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Protein Microinjection by Protease Permeabilization of Fibroblasts

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Exposure of cultured diploid fibroblasts to protease solutions induces a hyperpermeable state which permits entry of exogenous macromolecules directly into the cytosol. We have exploited this finding to devise a microinjection method whose chief advantages are simplicity and good retention of cell viability. Proteins successfully injected by this technique range from insulin to thyroglobulin. The amounts injected range from 4×10^5 to 5×10^6 molecules/cell.

KEY WORDS: microinjection; fibroblasts; permeabilization; proteolysis; protein; cystinosis.

Delineation of the loci and regulatory mechanisms of cellular proteolysis is assisted by the direct introduction of a labeled protein into the cytosolic compartment of cultured cells. A number of strategies have been devised to achieve this end. They have in common the production of transient defects in the plasma membrane, followed by entry of labeled protein into the cytosolic compartment. Successful methods include erythrocyte ghost loading (1,2), hypotonic–osmotic lysis (3), scrape loading (4), detergent permeabilization (5), and electroporation (6). In our experience, all of the above lead to poor cell viability and/or small amounts of protein injected. In this paper we describe the technique of protease permeabilization of cultured fibroblasts. This method, which employs standard protease solutions used in passaging cells, permits introduction of labeled protein directly into the cytosolic compartment with cell viability after treatment equal to that of simple passaging. The procedure requires no special techniques or equipment and appears superior to previously described methods.

MATERIALS AND METHODS

Diploid fibroblast cultures were derived from skin biopsies or necropsy material of patients with nephropathic cystinosis and maintained in Coon's-modified Ham's F_{12} medium (GIBCO), supplemented with 15% (v/v) fetal bovine serum and incubated at 37°C in a humidified incubator flushed with an air:CO_2 (19:1) mixture. DFL is a fetal lung fibroblast line from a cystinotic fetus. Control normal fibroblasts (GM 0010) and cystinotic fibroblasts (GM 0090) were purchased from the Human Genetic Mutant Cell Repository. Cells were harvested by trypsin treatment and cell number determined by a Model Z_F Coulter counter (Coulter Instruments, Inc., Hialeah, FL). ^{125}I was determined in a Tracor gamma counter. Counting efficiency for ^{125}I was 65%. ^{125}I lysozyme (sp act, 219 μCi/mg) was a custom purchase from ICN; the other radiochemicals were purchased from New England Nuclear. The specific activity of radiolabeled compounds was BSA,[3] 82.2 Ci/mmol;

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[3] Abbreviations used: BSA, bovine serum albumin, 5-IAF, 5-iodoacetamide fluoresceine; SDS, sodium do-

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insulin, 5 Ci/mg; thyroglobulin, 5.4 mCi/mg; [^{14}C]leucine, 348.3 mCi/mmol; and [^{3}H]mannitol, 19.1 Ci/mmol.

Pronase (activity, 87,110 puk/g) was purchased from Calbiochem-Behring Corp. Collagenase (207 u/g) was the product of Worthington Diagnostic Systems, Inc. 5-Iodoacetamide fluoresceine was purchased from Molecular Probes. 5-IAF–BSA was prepared as described (7). All other reagents were purchased from Sigma Chemical Co. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli utilizing a 4% stacking gel and a 10% analytical gel (8). Autoradiography was performed by exposing the dried gel to X-ray film at −70°C.

