THE LOCALIZATION OF TRYPsin IN CULTURED MAMMALIAN CELLS

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SUMMARY

The localization of trypsin in HeLa and CBM17 baby mouse kidney cells was studied using fluorescent and electron-microscope autoradiographical techniques. Intracellular uptake of trypsin, as well as cell surface localization, was demonstrated by the use of direct FITC- and tritium acetylated-labelled trypsin and immunoreactive procedures. Intracellular penetration of the enzyme was temperature dependent and while evident at 37 and 25 °C was negligible at 4 °C. Higher proteolytic activity could be demonstrated in the supernatants from disrupted trypsin-treated cells than in supernatants from disrupted PBS-treated cultures. Treatment of trypsin with serum, whilst depressing enzyme protease activity, did not modify intracellular uptake of the enzyme and intracellular localization of trypsin persisted in cultured cells for up to about 48 h. The results, while not accounting for the primary event in cell alteration following exposure to trypsin, clearly suggest that consideration must be given to the fact that intracellular penetration of the enzyme may affect certain intrinsic processes of the cell.

INTRODUCTION

Trypsin and other proteolytic enzymes are widely employed for dissociating avian and mammalian tissues in the preparation of cell suspensions, in the separation of intact tissue layers, and in experimental embryology (see reviews by Moscona, 1962; Moscona, Trowell & Willmer, 1965; Steinberg, 1967; Bernfield & Wessells, 1970; Bernfield, Banerjee & Cohn, 1972). Although treatment of cells with trypsin does not affect cellular viability to any great extent (Rinaldini, 1959; Moscona, 1962), trypsinization has been reported to alter certain properties of the cell, including the following: modification of adhesive properties (Ambrose & Easty, 1960; Feldman, 1955); modification in electrophoretic mobility (Ponders, 1951); liberation of glycoproteins and sugars from the cell surface (Cook, Heard & Seaman, 1960; Uhlenbruck, 1961; Moscona & Moscona, 1952; Rinaldini, 1958; Laws & Strickland, 1961; Gasic & Gasic, 1962; Shenn & Ginsburg, 1969); unmasking or loss of antigenic surface material (Weiss & Coombs, 1963; Sorkin, 1963; David, Lawrence & Thomas, 1964; Osunkoya, Mottram & Isouri, 1969); modification of cell agglutination sites (Burger, 1969, 1971); modification of insulin-binding capacity of fat cells (Kono & Barham, 1971); alteration in cell membrane permeability (Levine, 1960); loss of cellular dry mass (Weiss, 1958); increased cytoplasmic basophilia (Moscona & Moscona, 1952); chromatid breaks (Levan & Biesele, 1958); altered fertilization of sea-urchin eggs (Runnstöm, Hagstorn & Perlmann, 1959); ultrastructural alteration of the cell surface (Edward & Fogh, 1959; Weiss, 1963; Boeryd, Lundin, Norby & Scheli, 1968); increased cell deforma-
bility (Weiss, 1966); depression of mitotic activity (Chang, 1968); activation of cell division (Peck & Byvoet, 1965; Burger, 1970, 1971); modification of contact inhibition of growth (Burger, 1970); transient modification of polyribosome ratios (Hosick & Strohman, 1971); alteration in radiosensitivity of cells (Berry, Evans & Robinson, 1966; Phillips & Tolmach, 1964; Nias & Lajtha, 1964); transient suppression of [3H]thymidine incorporation into DNA (Berry et al. 1966); inactivation of acetylcholinesterase activity in human erythrocytes (Martin, 1970). Whether such changes in cell behaviour represent alterations in the physicochemical properties of the cell surface and/or intracellular penetration of the enzyme affecting intrinsic processes of the cell has not been shown. Direct information on the cellular localization of trypsin is lacking, though indirect evidence suggests that traces of trypsin may remain attached to the cell surface for a certain period of time (Rothen, 1948; Raut-Hebb & Chu, 1960; Wallis, Ver & Melnick, 1969; Rosenberg, 1960; Moscona et al. 1965; Barnard, Weiss & Ratcliffe, 1969; Poste, 1971).

