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# CORRELATION BETWEEN THE REGENERATION OF CELL SURFACE GLYCOPROTEINS AND THE CELL RE-ADHESION TO THE SUBSTRATUM IN TRYPSIN-TREATED CHICK FIBROBLASTS AT VARIOUS STAGES OF EMBRYO DEVELOPMENT

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## Summary

The ability of fibroblasts from 8- and 16-day-old chick embryos to adhere to a substratum was altered by trypsin treatment. The consequences of this treatment were investigated on cell re-adhesion to the substratum and cell morphology in relation to the regeneration of cell surface glycoproteins as estimated by the incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine.

Cell re-adhesion, cell shape and restoration of cell surface glycoproteins of the fibroblasts from chick embryos were markedly alike for each stage of embryo development.

Age-dependent differences were noted. The fibroblasts from 8-day-old embryos re-adhered progressively more rapidly than fibroblasts from 16-day-old embryos. The fibroblast morphology appeared to be dependent on the re-adhesion of cells to the substratum. Parallel to the re-adhesion, the cell surface glycoprotein recovery reached at least 90% in fibroblasts from 8-day-old embryos and only about 70% in fibroblasts from 16-day-old embryos after a 4 h culture as compared to the control cultures. These percentages coincided with 73% (fibroblasts from 8-day-old embryos) and 40% (fibroblasts from 16-day-old embryos) adhesion recovery.

The results are discussed in terms of a possible mechanism for cell surface recovery.

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## Introduction

It is well known that while many cell lines grow in suspension, other lines and most primary cells require a substratum on which they can adhere and spread for growth [1—4]. The adhesion of the cells to the substratum appears to be mediated by cell membrane glycoproteins [5—7]. The attachment between the cell surface and the substratum is broken by trypsin treatment and the destruction of binding forces causes the flattened cells to take on a spherical structure. Moreover, trypsin treatment releases cell surface glycoproteins, studies of which are well documented [8]. After trypsin treatment, several hours are necessary for cell surface recovery. Repair is confirmed using indirect immunofluorescence. Specific antigens have been shown to require a certain recovery time after trypsinization for restoration of function [9,10].

Embryo cells are especially interesting, since they exhibit differential cell surface properties in relation to the stage of embryo development, such as cell growth [11], cell aggregation [12] and interaction with lectins [13—15].

The aim of the studies presented in this paper was to investigate the consequences of the trypsin treatment of chick fibroblasts at various stages of embryo development, on cell adhesion to the substratum and cell morphology in relation to the regeneration of cell surface glycoproteins. This relationship was investigated over short periods of time following trypsin treatment of the cells.

## Materials and Methods

*Cells.* Chick embryo fibroblasts were obtained from 8- and 16-day-old embryos according to the method of Rein and Rubin [16], modified as previously reported [11].

*Cell cultures.* Cells were cultured in Nunclon flasks in 40 ml of Eagle's minimal essential medium containing 10% fetal calf serum, 1% glutamine and 1% antibiotics. Cultures were grown in humidified air with 5% $CO_2$ at 37°C. The initial seeding concentration was $10^6$ cells/ml. After reaching confluency, the cultures were used for the following experiments.

*Treatment of cells with trypsin.* Cultures were washed three times with phosphate-buffer saline (pH 7.4) and then treated with trypsin (Trypsin-TPCK, Worthington, in phosphate-buffered saline, $Ca^{2+}$ and $Mg^{2+}$-free, pH 7.4, at a final concentration of 50 $\mu$g/ml at 37°C). After 10 min of treatment, the effect of trypsin was blocked using the soybean trypsin inhibitor (Sigma Co, in phosphate-buffered saline, pH 7.4, at a final concentration of 100 $\mu$g/ml).

*Determination of cell adhesion to the substratum.* After treatment of the cells with trypsin, the cells were replated in 50-mm tissue culture dishes in 3 ml of Eagle's minimal essential medium ($10^6$ cells/ml). The number of cells adhered to the bottom of the tissue culture dish was determined using a hemocytometer after the cells had been harvested from tissue culture dishes following trypsin treatment as described above.