MICROINJECTION PROCEDURE

Confluent tissue culture plates of diploid fibroblasts were washed once with phosphate-buffered saline, pH 7.4, containing 0.1 g/liter calcium chloride, rinsed with trypsin (Difco 1:250, 2.5 g/liter in PBS with 0.201 g/liter EDTA), and incubated at 37°C for approximately 5 min (the optimum time varied with cell line between 2 and 6 min). The cells were then detached from the dish by pipetting in 5 ml of Coon's-modified Ham's F_{12} medium supplemented with 15% fetal calf serum. The cells were removed from the F_{12} medium by centrifugation for 4 min at 600*g*. The pelleted cells were resuspended in 1 ml of F_{12} without fetal calf serum, containing the protein to be injected, and after a gentle vortexing, incubated in suspension in a 37°C water bath for periods of time as described. At the end of the 37°C incubation the cells were collected by centrifugation at 600*g* for 4 min and then washed twice by vortexing in 10 ml of phosphate-buffered saline containing calcium chloride and 5 mg/ml of bovine serum albumin. After resuspension in F_{12} medium, an aliquot of cells was removed for radioactivity and cell number determination, and the cells plated in culture dishes. Cell survival was determined by retrypsinizing the cells after a 2-h period during which they were allowed to attach to the culture dish. The number of cells recovered at this point, divided by the number of cells present after the initial exposure to trypsin for microinjection, was taken as a measure of cell viability. Line GM 0010 was unusually sensitive to trypsin, with poor survival after a standard 5-min exposure. Experiments using this line were routinely exposed to trypsin for only 2 min.

Cells microinjected by permeabilization with pronase or collagenase were exposed to 0.5 mg/ml collagenase in 0.25 M sucrose, 10 mM Tris, pH 7.4, with 5 mM $CaCl_2$, or to 0.025 mg/ml pronase in PBS. The optimal incubation period was 15–20 min for collagenase and 2 min for pronase. After this variation, the remainder of the microinjection procedure was as for trypsin.

Unless otherwise specified, all microinjection experiments described in this report used a standard 5-min exposure to trypsin followed by a 20-min incubation at 37°C for microinjection. To permit comparison between experiments which employed differing concentrations of radioactivity, uptake is expressed as the volume of medium whose contained substrate had been captured, per 10^6 cells (μl/10^6 cells) (9,10).

RESULTS

After exposure to trypsin, fibroblasts become hyperpermeable to proteins in the medium in which they are suspended. In Fig. 1 is shown the mean uptake of ^{125}I-BSA in three cells lines as a function of prior exposure to trypsin. After trypsin treatment for the indicated time, they are transferred to microinjection medium and incubated suspended in that medium for 20 min as described under Materials and Methods. Uptake increases after an initial lag period of about 2 to 3 min. At this point the cells have

decyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEG, polyethylene glycol; IgG, immunoglobulin G.

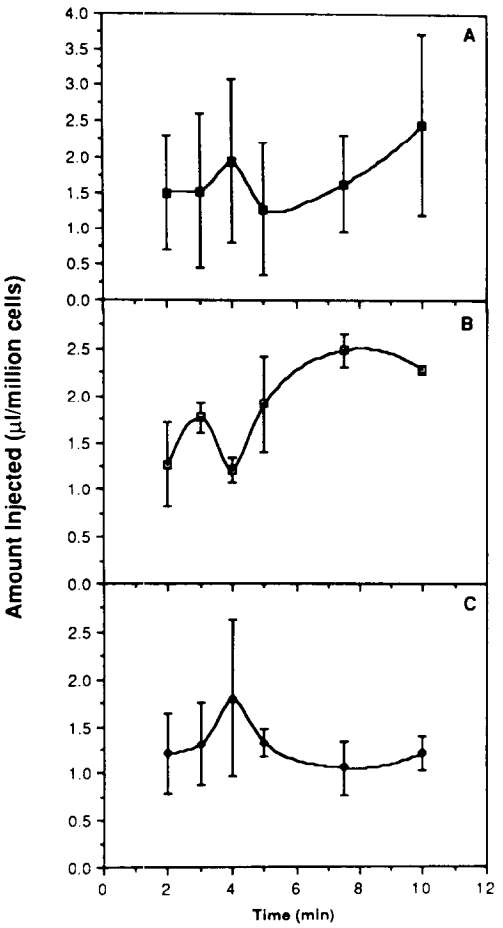


FIG. 1. The effect of duration of incubation in trypsin on subsequent uptake of ^{125}I-BSA. Cells were incubated in trypsin as described under Materials and Methods and removed from the tissue culture dish at the intervals shown, after which they were suspended in medium containing 2×10^6 cpm/ml of ^{125}I-BSA for 20 min. The cells were then washed twice with PBS containing 5 mg/ml unlabeled BSA, and aliquots were removed for cell number and radioactivity determination. Cell lines were (A) GM 0090, (B) DFL, and (C) GM 0010. Error bars are means ±1 SD for (A) and (C); (B) shows the extremes of duplicate plates.

