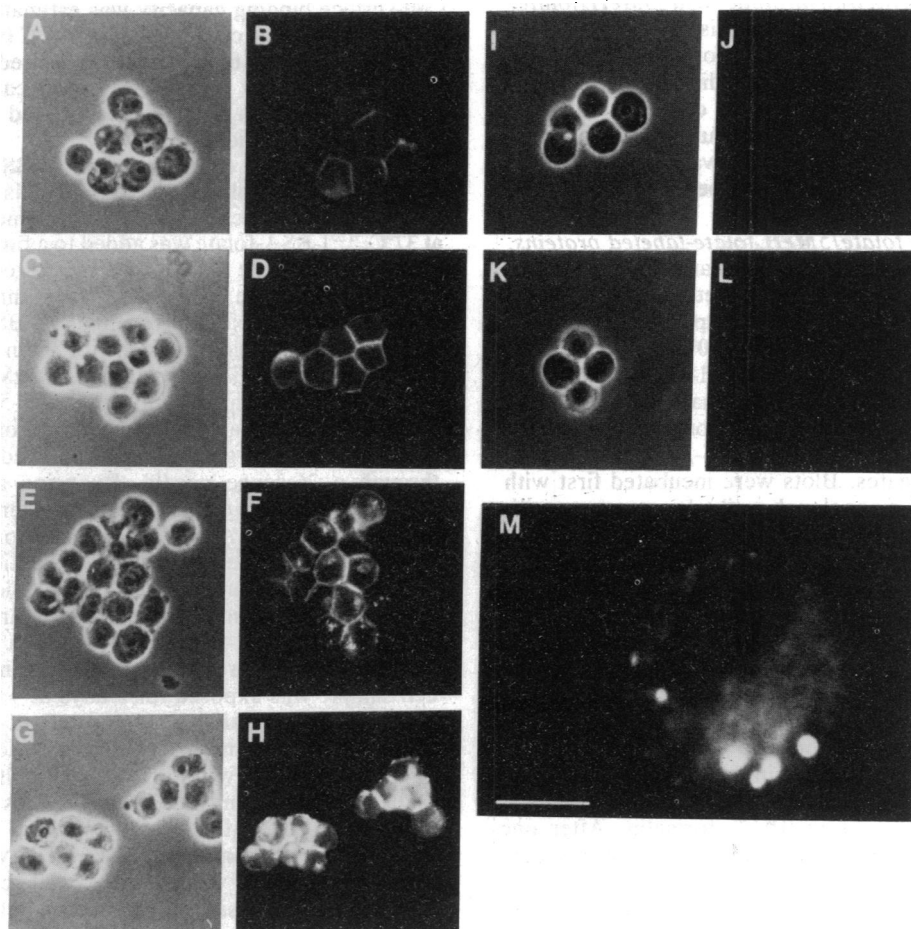


FIG. 1. Uptake of fluorescent folate-labeled proteins by KB cells. (*A*, *C*, *E*, and *G*) Phase-contrast micrographs of KB cells incubated with bovine IgG-FITC-folate ($25 \mu\text{g}/\text{ml}$) for 5, 30, 60, and 360 min, respectively. *B*, *D*, *F*, and *H* are their corresponding fluorescent micrographs. (*I* and *K*) Phase-contrast micrographs show KB cells that were incubated for 5 and 360 min, respectively, with bovine IgG labeled to the same extent with FITC but lacking any conjugated folate. *J* and *L* are their corresponding fluorescent micrographs. (*M*) Confocal image of a single KB cell incubated for 30 min with RNase A-RITC-folate ($25 \mu\text{g}/\text{ml}$). (*A–L*, $\times 30$; *M*, bar = $5 \mu\text{m}$.)

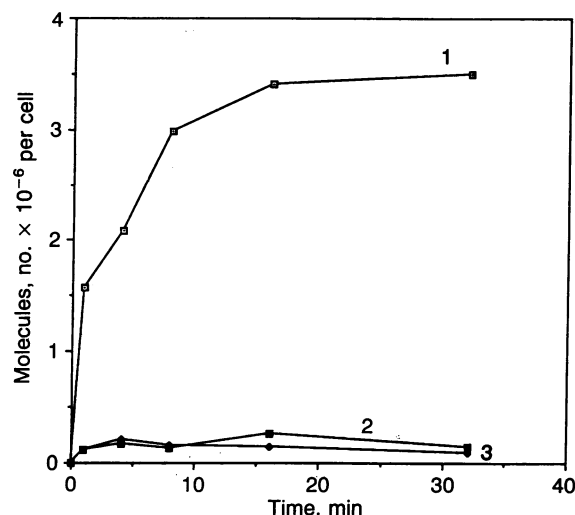


FIG. 2. Time-dependent cell association of ^{125}I -BSA-folate with cultured KB cells. KB cells were incubated for the indicated times with ^{125}I -BSA-folate ($5\text{ }\mu\text{g/ml}$; curve 1), ^{125}I -BSA ($5\text{ }\mu\text{g/ml}$; curve 2), or ^{125}I -BSA-folate ($5\text{ }\mu\text{g/ml}$) plus 100-fold molar excess of free folate (curve 3).