*Cell viability and integrity.* Cell viability was greater than 95% as estimated by trypan blue exclusion. In addition, the total lactate dehydrogenase levels

in the cells and the trypsinate were determined according to the method of Wroblewski and Ladue [17].

*Cell shape.* The cell shape of adhered cells was observed in tissue culture dishes using a Zeiss universal microscope.

*Incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine.* After treatment of the cells with trypsin, the cells were replated in 50-mm tissue culture dishes in 3 ml of Eagle's minimal essential medium ($10^6$ cells/ml) containing 1 $\mu$Ci/ml of [$^3$H]leucine (spec. at. 55 Ci/mmol, Radiochemical Centre, Amersham) and 0.85 $\mu$Ci/ml of [$^{14}$C]glucosamine (spec. act. 52 mCi/mmol, Radiochemical Centre, Amersham). At various intervals, the cells were washed three times with phosphate-buffered saline (pH 7.4) and were again treated with trypsin under the same conditions as described above. After centrifugation at $500 \times g$ for 5 min to remove the supernatant fraction (subsequently referred to as 'trypsinate'), the cell pellets were washed twice with phosphate-buffered saline (pH 7.4) and allowed to precipitate overnight in 10% (w/v) trichloroacetic acid (3 ml). The precipitate was washed twice with 5% trichloroacetic acid, dissolved in 10 ml of PCS scintillator (Amersham, Searle) and then counted for radioactivity in a liquid scintillation spectrometer (Intertechnique SL 30). The trypsinates were dialyzed against water overnight and the non-diffusible material recovered and counted for radioactivity.

The cell protein content was estimated by using the method of Lowry et al. [18] on the cell pellet.

Control cells were obtained usually by mechanical dispersion as previously described [11] and then cultured for 24 h before the experiments. These cells were not treated with trypsin and the precursors were added on attached cells. The incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine was investigated in the trypsinate and in the intracellular material of control cells.

*Determination of sialic acid content.* The fibroblasts were treated with *Vibrio cholerae* neuraminidase (Behringwerke; 50 U/ml in phosphate-buffered saline, pH 7.4, for 30 min at 37°C).

The neuraminidase was tested for protease activity using the azocoll test [19]. No such activity was found. The amount of sialic acid available from the cell surfaces was estimated by using the method of Warren [20]. The total sialic acid content was measured after acid hydrolysis in 0.01 M HCl at 80°C for 1 h. After purification on Dowex 1, the total sialic acid content was measured according to the method of Warren [20]. Corrections were applied for ribose and other contaminants [20].

*Determination of total hexosamine content.* The fibroblasts were treated with trypsin (TPCK, Worthington; 50 $\mu$g/ml of phosphated-buffered saline, $Ca^{2+}$ and $Mg^{2+}$-free, pH 7.4, for 10 min at 37°C). The amount of total hexosamines in the trypsinates of control and replated cells was measured by using the method of Elson and Morgan [21] after acid hydrolysis (3 M HCl for 4 h at 100°C).

*Treatment of cells with cycloheximide.* The effects of cycloheximide, which is a specific inhibitor of protein synthesis [22—24], were investigated on both the cell re-adhesion and the regeneration of cell surface glycoproteins.

The trypsin-treated fibroblasts were replated with cycloheximide at a final concentration of 3 $\mu$g/ml and then incubated for 1 and 4 h at 37°C.

*Control experiments for radioactive precursor incorporation.* Trypsinates from [$^3$H]leucine- and [$^{14}$C]glucosamine-labelled replated cells were dialyzed and then evaporated and dissolved in 1 ml 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1, containing 0.1% sodium dodecyl sulfate (SDS). Samples were applied to a column (1.2 × 53 cm) of Sephadex G-25 fine, equilibrated in 0.1% SDS in 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1. Elution was carried out in the same buffer, Fractions of 1 ml were collected and aliquots counted for radioactivity in a liquid scintillation counter.