In the present study an attempt has been made to gain information on the cellular localization and enzymic activity of trypsin using immunofluorescence and electron-microscope autoradiographical techniques.

MATERIALS AND METHODS

Maintenance of cell cultures

Monolayers of HeLa cells (obtained from Burroughs Wellcome, 1962) and of CBM17 baby mouse kidney cells (Franks & Henzell, 1970) were grown in 225-ml Pyrex baby’s feeding bottles at 36.5 °C in a gas phase of 5% CO₂ in air in Waymouth’s medium MB 752/1 (Waymouth, 1959) (Wellcome Reagents), supplemented with either 4% human serum (for HeLa cultures) or 5% calf serum (for CBM17 cultures). HeLa cultures were transferred routinely by syringing free the cells; proteolytic enzymes were not used. CBM17 cultures were routinely transferred by washing the cell monolayer twice with Ca⁺⁺-Mg⁺⁺-free phosphate-buffered saline (PBS), treating with 2 ml of 0.014% trypsin (Tryptar, Armour Pharmaceutical Co.) solution in PBS for approximately 5 min at 36.5 °C, adding 8 ml of culture medium, pipetting off the cells and transferring into new bottles. HeLa and CBM17 cells were inoculated in baby’s bottles at a density of approximately 1·2 x 10⁶ cells per ml.

Preparation of trypsin

(a) Bovine pancreatic trypsin (twice-crystallized, salt free, activity 3000 N.F. units/mg; Tryptar, Armour Pharmaceutical Co., Lot no. RD 2101) was prepared at a concentration of 0.014% (protease activity 130 BAPA mU./ml) in Ca⁺⁺-Mg⁺⁺-free phosphate-buffered saline, pH 7.2 (Dulbecco & Vogt, 1954), and stored in aliquots at −20 °C. Such preparations are used routinely in the department for cell transfers by trypsinization. Trypsin solutions at concentrations of 0.028 % (protease activity 280 BAPA mU./ml) and 0.1% (protease activity 1440 BAPA mU./ml) were also prepared.

(b) [3H]Acetyl trypsin was prepared and stored as previously described (Livingston, 1969) using bovine pancreatic trypsin (Worthington Bioch. Corp., preparation TRL, twice-crystallized).

(c) Fluorescein isothiocyanate (FITC) trypsin was prepared by dissolving 125 mg trypsin (Tryptar, Armour, Lot no. RD 2101) in 5 ml of 0.05 M sodium carbonate–bicarbonate buffer of pH 9.0 and adding 40 mg of 10% FITC in Celite (Calbiochem., Lot no. 70253) to the trypsin solution, with stirring maintained for 1 h at room temperature. Particulate matter was then removed by centrifugation for 5 min. Unconjugated dye in the supernatant was removed by passing the reaction mixture at 18 °C through a G25 Sephadex column (30 x 2.5 cm) using

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Ca\(^{2+}\)- Mg\(^{2+}\)-free phosphate-buffered saline, pH 7.2, as the eluant. Fractions of 5 ml were collected and trypsin activity was estimated in each fraction by the method of Erlanger, Kokowsky & Cohen (1961). The stability of the conjugated trypsin was determined at 30 min, 2 and 24-h intervals by thin layer chromatography on Sephadex G 200 dextran gel (Brighton, 1966). Free fluorochrome was not in evidence after 30 min or 2 h incubation but was present after 24 h incubation.