binding of 1.3×10^6 molecules per cell. Because endocytosis is blocked at this low temperature, we hypothesized that this population of bound molecules was folate receptor associated but not internalized. To confirm this point, the cells were briefly treated with acid saline, a procedure commonly used to remove cell surface folate without rupturing the cells (40–42). As anticipated, the acid wash released essentially all of the BSA-folate associated with cells incubated at 4°C (see Fig. 3). In contrast, if the suspension was maintained at the growth temperature of 37°C for the full 2-hr incubation, a maximum of 4×10^6 molecules of BSA-folate became tightly associated with the cell. This saturating amount of cell-associated BSA-folate lies within the range of values reported for folate and folate analogue association with KB cells under similar conditions (38). More importantly, washing of these cells with acid saline to remove the surface bound protein-folate conjugates still left roughly half of the radioactivity associated with the cell (data not shown). Based on the

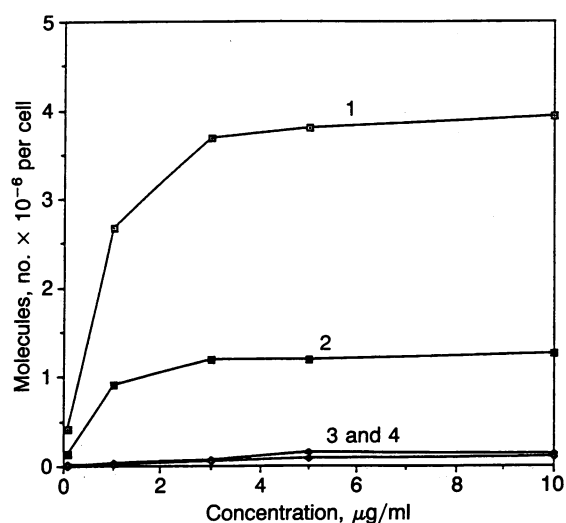


FIG. 3. Concentration-dependent association of ^{125}I -BSA-folate conjugate with KB cells. Cells were incubated for 2 hr with the indicated concentrations of ^{125}I -BSA-folate conjugate at 37°C (curve 1), ^{125}I -BSA-folate conjugate at 4°C (curve 2), ^{125}I -BSA at 37°C (curve 3), or ^{125}I -BSA-folate conjugate at 4°C followed by three acid-saline washes to remove bound folates (curve 4).

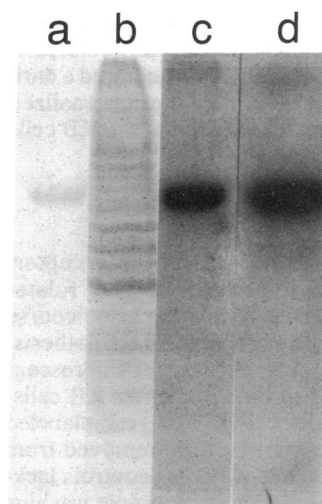


FIG. 4. Autoradiography of internalized ^{125}I -BSA-folate after SDS/polyacrylamide gel electrophoresis. Lanes: a and c, stock ^{125}I -BSA-folate ($10\text{ }\mu\text{g/ml}$); b and d, KB cells solubilized in SDS solution after three acid-saline washes. Lanes a and b are photographs of the Coomassie blue-stained gels. Lanes c and d are the autoradiographs of lanes a and b, respectively.

known uptake of folate by KB cells (38, 44, 45) and our observation of internalized protein conjugates in Fig. 1, we suggest the acid-inaccessible radioactivity corresponds to the endocytosed population of the folate conjugates.

Autoradiography of Internalized ^{125}I -BSA-Folate. To ensure that the internalized BSA-folate was not carried into the cells as digested fragments or peptides of BSA, the KB cells were allowed to associate with the BSA-folate for 6 hr (i.e., sufficient time for much of the folate-conjugated protein to enter the cell and to potentially be digested) and then washed with the acid-saline stripping solution to deplete the cells of surface bound conjugate. When the washed cells were then solubilized in SDS and separated electrophoretically on polyacrylamide gels, the resulting autoradiograph showed no evidence of BSA degradation (Fig. 4). We, therefore, conclude that the BSA-folate entering the cells does so as the intact macromolecule.