begun to "round up" on the culture dish. Continued incubation in trypsin further enhances uptake; however, cell viability declines after 7 min. For most cell lines, 4 to 5 min in trypsin yielded the best compromise between uptake and viability.

After a 5-min exposure to trypsin, resuspended cells take up the labeled protein in the injection medium for 30 min (Fig. 2). Cell viability declined at longer periods of incubation; hence 20 min was selected as the best compromise between cell viability and injection. Enhanced uptake of ^{125}I-BSA is not limited to trypsin treatment alone. A similar increase in uptake was obtained after exposure of cultured cells to pronase and collagenase (see Fig. 2). Pronase (ex. *Streptococcus griseus*) is a proprietary product with wide protease activity. It rapidly detached fibroblasts from the culture dish and led to cell death after more than 2 min of exposure. Collagenase had a much slower effect, with detachment not occurring until 15–20 min of incubation.

To demonstrate that the protein is entering directly into the cytosolic compartment and not merely binding to the cell surface or being pinocytosed at a greatly enhanced rate, trypsin-treated cells were exposed to culture medium containing 3 mg/ml of fluorescein-labeled albumin for 20 min, fixed in Karnofsky's buffer, and examined by fluorescence microscopy. Additional trypsin-treated cells were plated and fixed 2 h after reattachment. Non-trypsin-exposed control cells were fixed on the culture slide after a 20-min exposure to fluorescein–BSA. In Fig.

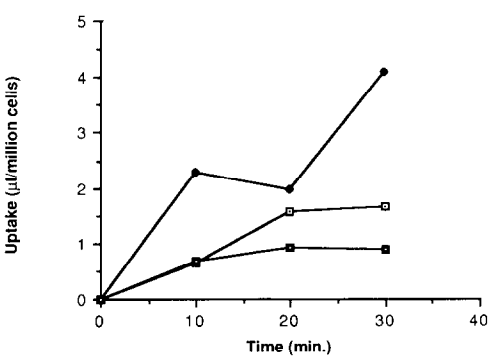
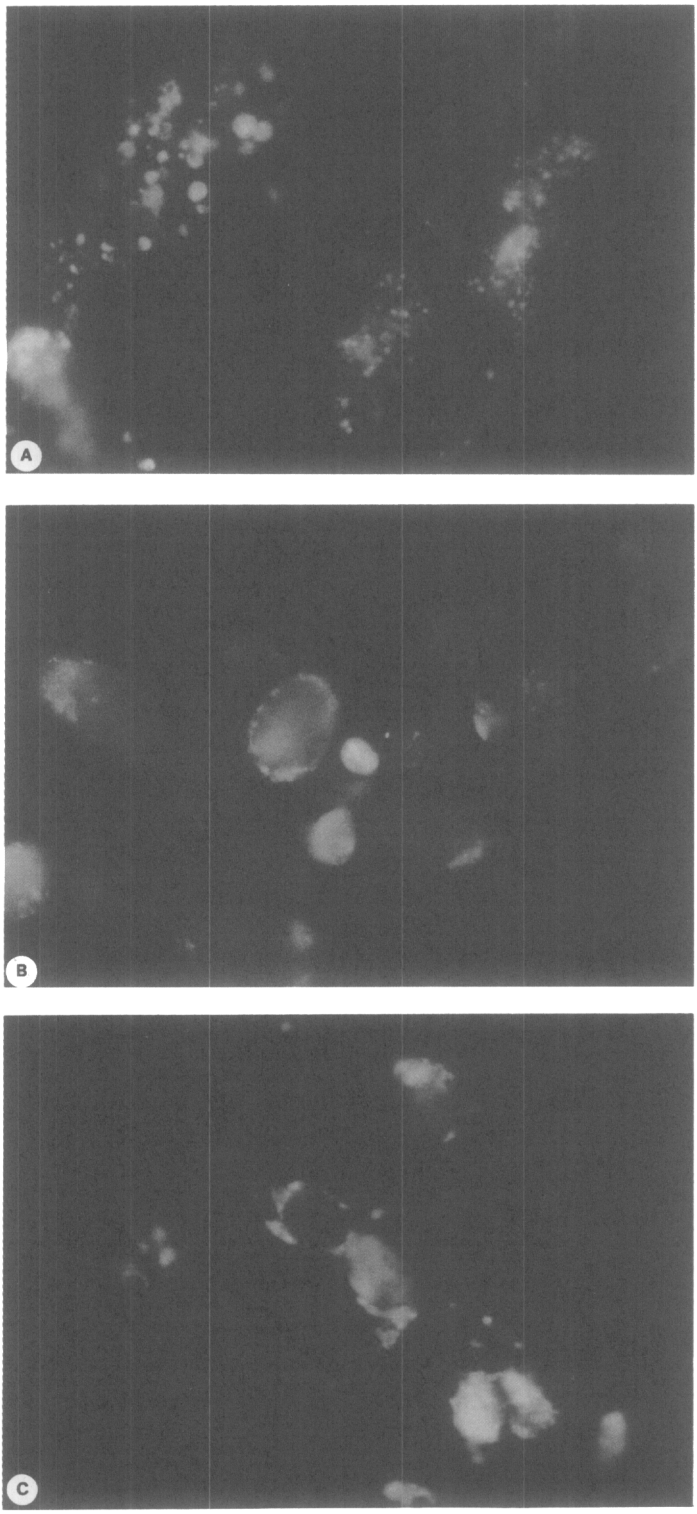