(d) Insolubilized trypsin was prepared as follows. Enzite CMC-hydrazide (Miles Seravac) (0.5 g) was suspended in 0.5 N hydrochloric acid (75 ml) and stirred at 0 °C for 30 min. 5.0 ml of a cold 5 % aqueous sodium nitrite solution were added dropwise over a period of 30 min. The insoluble azide was filtered off, washed well with cold 1 M sodium chloride and water. The azide was re-suspended in phosphate-buffered saline (50 ml) at 0 °C and the pH adjusted to 8.7 with 0.1 N sodium hydroxide solution. Lyophilized trypsin (Tryptar, Armour Pharmaceutical Co., Batch no. RD 2101, 148 mg) was added, the suspension was well stirred and the pH maintained at 8.7 for 2 h. The product was filtered, washed well with cold phosphate-buffered saline (10 ml) and stirred for 30 min. Finally, the material was filtered and again washed with buffer. The preparations were stored at 5 °C under buffer until required. It was customary to prepare the material immediately prior to use.

Assay of trypsin

The protease activity of bovine trypsin was measured with 4-N-benzoyl-DL-arginine-\(\beta\)-nitroanilide (BAPA) (Boehringer Corp.) as substrate. The rate of hydrolysis of the amide (BAPA) was followed spectrophotometrically as described by Erlanger et al. (1961). Optical density estimations were carried out in a Gilford Model 240/2441 spectrophotometer using 10 x 150 mm cuvettes.

Preparation of anti-trypsin serum

Anti-trypsin serum was prepared by Wellcome Reagents Limited by a method based on that of Arnon & Schechter (1966). Rabbits were immunized by 3 intramuscular injections at 10-day intervals. Each injection contained 15 mg of trypsin (Tryptar) in 3 ml Freund's complete adjuvant. A test bleed was carried out 8 days after the last injection. A fourth injection of trypsin in Freund's adjuvant was given 1 month later and the rabbits bled-out after a further 10 days. The serum was dispensed in 1-ml lots and stored at –20 °C.

Absorption of anti-serum

One-millilitre aliquots of anti-trypsin anti-serum were absorbed with insolubilized trypsin. The serum was circulated by means of a peristaltic pump (LKB) through a glass column (100 x 5 mm tube) packed with 0.5 g trypsin for 1 h at 5 °C.

Experimental procedures

FITC-trypsin. Confluent monolayers of HeLa cells were washed twice with PBS and 5 ml of fresh PBS (warmed to 36.5 °C) were then added to one bottle of cells, while 5 ml of FITC-trypsin at 36.5 °C (diluted to a concentration of 70–90 BAPA mU./ml) were added per bottle to a group of 4 bottles. At the end of 1, 5, 10 and 30 min of incubation at 36.5 °C in FITC-trypsin and of 30 min in PBS the cells still remaining on the glass surfaces were scraped off using a silicone-rubber-coated policeman, the cell suspensions centrifuged at 700 g for 2 min at 4 °C, washed once with PBS and re-centrifuged. The cell pellets were fixed in Carnoy's fixative for 30 min at room temperature following gentle dispersion of the cells in the fixative, and after centrifugation re-suspended in 70 % ethanol, washed twice with distilled water, lyophilized in a Pearse-Edwards freeze-drying apparatus overnight and embedded in Paraplast (Sherwood Medical Industries); sections were cut at 5 μm. The sections were mounted in glycerol-PBS (9:1 v/v), and examined using a Zeiss W.L. microscope with a HBO 200 mercury vapour lamp, UGI-3 exciter filter and 41/65 barrier filters.

Radioactive trypsin. Lyophilized \([\text{H}]\)acetetyl trypsin was dissolved in 30 ml cold distilled water, dialysed against PBS (pH 7.2 at 4 °C for 24 h) to remove possible free acetic acid, diluted in...
PBS to obtain a trypsin activity of the order of 70 BAPA mU./ml and used immediately. (Preliminary experiments showed that [H]acetyl trypsin possessed approximately half the proteolytic activity of unlabelled acetyl trypsin.) HeLa cells were treated either with (a) [H]acetyl trypsin, (b) [H]acetyl trypsin previously diluted in PBS containing 10% calf serum (trypsin activity approximately 4 BAPA mU./ml), or (c) control buffer solution (PBS) according to the procedure described above with FITC-trypsin. At the end of the different incubation periods an equal volume (i.e. 5 ml) of 5% glutaraldehyde in 0.1 M sodium cacodylate was added to the bottles. The cells were fixed for 2 h at 4°C, scraped from the glass surface, centrifuged 5 min at 700 g and washed 4 times over a 24-h period with 0.1 M sodium cacodylate buffer, pH 7.1, at 4°C. For electron autoradiography the cells were postfixed in Palade’s buffered osmium tetroxide fixative, pH 7.4, for 1 h, rinsed briefly in distilled water, dehydrated and embedded in Araldite (Glauert, 1965). Processing for autoradiography was according to Budd & Pelc (1964). Exposure times varied from 8 weeks to 6 months. Sections were examined in an Hitachi HS7S electron microscope.

