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The Release of Radioactive Nucleic Acids and Mucoproteins by Trypsin and Ethylenediaminetetra-acetate Treatment of Baby-Hamster Cells in Tissue Culture

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Monolayers of baby-hamster kidney cells were grown on glass in tissue culture and harvested with trypsin or EDTA in order to investigate the cell surface macromolecules removed by these cell-disaggregating agents. The release of nucleic acids from the cells during the harvesting procedure was monitored by labelling the cellular RNA with [5-^{3}H]uridine and the cellular DNA with [2-^{14}C]thymidine. Treatment of the cells with EDTA was found to cause an increase in the permeability of the plasma membrane with 7.6% of the cellular RNA, but less than 1% of the cellular DNA, being released. Moreover, 61% of the cells harvested with EDTA were permeable to Trypan Blue. With crude trypsin, lysis of the cell occurred with the release of similar amounts of RNA and DNA amounting to about 11% of the total cellular nucleic acid. In contrast, crystalline trypsin released only 1% of the cellular nucleic acids. Since virtually all the cells (99%) after harvesting in crystalline trypsin were impermeable to Trypan Blue, this method was suitable for obtaining cell surface macromolecules without contamination by intracellular damage. [1-^{14}C]Glucosamine was incorporated by the cells only into bound hexosamines and sialic acids. [By monitoring the release of radioactivity in high-molecular-weight material in such experiments a measure of the release of macromolecules containing amino sugars was obtained.] Of the total macromolecules containing amino sugars in the cells 33%, 24% and 13% were released when the cells were harvested with crude trypsin, crystalline trypsin or EDTA respectively. Crystalline trypsin also released 39% of the total sialic acid of the cell, whereas less than 1% of the cellular sialic acid was present in the EDTA-treated fraction. It is concluded that the macromolecules containing amino sugars released with crude trypsin and EDTA are likely to be heavily contaminated with intracellular material. However, the macromolecules released by crystalline trypsin appear to come from the cell surface.

The molecular structure of the cell surface is responsible for important biological properties such as cell adhesion (Curtis, 1967), the contact behaviour of cells and changes in such behaviour on viral transformation (Stoker, 1967), and immunological properties (Watkins, 1966). A suitable system for investigating the biochemistry of cell adhesion and cell contact is the study of monolayers of cells grown on a glass surface by tissue culture. One approach with this system is to release surface macromolecules by treatment of the cells with disaggregating agents such as trypsin or EDTA. Macromolecules, particularly mucoproteins, have been shown to be released when cells of intact tissue or in tissue culture are treated with trypsin (Langley & Ambrose, 1967; Winzler, Harris, Pekas, Johnson & Weber, 1967; Kraemer, 1967) or EDTA (Beierle, 1968). In determining whether such high-molecular-weight materials are indeed from the cell surface it is necessary to know the extent of cell lysis and increased cell permeability during the disaggregating procedure. Methods used for studying cell integrity and the effects on it of trypsin or EDTA have included counting the total number of cells (De Luca, 1965), measurement of the increase in cell permeability by the uptake of dyes (Langley & Ambrose, 1967; Beierle, 1968) and measurement of changes in the electrophoretic mobility of cells (Barnard, Weiss, & Ratcliffe, 1969). The present paper describes quantitatively the release of nucleic acids and macromolecules containing amino sugars when trypsin or EDTA are

used to harvest monolayers of baby-hamster kidney cells grown on glass. It also demonstrates that radioactive nucleic acids provide a useful method for estimating cell integrity. A preliminary report of this work has already been published (Allen & Snow, 1970).