FIG. 2. The effect of exposure to three proteases on the subsequent uptake of ^{125}I-BSA by cell line GM 0090. The cells were incubated in trypsin (□), pronase (□), or collagenase (♦) as described under Materials and Methods. then washed and suspended in ^{125}I-BSA-containing medium, and harvested at the indicated times for cell count and radioactivity determination.


A
B
C

3A is shown the typical pattern of pinocytic vesicles resulting from pinocytosis of the fluorescein–BSA by attached cells during the 20-min incubation. In contrast, Fig. 3B shows a diffusely fluorescent cytoplasm in the microinjected cells, consistent with permeation of the fluorescent albumin into the cytosol. Two hours after microinjection, the cells have reattached (C) but still retain diffuse fluorescence.

The cytosolic localization of injected material was further verified by microinjecting ^{125}I-lysozyme. Lysozyme has been shown to be preferentially degraded in the cytosol after conjugation to ubiquitin. Ubiquitination of lysozyme prior to degradation leads to a "ubiquitin ladder" on PAGE representing increasing molecular weight of the substrate as it is conjugated to multiple ubiquitin molecules (11–13). ^{125}I-lysozyme-injected cells were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography as shown in Fig. 4. In lane 2, a number of iodinated species of molecular weight greater than that of the injected lysozyme are seen, consistent with polyubiquitination. One and four hours later, all the higher molecular weight forms are absent, and less lysozyme is present, presumably due to proteolysis (lanes 3 and 4). No higher molecular weight forms are seen when the same medium is placed on cells not previously exposed to trypsin (lanes 5–7). Since an equal number of cells is represented in each lane (10^6), Fig. 4 also demonstrates the much greater uptake of protein by trypsin-treated cells (lanes 2–4) versus pinocytosis (lanes 5–7).

Known inhibitors of pinocytosis (NaCN, colchicine) (10,14) were found not to inhibit the uptake of ^{125}I-BSA after trypsin treatment, further supporting the contention that protease treatment results in cytosolic rather than pinocytic localization of the injected material (data not shown).