Cytochemical Staining of Internalized HRP-5MeH₄folate. Finally, to unequivocally establish that a functional macromolecule can be delivered into a living cell by the folate endocytosis pathway and to concurrently explore whether another form of folate might also mediate protein endocytosis, HRP was either conjugated to 5MeH₄folate or left underivatized and then added to a KB cell suspension. After 37°C incubation, washing, and fixation, the cells were stained with H_2O_2 and 4-chloro-1-naphthol to visualize the location of

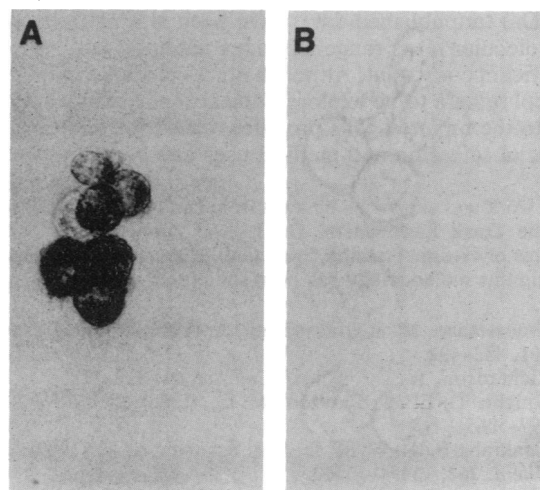


FIG. 5. Cytochemical staining of internalized HRP-5MeH₄folate. KB cells were incubated for 6 hr at 37°C with HRP-5MeH₄folate ($10\text{ }\mu\text{g/ml}$) (A) or HRP alone ($10\text{ }\mu\text{g/ml}$) (B) and were washed and stained for enzyme activity using 4-chloro-1-naphthol.

the catalytically active HRP. As seen in Fig. 5, cells incubated with unconjugated HRP remained essentially colorless, whereas cells treated with HRP-5MeH₄folate acquired a dark purple stain. We conclude from these data that internalized HRP retains its catalytic function upon entering the KB cells by the folate-mediated pathway.

DISCUSSION

Since folate is internalized into KB cells by a receptor-mediated event (38, 44), we hypothesized that folate-conjugated proteins should likewise follow the same course of uptake, namely endocytosis. In support of this hypothesis, we can now list the following observations: (i) fluorescent folate-labeled proteins are seen to bind and enter KB cells, whereas folate-free proteins are not (Fig. 1), (ii) radiolabeled proteins conjugated to folate cannot be fully removed from KB cells even by acid-saline washes, whereas controls lacking folate could be cleared of radioactivity by simple washing in PBS (Fig. 3), (iii) exogenous HRP activity could be detected inside cells treated with 5MeH₄folate-conjugated enzymes but not in cells treated with free enzyme (Fig. 5), (iv) binding and uptake of folate-conjugated protein could be competitively blocked by free folate (Fig. 2), and (v) the time and concentration dependence of folate-conjugated protein uptake follows closely the uptake of free folate by the same cell line (Figs. 2 and 3).

It is also apparent from our data that the folate conjugates are internalized in a nondestructive manner and that the macromolecules conjugated to folate remain in an active state (Figs. 4 and 5). These data support previous claims that folate is internalized into cells by a nonlysosomal pathway (34–36).

The studies displayed in the above figures were all conducted on KB cells largely because folate uptake by these cells had already been substantially characterized (36, 38, 40, 44, 45). However, in the course of our research many experiments were also conducted on HeLa, XC, MCF7, and PC12 cells in culture. Furthermore, in some studies (e.g., Fig. 5) 5MeH₄folate was substituted for folic acid. Except for differences in rates and magnitudes of uptake (XC, PC12, and MCF7 cells internalize conjugates much slower than KB and HeLa cells), the results from all of the above studies were similar. It would, therefore, appear that many cells have the ability to internalize folate-conjugated macromolecules in reasonable quantities by a folate receptor-mediated mechanism. Since proteins ranging in size from RNase (13.7 kDa) to HRP (40 kDa) to BSA (68 kDa) to IgG (160 kDa) to ferritin (443 kDa) (unpublished data) have been internalized, within this molecular mass range size does not appear to represent a significant constraint. Although practical applications of the protocol remain to be explored, the absence of any apparent harm to the targeted cells provides reason for optimism that beneficial scientific and medical uses can be identified.

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