METHODS

Enzymes. Bovine pancreatic ribonuclease (40–50 Kunitz units/mg; BDH Chemicals Ltd., Poole, Dorset, U.K.) was heated for 15min at 90°C in 0.4M-NaCl to remove deoxyribonuclease activity. Bovine pancreatic deoxyribonuclease (2000 Kunitz units/mg) was type 1 from Sigma Chemical Co., St Louis, Mo., U.S.A. Crude trypsin was obtained from Difco Chemical Co., Detroit, Mich., U.S.A.; crystalline trypsin was type 1 from Sigma Chemical Co. The activity of the two trypsin preparations was assayed, under the conditions used in the harvesting experiments, by the method of Rick (1965) with benzoyl-arginine ethyl ester as substrate. The activity of the crude trypsin (920 units/mg) was 35% that of the crystalline trypsin (2600 units/mg) by this assay and during a 1h incubation at 37°C these activities decreased by 3 and 8% for the crude and crystalline trypsin preparations respectively.

Radioactive compounds. These were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and had the following specific radioactivities: [5-^{3}H]uridine, 5.0Ci/mmol; [2-^{14}C]thymidine, 50mCi/mmol; [1-^{14}C]-glucosamine, 50mCi/mmol. Radioactivity was measured by using a Beckman LS 100 liquid-scintillation counter at ambient temperature.

Radioactive nucleic acids. These were isolated for measurement of radioactivity by precipitation with ice-cold trichloroacetic acid at a final concentration of 5% (w/v). After 30min at 4°C the precipitate was filtered off on an Oxoid filter (0.45μm pore size), the filter was washed with a further 5vol. of trichloroacetic acid, dried and counted for radioactivity in scintillation fluid containing 6g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l of toluene (BDH Chemicals, sulphur-free).

Macromolecules containing radioactive amino sugars. These were isolated by three different methods: (i) by precipitation with 3vol. of ethanol plus 1% $CaCl_2$; (ii) by precipitation with ice-cold trichloroacetic acid [5% (w/v) final concentration]; (iii) by dialysis of the cells against running water for 3 days. The precipitates from addition of ethanol and trichloroacetic acid were re-suspended in water and counted for radioactivity in Herberg's scintillation fluid (Herberg, 1960). The dialysed high-molecular-weight material was added directly to the Herberg's scintillation fluid and counted for radioactivity. Sialic acids in the ethanol precipitate were isolated by hydrolysis in 0.2M-H_2SO_4 at 80°C for 1h, followed by separation on an Amberlite CG400 column (Whitehouse & Zilliken, 1960). Sialic acids were assayed quantitatively by the thiobarbituric acid reaction (Aminoff, 1961) and qualitatively by paper chromatography (Allen & Kent, 1968*a*). Other amino sugars, neutral sugars and amino acids were analysed by acid hydrolysis followed by paper chromatography (Allen & Kent, 1968*a*).

Tissue culture. The cells used in all the experiments were from a permanent cell line of baby-hamster kidney cells C13 21 (Stoker & Macpherson, 1961). The cells were grown in the Dulbecco–Vogt modification of Eagle's medium plus 10% (v/v) calf serum in 20oz. medical flats, each containing 50ml of growth medium. Routine checks for contamination by pleuropneumonia-like organisms were made by staining (Fogh & Fogh, 1964). Contamination by yeast and bacteria was checked for by microscopic observation and inoculation of brain–heart infusion broth with samples.

Harvesting cells. Cells, seeded out at a density of 3×10^6 cells/bottle, were grown for 48h. This resulted in a fivefold increase in cell number and a 95% confluent monolayer of cells. In some experiments cells were harvested after 24h and after 72h, when the cells were 40–50 and 100% confluent respectively. Cells were harvested as follows.

(*a*) The growth medium was poured off.

(*b*) Cells were washed with 2×40ml and 4×10ml of tris-buffered saline (NaCl, 8.0g; KCl, 0.38g; Na_2HPO_4, 0.1g; $CaCl_2$, 0.1g; $MgCl_2$, $6H_2O$, 0.1g; Sigma 7–9 tris base, 3g/l; adjusted with HCl to pH7.4). The final 10ml washes of cells grown in the presence of radioactive precursors contained only background levels of radioactivity.