A direct comparison of the amount of protein microinjected with that pinocytosed in the same interval was accomplished by parallel experiments in which the same medium was placed on tissue culture plates not exposed to trypsin and on trypsin-permeablized cells in suspension. In these experiments the mean uptake in 20 min of ^{125}I-BSA was 0.31 μl/million cells in line DFL after trypsin treatment, but only 0.04 μl/million cells in those not trypsin-treated. The corresponding values for trypsin versus non-trypsin-treated cells are 3.58 versus 0.32 in line GM 0090 and 1.48 versus 0.19 in line GM 0010. The difference between trypsin-treated and control cells in the uptake of BSA for all cell lines is significant at $P = 0.02$ by Student's paired t test. There is significant line to line and day to day variation in the amount of ^{125}I-BSA taken up; however, in all lines the trypsin-treated cells took up 7 to 10 times as much protein as the control cells.

To ascertain if the cells were permeabilized to small molecules as well, the uptake of [^{3}H]mannitol and [^{14}C]leucine was measured after trypsin treatment. Uptake of [^{14}C]leucine by trypsin-treated cells showed no saturation at concentrations of leucine of up to 20 mM (see Fig. 5). When [^{3}H]mannitol uptake was studied, a rate of 0.20 ± .07 μl/10^6 cells/min was obtained. Previously established rates of uptake of membrane impermeant solutes into substrate anchored cells are between 0.002 and 0.02 μl/10^6 cells/min (15).

A number of fibroblast cell lines and proteins have been shown to be susceptible to this technique (Table 1). The mean uptake of ^{125}I-BSA in three fibroblast lines (GM 0090, GM 0010, and DFL) varied between 0.86

FIG. 3. Fluorescent (A, B, C) photomicrographs of fibroblasts exposed to fluorescein–BSA for 20 min while substrate attached (A), following trypsinization (B), or 2 h after reattachment (C). Note pinosomes in attached spindle-shaped cells (A and C), as opposed to the diffuse cytoplasmic staining in spherical cells in suspension (B). After reattachment the cells regain the spindle shape (C). Final magnification before photographic enlargement: (A) 650×; (B) and (C) 260×.

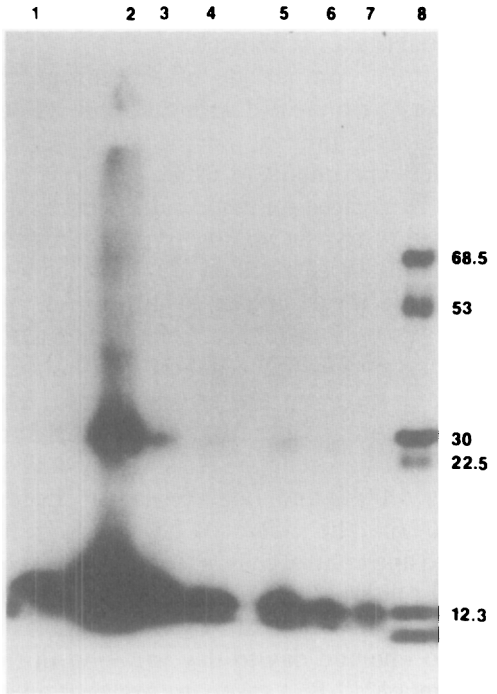


FIG. 4. The fate of lysozyme microinjected by trypsin permeabilization compared to pinocytosed lysozyme. Cultures of GM 0010 were exposed to trypsin for 2 min, detached from the culture dish, and suspended in medium containing 2.98×10^7 cpm/ml of ^{125}I-lysozyme at 37°C for 20 min. The cells were then washed twice in PBS containing 5 mg/ml unlabeled lysozyme and plated. At 0 time and 1 and 4 h after plating, the cells were removed from culture, pelleted, and dissolved in running buffer and an aliquot equal to 10^6 cells was analyzed by SDS–PAGE. The dried gel was then subjected to autoradiography as described under Materials and Methods. Control cells were identical except prior trypsin exposure was omitted. Lane 1, ^{125}I-lysozyme medium. Lanes 2–4, cells preexposed to trypsin at 0, 1, and 4 h after the incubation in suspension. Lanes 5–7, controls cells at 0, 1, and 4 h. Lane 8, molecular weight standards. A naturally occurring dimer is seen at M_r 28,000 in the initial medium and cells at all time points.