**Immunofluorescence tracing of trypsin.** In a first series of experiments HeLa cells and CBM17 cells grown to confluency, were washed once with pre-warmed PBS and treated for 30 min at 36.5 or 4°C with (i) 5 ml of PBS, (ii) 5 ml of 0.1% or 0.014% trypsin solution, or (iii) 5 ml of 0.028% trypsin solution (diluted 50% with calf serum and the mixture previously left for 30 min at 25°C: protease activity 13.2 BAPA mU./ml). At the end of the incubation period cells still adhering to the glass surface were scraped off, the cell suspension centrifuged at 700 g for 5 min at 4°C, washed once in PBS and recentrifuged. The pellet of cells was fixed in Carnoy’s fixative for 30 min at room temperature, processed routinely, embedded in Paraplast and 5-µm sections cut.

In a second series of experiments confluent monolayers of HeLa and CBM17 cells were washed once with PBS and individual baby’s bottles treated in one of two ways. (a) With 5 ml of 0.1% trypsin solution for 30 min at 36.5°C or with 5 ml PBS. Following centrifugation at 700 g for 5 min at 4°C and removal of the trypsin supernatant, the cells were resuspended in medium, distributed in bottles, and cultured at 36.5°C. Cell pellets were prepared and fixed in Carnoy’s fixative at 0, 2 and 24-h intervals and processed as above. Or (b) cells were treated with 2 ml of 0.014% trypsin solution for about 5 min at 36.5°C until rounding of cells was observed. Routine cell transfer procedure was followed and 8 ml of medium were added per bottle and cells adhering to the glass surface syringed off before distribution of the cell suspension. The cells were cultured as above and cell pellets fixed at 0, 2, 24, 48, 72 and 96-h intervals.

For studies using the indirect immunofluorescence procedure, dilutions (1:5) of anti-trypsin rabbit serum, trypsin-absorbed anti-trypsin rabbit serum, and of negative control serum (normal pre-immune rabbit serum), were made in PBS. Replicate slides were covered with the appropriate serum and incubated for 30 min in a moist chamber at room temperature. The serum was then quickly rinsed off and each slide washed twice for 10 min in PBS and then drained until no visible moisture was apparent. Sheep anti-rabbit immunoglobulin (IgG) fluorescein isothiocyanate (FITC) (Wellcome Reagents Limited), diluted 1:5 in PBS, was applied and incubated for 30 min. The slides were drained of conjugate, washed twice for 10 min in PBS, drained of visible moisture and mounted in glycerol-PBS (9:1 v/v).

**Estimation of intracellular protease activity.** Suspensions of CBM17 cells obtained following treatment with PBS or 0.1% trypsin solution for 30 min at 36.5°C were centrifuged at 1000 g for 5 min at 4°C. The cell pellets were washed 4 times with 10 ml of 0.154 M KCl, and centrifuged at 1000 g for 5 min. Lysis of the cells was based on a method of Prescott et al. (1966). The cells were resuspended in 5 ml 0.1% Triton X-100 (prepared v/v in double-distilled water) and disruption of the cells accomplished by agitation for 2 min on a Vortex Whirlimixer; 0.1-ml aliquots of the suspension were stained with 1 ml of Leishmann’s stain to determine whether the nuclei were free of cytoplasmic contamination. The nuclei were then centrifuged off at 700 g for 20 min at 4°C. The supernatants from all centrifugations were stored at −20°C prior to assaying for protease activity.
Table 1. Immunofluorescence tracing of trypsin