(*c*) The cells were harvested from the glass surface by incubation in 20ml of one of the following: (i) 0.5mM-EDTA for 10min; (ii) tris-buffered saline without Ca^{2+} or Mg^{2+} for 20min; (iii) 0.1% crude trypsin or 0.04% crystalline trypsin in tris-buffered saline (with Ca^{2+} and Mg^{2+}) for 10min. With the trypsin solutions and the Ca^{2+} and Mg^{2+}-deficient buffer the incubation times were the minimum required to remove all the cells from the glass. With EDTA, however, all the cells came off the glass after 2min of incubation. Experiments were also done by incubating the cells for 10min in 0.001% crystalline trypsin or for 20min in tris-buffered saline (with Ca^{2+} and Mg^{2+}). In neither of these cases did cells come off the glass surface, nor were any morphological changes seen.

(*d*) The harvesting medium was immediately centrifuged at 1500*g* for 10min to give a cell pellet and cell-free harvesting medium.

RESULTS

Release of radioactive nucleic acids. The cellular RNA and DNA were labelled by growing the cells in medium containing both [5-^{3}H]uridine (10μCi/bottle) and [2-^{14}C]thymidine (2.5μCi/bottle). After 48h the sum of the radioactivities in the trichloroacetic acid precipitates of the culture medium, washings and cells represented a total of 12% (2.6×10^6c.p.m./min) of the added ^{3}H counts and 8% (4.4×10^5c.p.m./min) of the added ^{14}C counts. Digestion of the culture medium, harvesting medium and cells with ribonuclease and deoxyribonuclease, followed by precipitation with trichloroacetic acid showed that the ^{3}H label from [5-^{3}H]-uridine was incorporated solely into RNA and the ^{14}C from [2-^{14}C]thymidine label was incorporated solely into DNA (Table 1). The decrease in the number of ^{3}H counts in the precipitate after

deoxyribonuclease treatment indicates some contaminating ribonuclease activity in this enzyme preparation. In all further experiments done under these conditions the ^3H and ^{14}C counts precipitated by trichloroacetic acid were taken as a measure of cellular RNA and DNA respectively.

The distribution of the radioactive nucleic acids in solutions obtained by harvesting the cells in EDTA or tris-buffered saline without Ca^{2+} or Mg^{2+} is given in Table 2. Less than 2% of the ^3H-labelled RNA and of the ^{14}C-labelled DNA was removed with the culture medium and washings. When the cells were harvested with 0.5mM-EDTA however, 7.6% of the ^3H-labelled RNA but only 0.7% of the ^{14}C-labelled DNA were released into the harvesting medium, the remaining nucleic acids being found in the cell pellet. A similar distribution of the cellular nucleic acids was observed when the cells were harvested with tris-buffered saline without Ca^{2+} and Mg^{2+} ions. The longer time necessary to remove the cells from the glass with the Ca^{2+}- and Mg^{2+}-deficient buffer, compared with EDTA, was associated with a larger release of ^3H-labelled RNA (13.2%, Table 2). Attempts to decrease the amount of RNA released by incubation of the cells for 2min in EDTA (cf. 10min in all other EDTA incubations) still released 7.7% of the total cellular ^3H-labelled RNA from the cells. Six successive 10min incubations of the cells with 0.5mM-EDTA, the cells being recovered by centrifugation after each incubation, extracted 66% of the total ^3H-labelled RNA but only 13% of the ^{14}C-labelled DNA from the cells (Table 3).

To examine the possible contribution of surface-bound RNA to the above results, cells labelled with ^3H-labelled RNA were washed twice with 40ml of tris-buffered saline and incubated for 10min with 20ml of the same buffer containing 20μg of ribonuclease/ml. In a control experiment under these conditions the ribonuclease solubilized 97% of radioactive *Escherichia coli* RNA as assayed by trichloroacetic acid precipitation. In the presence of ribonuclease no detachment of the cells occurred and no microscopic changes were observed in their morphology. After ribonuclease treatment the cells were again washed (4 × 40ml) with tris-buffered saline and incubated with 0.5mM-EDTA containing 50μg of non-radioactive yeast RNA/ml (the non-radioactive yeast RNA was used to swamp the small amount of ribonuclease activity remaining after washing). No significant difference (two experiments) was observed between the ^3H-labelled RNA released from the cells that had been treated with ribonuclease (9.0% of the total RNA) and the ^3H-labelled RNA released in the control by incubating the cells with tris-buffered saline alone (8.3% of the total RNA).