Dialyzed trypsinates from [$^{14}$C]glucosamine-labelled replated cells were extensively digested with pronase (Calbiochem) (1 mg/ml in 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1, for 24 h at 37°C, three times). The pronase digest was re-dissolved and samples applied to a column (1 × 63 cm) of Sephadex G-25 fine and eluted with 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1. Fractions of 1 ml were collected and counted for radioactivity.

Paper chromatography was performed on [$^{14}$C]glucosamine-labelled trypsinate. Neutral sugars were released after a 2 M HCl hydrolysis for 2 h at 100°C), hexosamines by a 4 M HCl hydrolysis for 4 h at 100°C and sialic acid after a 0.01 M HCl hydrolysis for 30 min at 80°C. The chromatogram was developed on Schleicher and Schüll 2043 B paper during 40 h using butanol/pyridine/hydrochloric acid (5 : 3 : 2, v/v/v) for hexosamines and ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, v/v/v/v) for sialic acid. After drying, the paper was cut into pieces and counted for radioactivity.

## Results

*Cell re-adhesion and cell shape of chick embryo fibroblasts following trypsin treatment*

The trypsin-treated fibroblasts were replated and cell re-adhesion to the substratum and recovery of cell shape were investigated at various times following treatment. Although the cell viability was not affected, the cells were spherical and did not adhere to the substratum immediately after treatment with trypsin (Table I). From 0 to 20 min following trypsin treatment, the cells were only adsorbed to the substratum and were easily detached by washing.

After 30 min, 40% of the fibroblasts from 8-day-old embryos were attached to the substratum. After 30 min, the balance sheet of cells plated, cells failing to attach and cells recovered after the second trypsinization indicated that $1.7 \cdot 10^6$ cells did not attach to the substratum. This result showed that the second trypsin treatment did not digest a fraction of re-adhered cells. With time, this percentage increased, reaching 66% after 1.5 h in fibroblasts from 8-day-old embryos; after a 4 h culture, the number of re-adhered cells reached 73% in fibroblasts from 8-day-old embryos (Table I). In contrast to the younger fibroblasts, the number of the fibroblasts from 16-day-old embryos re-adhering to the substratum was only 23% after a 30 min culture and thereafter this increased slightly to a plateau level of 40% from 3 to 4 h. The fibroblasts from 16-day-old embryos therefore exhibited the least cell re-adhesion as compared to younger fibroblasts (Table I).

The attached fibroblasts were essentially elongated (80%) after a 4 h culture;

TABLE I

RE-ADHESION TO THE SUBSTRATUM OF REPLATED FIBROBLASTS FROM 8- AND 16-DAY-OLD EMBRYOS FOLLOWING TRYPSIN TREATMENT

Each result gives the average of values from four separate experiments. Standard error does not exceed 10%. The values given in parentheses represent the percentage of cell re-adhesion as compared to the initial seeding concentration ($3 \times 10^6$ cells/dish). Cells detached from a subconfluent monolayer with trypsin (50 μg/ml) for 10 min were replated ($3 \times 10^6$ cells/dish) in Eagle's minimal essential medium supplemented with 10% fetal calf serum and incubated at 37°C. At different times, the medium was poured off and attached cells were counted after their detachment by trypsin. From 0 to 20 min, no cell was re-adhered. A pre-treatment for 4 h with cycloheximide before the re-adhesion assay blocked the re-adhesion of fibroblasts from 8- and 16-day-old embryos.

| Age of embryos (days) | Cyclo heximide * (3 μg/ml) | Number of attached cells ($\times 10^6$)/dish at various times (h) | | | | |
|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 1.5 | 3 | 4 |
| 8 | — | 1.2 (40) | 1 (40) | 2 (66) | 2 (66) | 2.2 (73) |
| | + | — | 1.1 (40) | — | — | 2.1 (73) |
| 16 | — | 0.7 (23) | 0.9 (30) | 0.9 (30) | 1.2 (40) | 1.2 (40) |
| | + | — | 0.9 (30) | — | — | 1.1 (40) |

* In these experiments cycloheximide was added at time 0 of the re-adhesion assay.

however, 20% of attached cells remained spherical indicating that their fibroblast shape was not totally recovered after this time.