<table>
<thead>
<tr>
<th>Cell</th>
<th>Treatment</th>
<th>Culture period prior to fixation, h</th>
<th>Anti-trypsin serum</th>
<th>Pre-immune serum</th>
<th>Trypsin-absorbed anti-trypsin serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>PBS</td>
<td>0</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>CBM17</td>
<td>0.1% trypsin (30 min at 36.5 °C)</td>
<td>0</td>
<td>+</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>+ (+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.14% trypsin (5 min at 36.5 °C)</td>
<td>0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBM17</td>
<td></td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>(±)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>o</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>o</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBM17</td>
<td>0.14% trypsin (30 min at 4 °C)</td>
<td>o</td>
<td>o to (±)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBM17</td>
<td>0.28% trypsin + 50% calf serum (30 min at 36.5 °C)</td>
<td>o</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

o, No specific fluorescence; +, specific fluorescence.

RESULTS

Localization of FITC-trypsin

The majority of the cells were characterized by a diffuse fluorescence of the cytoplasm and the nucleolus, while intensely fluorescent areas were noted over dead cells and cell debris. Aggregates of fluorescent material were occasionally found localized irregularly over the outer cell surface (Fig. 1). There was no apparent difference in the localization of fluorescent material in cells exposed to FITC-trypsin for either 1 or for 30 min.

Localization of [3H]acetyl trypsin

After 1 min exposure to [3H]acetyl trypsin aggregates of silver grains were found on the outer cell surface associated with the cytoplasmic processes (Fig. 2). There was slight localization of labelled compound in the cytoplasm and to a lesser extent in the nucleus (Figs. 3, 4). Heavier incorporation of label in the cytoplasm and the nucleus was found following longer exposure of the cells to [3H]acetyl-labelled trypsin. A gradient in the intracellular pattern was seen similar to that of the 1-min treated cells, with the majority of the silver grains localized extracellularly but with significant label in the cytoplasm and some incorporation of label in the nucleus. There appeared to be no specific intracellular localization of the labelled radioactive material and there was no apparent difference in the labelling pattern between cells exposed to serum-
Table 2. Protease activity in cell supernatants from CBM17 cells

<table>
<thead>
<tr>
<th></th>
<th>Trypsin-treated group, BAPA mU./ml</th>
<th>PBS-treated group, BAPA mU./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Trypsin solution</td>
<td>365</td>
<td>0.6</td>
</tr>
<tr>
<td>Medium from cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washings from treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>3.07</td>
<td>1.6</td>
</tr>
<tr>
<td>2nd</td>
<td>3.2</td>
<td>1.2</td>
</tr>
<tr>
<td>3rd</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>4th</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant from lysed cells</td>
<td>9.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Packed cell volume, ml estimated at 4th wash</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Total cell count</td>
<td>4.62 x 10^8</td>
<td>5.02 x 10^8</td>
</tr>
</tbody>
</table>

treated and non-serum-treated [PH]acetyl trypsin. Considerable amounts of label were seen in the dead cells (Fig. 5).

Immunofluorescence tracing of trypsin

The results of these experiments are summarized in Table 1. Cells with a positive immunoreaction showed an intense fluorescence localized in the cytoplasm and in the nucleolus (Fig. 6). A progressive loss in intensity of fluorescence was observed in the cultured cells and the presence of fluorescent material was difficult to detect after a culture period of 48 h. Addition of serum to the trypsin solution, whilst depressing protease activity, did not modify the intracellular uptake of the enzyme. Penetration of trypsin into the cells was affected by temperature and at 4 °C little or no fluorescent material could be resolved.