Table 1. *Ribonuclease and deoxyribonuclease digestion of the contents of radioactive cells and medium*

Cells were labelled with [^3H]uridine and [^{14}C]thymidine for 48h. The cells were disrupted by freezing and thawing and then suspended in tris-buffered saline (see the Methods section). The enzymes were added to the suspension and incubated at 37°C for 1h.

	Radioactivity in trichloroacetic acid precipitate after enzymic digestion (% of control without enzyme)	
Enzyme	^3H	^{14}C
Ribonuclease (67μg/ml)	1.1	99
Deoxyribonuclease (3.4μg/ml)	77	1.5

Table 2. *Nucleic acids released from cells when harvested by depletion of bivalent cations*

The conditions were as described in the Methods section.

	Radioactive nucleic acids (% of total formed by cells)		
	^3H-RNA	^{14}C-DNA	No. of experiments
Growth medium	1.5±1.5	1.5±1.4	9
Pooled washings	0.9±0.9	0.4±1.0	9
Harvesting medium:			
(i) 0.5mM-EDTA for 10min*	7.6±2.9	0.7±0.5	6
(ii) Tris-buffered saline without Ca^{2+} and Mg^{2+} for 20min*	13.1±1.4	0.3±0.1	2
(iii) Tris-buffered saline with Ca^{2+} and Mg^{2+} for 20min†	0.5±0.4	0.1±0.1	3

* Remaining counts are in the cell pellet after centrifugation.
† Remaining counts are on the glass.

When crude trypsin was used to harvest the cells approximately equal amounts (about 11%) of ^{3}H-labelled RNA and ^{14}C-labelled DNA were released into the medium (Table 4). With 0.04% crystalline trypsin however, much smaller amounts (about 1%) of the nucleic acids remained in the harvesting medium after the cells had been removed. Similar amounts of nucleic acids were released by treatment with 0.001% trypsin (about 1.5%); however, under these conditions less than 3% of the cells were removed from the glass. Fig. 1 shows that the release of nucleic acid from the cells incubated with crude trypsin for 1h is negligible after the first 10min when the cells have come off the glass.

Very similar results were obtained on the release of cellular nucleic acids by trypsin- or EDTA-treatment of cells grown for only 24h (40–50% confluent) or for 72h (completely confluent).

Dye-exclusion studies. The results of dye-exclusion studies (Paul, 1965) on cells harvested by different methods are shown in Table 5. Only after trypsin treatment were the cells impermeable to Trypan Blue and obtainable as a suspension of single cells. Considerable uptake of Trypan Blue was observed when EDTA (61% staining) or tris-buffered saline without Ca^{2+} or Mg^{2+} (77% staining) were used and large clumps of up to 100 cells were observed in the suspension of harvested cells. Similar large clumps of cells (72% staining) were seen in cell preparations prepared by scraping the monolayers off the glass surface with a glass rod. Less than 0.1% of the cells stained when monolayers of cells, washed with tris-buffered saline, were incubated still adhering to the glass in the same buffer containing 0.1% Trypan Blue.

Release of macromolecules containing radioactive amino sugars. The high-molecular-weight material in the different fractions from cells grown for 48h in the presence of [1-^{14}C]glucosamine (2.5μCi/bottle) was isolated by one of three methods. These were: precipitation with 3vol. of ethanol plus 1% $CaCl_2$; dialysis against running water for 3 days; precipitation with trichloroacetic acid (5%, w/v, final concentration). Controls for these experiments were performed by adding growth medium plus [1-^{14}C]glucosamine to a 95% confluent monolayer of non-radioactive cells. After 30min the control cells were harvested by the standard methods and the radioactivity in the precipitates or dialysed

Table 3. *Repeated incubations of cells in* 0.5mM-*EDTA*

The conditions were as described in the Methods section. After each incubation with EDTA the cells were separated from the solution by centrifugation at 1500*g* for 10min.