and 1.98 μl/million cells in a 20-min injection. The size of protein which can enter protease-permeabilized cells varied widely. As shown in Table 1, proteins taken up ranged from insulin (M_r 6000) to thyroglobulin (M_r 660,000). There was no apparent correlation between the amount taken up and the molecular weight of the protein. The number of molecules per cell of each protein injected is also shown in Table 1. This number varied between 4×10^5 and 5×10^6 molecules/cell.

Cell viability was assessed by harvesting cells after microinjection and attachment and comparing the number recovered to that present after initial trypsin treatment. In 15 separate experiments employing three fibroblast lines the mean survival expressed as above was 51 ± 2.3%. This measure of viability, attachment, is similar to that found after simply passing the cell using typsin. A recent paper reported 54.4% attachment after trypsinization (16).

DISCUSSION

Of the many techniques currently available for introducing exogenous macromolecules into the cytoplasmic compartment of cultured cells, none appears to fulfill all the desirable criteria. Some (e.g., erythrocyte ghost fusion, direct microinjection, electroporation) require large amounts of prepara-

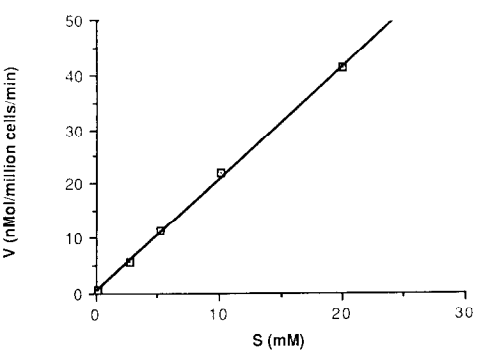


FIG. 5. The uptake of [^{14}C]leucine into trypsin-treated cells in suspension. The cells were suspended in 1.0 ml medium containing [^{14}C]leucine at a final concentration of 2.2 to 20.2 mM leucine and 100-μl aliquots taken at intervals of 0.5, 1, 2, 4, 6, and 8 min. The aliquot was immediately pipetted into 1 ml PBS at 4°C and centrifuged. The pellet was washed once in PBS at 4°C and then dissolved in scintillation fluor for radioactivity determination. Duplicate aliquots were used for cell number determination and the results expressed as nanomoles per 10^6 cells for each interval. Initial rates were used to calculate the velocity of uptake.

TABLE 1

PROTEASE-MEDIATED PROTEIN UPTAKE BY CULTURED FIBROBLASTS

A. Cell line		Amount injected (μl/10^6 cells)	
GM 0090		0.86 ± 0.36 (n = 9)	
GM 0010		1.98 ± 1.5 (n = 11)	
DFL		1.09 ± 0.35 (n = 5)	
B. Protein	(M_r)		Molecules/cell
Insulin	6,000	0.97	5.84×10^5
Lysozyme	14,000	3.27	4.93×10^6
BSA	68,500	0.52	3.6×10^5
Thyroglobulin	660,000	2.98	1.8×10^6

Note. In part A, the amount injected represents the mean ±1 SD of n experiments employing the standard exposure to trypsin and uptake of ^{125}I-BSA. In B, line GM 0010 was treated with trypsin and then exposed to the indicated proteins as under Materials and Methods. The data in part B are for single experiments with each protein. The concentrations of insulin, thyroglobulin, and BSA were each 1.0 μM; lysozyme was 2.5 μM.

tion and/or special equipment. Others (e.g., scrape loading, hypotonic osmotic lysis) yield poor cell viability, load relatively small amounts of protein, or introduce only a restricted range of molecules (2). Erythrocyte ghost loading in a specialized cell line has been reported to deliver 1.8×10^8 molecules of horseradish peroxidase per cell, and 1.4×10^7 molecules/cell of IgG (17). Scrape-loading has been reported to deliver 10^7 dextran molecules/cell (18), whereas the delivery of BSA by erythrocyte ghosts is reported at 2.7×10^6 molecules/cell, and that of IgG at $2\text{–}5 \times 10^5$ (1).