The immunoreaction seemed to be specific. No reaction was seen when the anti-trypsin serum was replaced by pre-immune rabbit serum or when the anti-trypsin serum was absorbed with insolubilized trypsin. In addition, no fluorescence was seen in control experiments using sections from PBS-treated cells.

Intracellular protease activity

Protease activities, estimated in supernatants from centrifugations taken at different stages in the process of cell disruption, are summarized in Table 2.

A progressive decrease in activity was observed in successive washings of the cells and PBS-treated cell supernatants showed absence of activity in the final wash and only a minimal activity remained in the final trypsin-treated-cell wash. Following disruption of the cells, increased protease activity was detected in the lysed cell supernatants and an approximately 3-fold difference in activity was noted between the trypsin-treated and control PBS-treated groups.
Trypsin localization in cultured cells

DISCUSSION

The findings of this study indicate that when cells are treated with trypsin the enzyme is not only bound to the cell surface but is also taken up into the cell. Localization of trypsin in the cytoplasm, nucleus and nucleolus can be demonstrated by immunoreactive procedures and by the use of direct FITC- and tritium acetlated-labelled trypsin. These data are in close agreement with the results obtained by Nordling & Mayhew (1966) in studies on the intracellular uptake of FITC-labelled neuraminidase. However, trypsin appears to be taken up in greater amounts in the cytoplasmic and nucleolar regions and nuclear localization of the enzyme, unlike that of neuraminidase, could not be demonstrated by fluorescent procedures. A possible artifact in the studies using direct-labelled trypsin is the cleavage of fluorescein or tritium-acetyl molecules from the trypsin molecules. While this possibility cannot be entirely eliminated, it seems unlikely, as the fluorescein-conjugated trypsin could be shown to be stable for a period of at least 2 h, a period considerably in excess of the maximum cell treatment period. Similarly, the presence of tritium-labelled acetic acid is also unlikely following 24-h dialysis of [3H]acetyl trypsin solutions to remove any unbound radioactive products (Livingston, 1969). Furthermore, evidence for a specific uptake of trypsin, or possibly of trypsin degradation products, is provided by data from the immunofluorescent studies.

How the trypsin enters the cell has not been determined, but it seems likely that it does so by endocytosis, according to the scheme demonstrated for a number of macromolecules (Chapman-Andresen, 1964; see in Dingle & Fell, 1969). The uptake of trypsin is temperature-dependent and intracellular penetration of the enzyme, whilst in evidence at 37 and 25 °C, is negligible at 4 °C. This suggests that trypsin is taken up into cells by endocytosis, a process which has been shown to be restricted at low temperatures (Jacques, 1969). Treatment of trypsin with serum reduces the protease activity of the enzyme but has no apparent restrictive effect on the cellular uptake of trypsin. Intracellular localization of trypsin can be shown, by immunofluorescence studies, to persist in cultured cells for up to about 48 h. Subsequent loss of positive fluorescent material may reflect a diluting effect resulting from cell division, or may indicate exocytosis of the trypsin or degradation of the protein moiety.

No evidence is available as to whether the enzyme is taken up in the cells as an intact molecule or whether activity of the trypsin molecule is affected. Trypsin is known to undergo autodigestion readily in solution at pH values higher than 3, but evidence that the degraded products retain trypsin activity is lacking (see Keil, 1971), although bovine trypsin seems to be able to autolyse one bond without losing activity (Desnuelle, Rovery & Maroux, 1967). It is probable that cells may be exposed to an enzyme solution constituted both of intact molecules and degraded polypeptide groups. Our attempt to show trypsin activity intracellularly, whilst not conclusive, does indicate a higher proteolytic activity in the supernatant from disrupted trypsin-treated cells. However, the substrate employed to demonstrate protease activity, \( \alpha-N \)-benzoyl-DL-arginine-\( \beta \)-nitroanilide, is not specific to trypsin and the increase in proteolytic
activity observed in trypsin-treated cells may reflect an indirect activation of other intracellular hydrolytic enzymes.