		Radioactive nucleic acids (% of total formed by cells)	
		^{3}H-labelled RNA	^{14}C-labelled DNA
Growth medium		1.7	2.3
Pooled washings		1.0	0.1
Successive incubations in 0.5mM-EDTA	no. 1	8.5	0.8
	no. 2	14.1	1.4
	no. 3	10.3	1.6
	no. 4	13.9	2.1
	no. 5	9.0	1.3
	no. 6	7.4	2.0
Cell pellet after EDTA incubation no. 6		34.1	88.4

Table 4. *Nucleic acids released from cells when harvested with trypsin*

The trypsin was dissolved in tris-buffered saline, pH7.4, and the cells were incubated for 10min at 37°C. The cells were removed by centrifugation at 1500*g* for 10min.

Harvesting medium	Radioactive nucleic acids in harvesting medium (% of total formed by cells)		Cells remaining on the glass (% of total cells)	No. of experiments
	^{3}H-labelled RNA	^{14}C-labelled DNA		
Trypsin (0.1%, crude)	10.4±3.9	11.4±4.0	5.1	10
Trypsin (0.04%, crystalline)	1.1±0.9	0.7±0.8	4.1	6
Trypsin (0.001%, crystalline)	1.5±0.7	0.8±0.8	97.6	6

material measured. There were no significant counts above background in samples prepared, by any of the three isolation methods, from the cells, the harvesting medium, or the pooled 4×10ml tris-buffered saline washes. However, the controls for the ethanol-precipitated and dialysed growth medium contained $7.5 \pm 2.5\%$ and $1.3 \pm 0.8\%$ respectively of the total added radioactivity. These very high backgrounds for the controls appear to be due to non-specific binding of the added [1-^{14}C]-glucosamine by a component in the calf serum added with the growth medium. Because of this high background the results for the growth medium were not considered further.

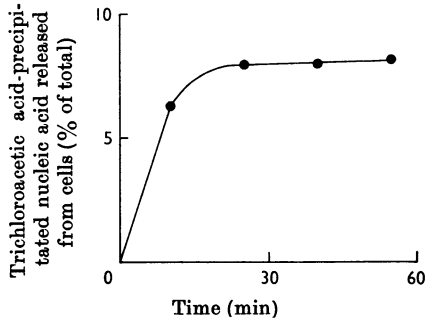

Fig. 1. Release of ^{3}H-labelled RNA and ^{14}C-labelled DNA by trypsin on monolayers of baby hamster kidney cells. Tris-buffered saline containing 0.1% of Difco trypsin was added to a monolayer of cells labelled with ^{3}H-labelled RNA and ^{14}C-labelled DNA. The cells were incubated at 37°C and samples withdrawn at timed intervals and assayed for radioactive nucleic acids. The cells were free in suspension after 10min of incubation.

The amounts of radioactivity in high-molecular-weight material incorporated by the cells, and released from the cells on harvesting, are shown in Tables 6 and 7. Of the radioactivity bound to the cells crude trypsin removes 32.6%, crystalline trypsin (0.04%) removes 23.6% whereas the low concentration of crystalline trypsin (0.001%) and EDTA remove 12.9 and 12.7% respectively. Dialysis of the radioactive material released from the cells by crystalline trypsin gave 93% of the amount of radioactivity precipitated from this fraction by ethanol. Addition of trichloroacetic acid to the fraction released by crystalline trypsin precipitated only 21% of the radioactivity precipitated by ethanol. The distribution of the ^{14}C radioactivity in the ethanol precipitates of the cells and harvesting medium from these experiments was investigated by acid hydrolysis and paper chromatography. Paper chromatography of the sialic acid fraction gave two radioactive, thiobarbituric acid-staining spots co-chromatographing with standard *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid. The sialic acid fraction accounted for about 10% of the total radioactivity incorporated. The remaining radioactivity in the ethanol precipitate was in a

Table 5. *Permeability of harvested cells to Trypan Blue*

The cells were harvested and centrifuged at 1500*g* for 10min. The pellet was resuspended in 0.1% Trypan Blue in tris-buffered saline, pH 7.4, with a wide-mouthed Pasteur pipette, and incubated for 10min.