Protease permeabilization of cultured fibroblasts compares favorably with the above, allowing the injection of 10^5 to 10^6 molecules/cell (Table 1) of a variety of proteins directly into the cytosolic compartment. We have shown that the uptake of protein is into cytosol both by functional (Fig. 4) and morphologic (Fig. 3) criteria. The amount microinjected corresponds to a captured volume of medium of between 0.5 and 3.0 μl/10^6 cells during the standard 20-min incubation depending on the cell line, the protein injected, and variation in the effect of the protease in producing channels in the plasma membrane.

Uptake of leucine into cultured human fibroblasts occurs primarily by System L, which has a K_m of 0.1 mM and V_{max} of about 1.2 nmol/min/mg protein (19). From our data on the uptake of leucine by trypsin-treated fibroblasts in suspension (Fig. 5), it can be seen that uptake is nonsaturable to concentrations of 20 mM. At 20 mM the rate of uptake is calculated to be about 460 nmol/min/mg protein.[4] This is a rate almost 400 times that of the V_{max} for System L. Similarly, when the uptake of [^{3}H]mannitol was studied, a rate of 10 to 100 times that of the normal pinocytosis rate was found (see Results). These findings are most consistent with permeabilization of the plasma membrane.

The large molecular weight range of proteins showing increased uptake after cells are trypsin permeabilized suggests that the pores formed after the procedure are larger than those produced by red blood cell ghost-mediated microinjection. That procedure is limited to molecules which can diffuse through pores of 20–50 nm (2). Since the red blood cell membrane is negatively charged, certain

[4] The cells used in these experiments contained 0.09 ± 0.03 mg protein/10^6 cells.

positively charged molecules adsorb strongly (e.g., lysozyme), limiting injection efficiency by this technique. Red blood cell fusion also alters the surface characteristics of the host cell by incorporating some of the red cell membranes into the host membrane. This, plus the effects of the fusogen (PEG, Sendai virus) may exert unpredictable effects on the host cell's metabolism (2).

With the scrape-loading technique, cells are physically detached from the culture dish with a rubber scraper and then transferred at 4°C to medium containing the protein to be injected. The injected material presumably enters the cell via rents induced in the plasma membrane by the physical force which detaches the cells from the substrate. In our experience, cells subjected to this method do not reattach to the culture dish after injection and are essentially nonviable. Hypotonic–osmotic lysis is an ingenious technique which allows co-pinocytosis of hypertonic sucrose-containing medium to which has been added the protein to be loaded. After a brief period of uptake, the cells are exposed to hypotonic medium and the newly formed pinosomes rupture, releasing the labeled protein directly into the cytosol. This technique is limited by the relatively low rate of pinocytosis (2). Electroporation uses a brief electrical discharge to produce defects in the plasma membrane through which the protein is injected. We have been unable to obtain sufficient numbers of viable cells to study proteolysis by this method. The same disadvantage applies to permeabilization of plasma membranes by detergents (for example, Brij 58).

In contrast, cells are routinely passaged by exposure to trypsin or other proteases with excellent viability after such exposures. It is surprising that the hyperpermeability induced by such treatment apparently has not been previously recognized. Permeabilization by protease treatment could occur by simple degradation of transmembrane proteins, leaving channels through which the exogenous protein can migrate. Alternatively, it has been shown that trypsin-treated cells round up, but remain attached to the culture dish by long cytoplasmic extensions termed "trypsinization retraction fibrils" (20). It is possible that during detachment of trypsin-treated cells from the culture dish the termini of these extensions rupture, producing in effect siphons which aspirate the culture medium. Physically this method may be analogous to scrape-loading, but detachment of the cells after protease treatment is gentler, leading to better cell viability.