A very extensive literature (briefly indicated in the Introduction) relates to the action of trypsin on cells and there is evidence that the enzyme can directly influence cell activity and, in particular, the properties of the cell surface. However, the present observations demonstrating intracellular localization of trypsin do suggest a reconsideration of the view that the action of trypsin is limited to the cell surface. A number of the studies reported in the literature suggest that alteration in certain cell functions could result from a primary intracellular effect of the enzyme. Thus, increases in mitotic activity and chromosomal abnormalities have been demonstrated in primary cultures prepared from embryonic mouse tissues subjected to prolonged (3 h) trypsinization (Levan & Biesele, 1958). Aneuploidy in 5–10% of the population of trypsinized embryonic cells has been reported after only 24 h in culture (Chu, 1962). It is, however, true that diploid strains derived from human foetal tissues have been serially transferred through as many as 50 passages by techniques involving the use of trypsin (Hayflick & Moorhead, 1961). Further indication of the effect on intracellular cell functions is provided by the study of Hosick & Strohman (1971) showing extensive degradation of polyribosomes and a net loss of ribosomes in primary cultures of chick embryo muscle dissociated with trypsin. It was also observed that following trypsinization a lag of approximately 15 h occurred in culture before any detectable net increase in ribosome number. Reaggregation studies on trypsin-dissociated cells showing reaggregation lag-times of 24–48 h, and delayed morphogenetic expression in embryonic rudiments following proteolytic enzyme treatment (see Grobstein, 1967), may well reflect, in addition to the already documented modifications in cell surface characteristics, significant intracellular changes following enzyme penetration.

Brief treatment of cells with low concentrations of trypsin has been shown to result in a growth-stimulating effect (Burger, 1969, 1970, 1971; Rubin, 1970). These authors concluded that growth stimulation was probably due to a proteolytic effect on the cell membrane, although the possibility of an intracellular action was not excluded. Weissmann, Troll, van Duuren & Sessa (1968) have suggested that growth control may be modified if proteases released following destabilization of cell lysosomal membranes get into the nucleus and release repressor materials. A further point that may have to be considered in relation to the effect of trypsin on the intracellular processes of the cell is that these effects may reflect the action of contaminating enzymes such as chymotrypsin and ribonuclease. The recent studies of Bernfield et al. (1972) emphasize the point that conclusions derived from studies of enzymically treated cells are dependent upon the purity of the enzymes.

Although it is not possible from the present studies to account for any primary event in cell alteration following exposure to trypsin, it is clear that consideration must be given to the fact that modification of certain cell properties may be a consequence of the intracellular penetration of the enzyme.

The authors wish to thank Dr W. D. Brighton and Dr S. Young for helpful discussion on immunofluorescence procedures, Mrs P. Wilson for assistance with electron-microscope autoradiography and Mr G. D. Leach and his unit for the processing of the photographs.
REFERENCES


Tryptin localization in cultured cells
Fig. 1. HeLa cell culture treated 1 min with FITC-trypsin, showing extracellular localization of intensely staining aggregates of fluorescent material. × 675.

Fig. 2. HeLa cell culture treated 1 min with [3H]acetyl trypsin, showing aggregates of silver grains on outer cell surface. × 25 000.

Fig. 3. HeLa cell culture treated 1 min with [3H]acetyl trypsin, showing incorporation of label in the cytoplasm. × 25 000.
Trypsin localization in cultured cells
Fig. 4. HeLa cell culture treated 1 min with [³H]acetyl trypsin, showing intranuclear localization of labelled radioactive material. × 25 000.

Fig. 5. HeLa cell culture treated 1 min with [³H]acetyl trypsin, showing considerable amounts of label in dead cell. × 25 000.
Trypsin localization in cultured cells
Fig. 6. CBM17 cell culture treated 30 min with 0.1% trypsin. Sections reacted with anti-trypsin serum and FITC-anti-rabbit IgG. There is specific green fluorescence in the cytoplasm and nucleolus. × 960.