Harvesting medium	% of total stained cells	No. of experiments
0.5mM-EDTA for 10min	61 ± 18	9
Tris-buffered saline without Ca^{2+} and Mg^{2+} for 20min	77 ± 12	6
Tris-buffered saline with Ca^{2+} and Mg^{2+} for 20min*	0.1	3
Trypsin (0.1%, crude; 0.04%, crystalline)	0.1	4
Cells scraped off in tris-buffered saline with Ca^{2+} and Mg^{2+}	72	2

* Cells were counted while still adhering to surface of growth vessel.

Table 6. *Radioactivity from* [1-^{14}C]*glucosamine incorporated into macromolecules containing amino sugars*

Cells were incubated for 48h at 37°C with growth medium containing 2.5μCi of [1-^{14}C]glucosamine.

	Incorporation of radioactivity into the cells and harvesting medium	
Isolation method	$10^{-4} \times$ Total counts (d.p.m.)	% of total added counts
Ethanol precipitation	7.3	3.3
Dialysis	6.9	3.1
Trichloroacetic acid precipitation	3.1	1.4

Table 7. *Macromolecules containing radioactive amino sugars released on harvesting cells*

Cells were grown for 48 h at 37°C in the presence of growth medium containing [1-^{14}C]glucosamine.

Harvesting medium	Counts in ethanol precipitate of harvesting medium (% of total counts in ethanol precipitate of cells and harvesting medium)	No. of experiments
0.5 mM-EDTA	12.9 ± 3.5	6
Trypsin (crude, 0.1%)	32.6 ± 12.2	7
Trypsin (crystalline, 0.04%)	23.6 ± 6.0	6
Trypsin (crystalline, 0.001%)	12.7 ± 3.5	6

single spot co-chromatographing with standard glucosamine and galactosamine. No radioactivity was found in the neutral sugars, particularly galactose, fucose and ribose, nor in the amino acids.

The total sialic acid in ethanol precipitates from the cells and harvesting medium was 25.8 μg/bottle as measured by the thiobarbituric acid reaction. It was necessary after acid hydrolysis, and before estimation, to isolate the sialic acids quantitatively with an anion exchange column. This is because the ethanol precipitates from the cells, without prior column treatment, contained material, other than sialic acid, which interfered with the thiobarbituric acid estimation, accounting for approx. 20% of the total colour. With crystalline trypsin 38.8 ± 5.2% (4 determinations) of the total cellular sialic acid was in the ethanol precipitate of the harvesting medium after the cells had been removed. The ethanol precipitate of the EDTA harvesting medium contained less than 1% of the total ethanol-precipitable bound sialic acids from the cells. The calf serum in the growth medium and the crude trypsin both contained large amounts of thiobarbituric acid-positive material which made analysis of these fractions for sialic acid of cellular origin impracticable.

DISCUSSION

To study the contact properties of cells it is necessary to study cells grown in intact tissue or monolayers of cells grown on glass in tissue culture. Most previous studies dealing with the effects of cell-disaggregating agents on cell integrity have been on cells in suspension (Kraemer, 1966; Barnard *et al.* 1969), or from intact tissue (Kemp, Jones, Cunningham & James, 1967; Kerkof, Smith, Gagné, Pitelka & Abraham, 1970). Further, methods so far described for disaggregating intact tissues have been shown to cause considerable damage to the cell (Kemp *et al.* 1967; Kerkof *et al.* 1970).

Our results show that monitoring the release of radioactive nucleic acids provides a good method for investigating the effects on cell integrity of disaggregating agents used to harvest monolayers of cells grown on glass in tissue culture. The incorporation of [5-^{3}H]uridine and [2-^{14}C]thymidine solely into RNA and DNA respectively provides a sensitive assay for cellular nucleic acids. Further, since the number of cells increases fivefold during the experiments, and the percentage of total counts incorporated is below 15% of the total added counts with each radioactive precursor, it is assumed that the specific radioactivity of the macromolecules is uniform in each case.