The implications of this finding are twofold: (i) Cells are hyperpermeable after passaging and will take up significant amounts of molecules present in the culture medium in the postpassage period prior to attachment. (ii) All substrate-anchored cells subjected to electroporation are initially detached by protease treatment. It appears possible that much of the injection attributed to electroporation is merely the result of incubating trypsin-permeabilized cells in suspension.

Recently, the study of cellular proteolysis has been markedly expanded by identification of a number of recognition sequences which permit selective proteolysis of specific proteins (21,22) and enhanced degradation of proteins under step-down conditions (23). Evidence has also been presented showing an apparent need for protein synthesis for lysosomal proteolysis (24). Such studies require identification of a specific protein located in the cytosolic compartment to allow observation of the regulation of catabolism (25,26). Protease-permeabilized microinjection appears to confer significant advantages in achieving this goal. It requires no special equipment, yields excellent cell viability, and accomodates a broad range of injectable proteins.

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REFERENCES

1. Celis, J. (1984) *Biochem. J.* **223,** 281–291.
2. McElligott, M., and Dice, J. (1984) *Biosci. Rep.* **4,** 451–466.
3. Okada, C., and Rechsteiner, M. (1982) *Cell* **29,** 33–41.
4. McNeil, P., Murphy, R., Lanni, F., and Taylor, D. (1984) *J. Cell Biol.* **98,** 1556–1564.
5. Schliwa, M.. van Blerkom, J., and Porter, K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4329–4333.
6. Knight, D., and Scrutton, M. (1986) *Biochem. J.* **234,** 497–506.
7. Wang, Y., and Taylor, D. (1980) *J. Histochem. Cytochem.* **28,** 1198–1206.
8. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
9. Pratten, M., Williams, K., and Lloyd, J. (1977) *Biochem. J.* **168,** 365–372.
10. Thoene, J., Forster, S., and Lloyd, J. (1985) *Biochem. Biophys. Res. Commun.* **127,** 733–738.
11. Finley, D., Ciechanover, A., and Varshavsky, A. (1984) *Cell* **37,** 43–55.
12. Hough, R., and Rechsteiner, M. (1986) *J. Biol. Chem.* **261,** 2391–2399.
13. Hough, R., Pratt, G., and Rechsteiner, M. (1986) *J. Biol. Chem.* **261,** 2400–2408.
14. Kaplan, J., Ward, D., and Wiley, H. (1985) *J. Cell Biol.* **101,** 121–129.
15. Dean, R. (1984) *Exp. Cell Res.* **151,** 563–566.
16. Giard, D. (1987) *In Vitro Cell. Dev. Biol.* **23,** 691–697.
17. Doxsey, S., Sambrook, J., Helenius, A., and White, J. (1985) *J. Cell Biol.* **101,** 19–27.
18. McNeil, P., Murphey, R., Lanni, F., and Taylor, D. (1984) *J. Cell Biol.* **98,** 1556–1564.
19. Longo, N., Franchi-Gazzola, R., Bussolati, O., Dall'Asta, V., Foa, P., Guidotti, G., and Gazzola, G. (1985) *Biochem. Biophys. Acta* **844,** 216–223.
20. Dalen, H., and Todd, P. W. (1971) *Exp. Cell Res.* **66,** 353–361.
21. Rogers, S., Wells, R., and Rechsteiner, M. (1986) *Science* **234,** 364–368.
22. Bachmair, A., Finley, D., and Varshavsky, A. (1986) *Science* **234,** 179–186.
23. Backer, J., and Dice, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5830–5834.
24. Thoene, J., Lemons, R., Boskovich, S., and Borysko, K. (1985) *J. Clin. Invest.* **75,** 370–376.
25. Carlson, N., and Rechsteiner, M. (1987) *J. Cell Biol.* **104,** 537–546.
26. Carlson, N., Rogers, S., and Rechsteiner, M. (1987) *J. Cell Biol.* **104,** 547–555.