The small amounts of RNA and DNA in the growth medium and the washings can be explained by the lysis of dead cells. The release by 0.5 mM-EDTA and Ca^{2+}- and Mg^{2+}-deficient tris-buffered saline of 7.6% and 13.6% of the total cellular RNA respectively is in keeping with an increase in permeability of the plasma membrane. Ca^{2+} ions have been shown to be essential for maintaining the permeability barrier of the plasma membrane, which in the absence of Ca^{2+} becomes permeable to molecules of high molecular weight (Lowenstein, 1966). That the removal of bivalent cations, in our experiments, is causing increased cell permeability rather than cell lysis is indicated by the lack of release of DNA (less than 1%) from the cells. In particular, with six successive incubations of the cells with 0.5 mM-EDTA, 65% of the RNA is released from the cells compared with only 13% of the DNA (Table 2). EDTA has been shown to attack ribosomes (Arnstein, Cox, Gould & Potter, 1965) and since ribosomal RNA is the major RNA species in the mammalian cell (e.g. Hadjiolov, 1967), part of the 65% of the cell RNA released by EDTA is presumably ribosomal. The absence of bound sialic acid in the material removed from the cells by EDTA is further evidence against significant lysis of the cells and liberation of their membrane components under these conditions. Sialic acids have been shown to be present on both the intracellular and plasma membranes of mammalian cells (Patterson & Tourster, 1962).

Dye-exclusion studies also show increased perme-

ability of the cells after harvesting with EDTA or Ca^{2+}- and Mg^{2+}-deficient buffer, when 61 and 72% of the cells respectively take up Trypan Blue. Beierle (1968) has reported the release of ribonuclease sensitive, 260nm-absorbing material from baby-hamster kidney cells when treated with EDTA (0.5mM for 20min). However, since, in contrast with our results, only 5% of the cells were permeable to Trypan Blue this RNA is proposed as coming from the cell surface. Electrophoretic studies on mammalian cells treated with ribonuclease have also been used to show the presence of RNA on the cell surface (Weiss, 1969). It is unlikely that such surface RNA makes an important contribution in our experiments since ribonuclease-treatment of the cells had no effect on the amount of RNA released by subsequent incubation in EDTA. Also, the large amounts of the cell RNA released by the depletion of bivalent cations, especially with successive incubations in EDTA, make a major contribution from surface RNA seem unlikely.

Of the methods tried, treatment with 0.04% crystalline trypsin was the least harmful to the integrity of the cells, releasing only 1.1% and 0.7% of the cell RNA and DNA respectively. Preparations of crude trypsin, however, released 10.4% of the RNA and 11.4% of the DNA from the cells on harvesting. The release of about equal amounts of the two nucleic acids from the cells indicates that with trypsin, in contrast with EDTA, lysis of the cells occurs. However, the remaining unlysed cells appeared to have their permeability barrier intact, less than 1% taking up Trypan Blue. The continued integrity of viable cells in the presence of trypsin is well established (De Luca, 1965; Kraemer, 1967). Our results confirm this but show (Fig. 1) that with crude trypsin there is some cell lysis on their detachment from the glass surface. It is not clear why about 10% of the monolayer of cells should be lysed by crude trypsin. No evidence of decreased viability of the cells was found by staining the monolayer with Trypan Blue while it was still attached to the glass (less than 0.1% staining). The more deleterious effect of crude trypsin, in contrast with crystalline trypsin, could be due to other enzymic activities contaminating this preparation. The low amounts of RNA and DNA in the supernatant from harvesting the cells with crystalline trypsin also show that centrifuging the cells at 1500 *g* for 10min is sufficient to precipitate over 99% of the cells in suspension without cell damage.

Various methods have been described for disaggregating cells in tissue culture, including trypsin in the absence (Barnard *et al.* 1969) or presence (Kraemer, 1966) of Ca^{2+} and Mg^{2+}, trypsin with EDTA (Stoker & Macpherson, 1961) and EDTA alone (Beierle, 1968; Burger, 1969; Richmond, Glaeser & Todd, 1968). Although for routine tissue-culture techniques a small amount of cell damage is not important, with studies on cell-surface materials released by these cell-disaggregating agents it is important to keep cell damage to an absolute minimum. From our results it would appear that when cells are harvested from glass with crystalline trypsin in buffer containing Ca^{2+} and Mg^{2+}, less than 2% of the cells are damaged and the harvesting medium, after removal of the cells by centrifugation, is likely to be free of significant amounts of intracellular material. Our observations also suggest that trypsin is a more efficient disaggregating agent than EDTA under the conditions used. EDTA (0.5mM) removed sheets of cells from the glass within 2min at 37°C but even after 10min of incubation in EDTA large numbers of cells still remained clumped together. In contrast, trypsin provided a suspension of single cells, although 10min of incubation was necessary to remove the cells from the glass surface. These observations also point to differences between cell-to-glass and cell-to-cell adhesion.

The incorporation of [1-^{14}C]glucosamine solely into the hexosamines and sialic acids of high-molecular-weight materials acts as a marker for the macromolecules containing amino sugars, which include mucoproteins, mucopolysaccharides and mucolipids, produced by the cell. Both mucoproteins and mucolipids have been shown to be present on the cell surface of mammalian cells (Langley & Ambrose, 1967; Hakomori, Teather & Andrews, 1968). Further, both trypsin (Kraemer, 1967) and EDTA (Beierle, 1968) have been shown to release from cells in tissue culture high-molecular-weight material containing bound amino sugars. In our experiments 12.9, 32.6 and 23.6% of the total ethanol-precipitable macromolecules of the cell containing radioactive amino sugars were released when EDTA, crude trypsin and crystalline trypsin respectively were used to harvest the cells from the glass surface. Taken in conjunction with the results on the release of RNA and increased dye permeability of cells treated wih EDTA, all the macromolecules containing bound amino sugars released by EDTA could have come from an intracellular source. Kraemer (1968) has reported the existence of an intracellular heparin-like material in baby-hamster ovary cells. With crude trypsin about a third of the macromolecules containing amino sugars could have come from the intracellular contents of lysed cells. With 0.04% crystalline trypsin, however, since there is lysis of less than 2% of the cells, it would appear that over 90% of the bound amino sugars liberated on harvesting the cells are from the cell surface.

The nature of the material removed by treatment of the cells with crystalline trypsin is likely to be of

importance in explaining the biological properties of the cell surface. As well as such material being removed with cell disaggregation, differences in the material removed by trypsin from the cell surface have been shown to account for changes in the agglutinability of virus-transformed cells from their non-transformed counterparts (Burger, 1969; Hakomori *et al.* 1968). Trypsin has been shown to remove defined mucoproteins from the cell surface of Erhlich ascites-tumour cells (Langley & Ambrose, 1967) and from human erythrocytes (Winzler *et al.* 1967). It is reasonable to assume that mucoproteins form part of the material removed by trypsin in our experiments, although other materials, particularly proteins, may also be removed by trypsin. In the crystalline trypsin-treated fraction the radioactive macromolecules containing amino sugars precipitated by trichloroacetic acid amounted to only 21% of that precipitated by ethanol and 22% of that retained by dialysis. This suggests that a large proportion of these macromolecules removed by trypsin are neutral polysaccharides with relatively little protein attached.

The presence of both *N*-glycollylneuraminic acid and *N*-acetylneuraminic acid together in mammalian systems is well established (Allen & Kent, 1968*b*) and our results show that both are present in the baby-hamster kidney-cell line. Further, release of 39% of the total cell-bound, ethanol-precipitable sialic acid from the cells by crystalline trypsin shows that some of this material liberated from the cell surface has bound sialic acid. Also, since no sialic acid was released by EDTA-disaggregation of the cells, trypsin disaggregation must remove macromolecules not released by EDTA. It is noteworthy that Kraemer (1967), using baby-hamster ovary cells in suspension, found that trypsin removed radioactively labelled macromolecules containing amino sugars but virtually no sialic acid.

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