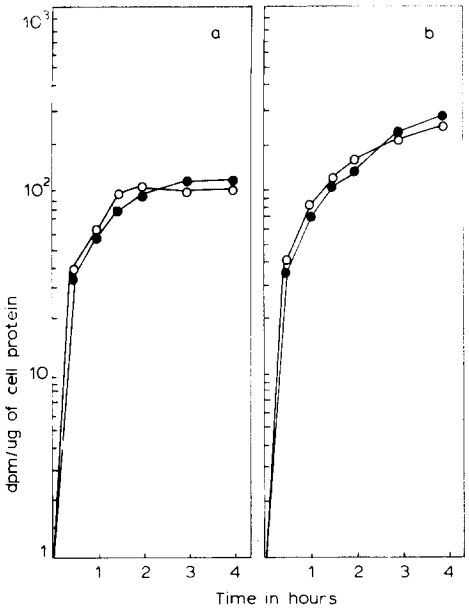
*Regeneration of cell surface glycoproteins in chick embryo fibroblasts following trypsin treatment*

(1) The regeneration of cell surface glycoproteins was estimated by the incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the replated 8- and 16-day-old embryos at various times after trypsin treatment. The incorporation was studied both in the trypsinate and in the intracellular material of replated cells. Parallel to the re-adhesion of the cells, the incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the trypsinate increased during the first hour of culture in fibroblasts from 8-day-old embryos. Thereafter, from 1.5 h, this incorporation almost reached a plateau (Fig. 1a), In the replated fibroblasts from 16-day-old embryos, the incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the trypsinate also increased with time but reached a plateau only after 3—4 h of culture (Fig. 1b). These results suggest that the regeneration of trypsin-sensitive cell surface glycoproteins, involved in the cell re-adhesion, occurred within 1.5 and 3—4 h following the trypsin treatment of fibroblasts from young (8-day-old) and old (16-day-old) embryos, respectively.

The intracellular incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the replated fibroblasts from 8- and 16-day-old embryos continued to increase with time up to 4 h (Fig. 2).

It was noted that the replated fibroblasts from 8-day-old embryos achieved control levels of incorporation at 4 h, while the re-plated fibroblasts from 16-day-old embryos reached about 70—80% of control values (Table II).

(2) The non-dialysable material removed from the cell surface by trypsin (trypsinate) contained an equal amount or more radioactivity than that incorporated into trypsinated cells. In all cases, excellent preservation of cell viabil-



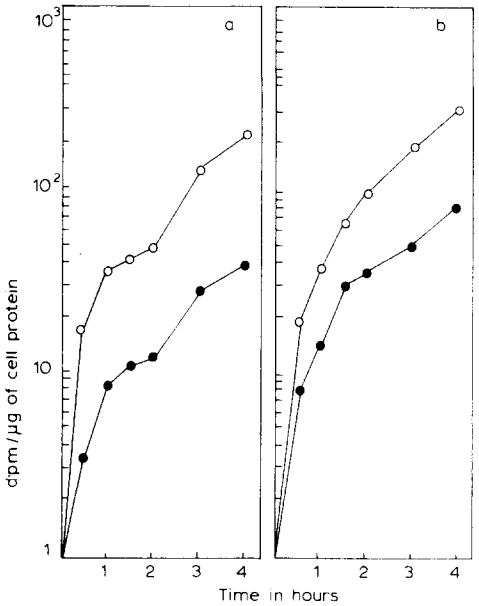




Fig. 1. Incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the trypsinate of fibroblasts from 8-day-old (a) and 16-day-old (b) embryos. Each point gives the average of values obtained from four separate experiments and the standard error does not exceed 10%. ●——●, [$^{14}$C]glucosamine; ○——○, [$^3$H]-leucine.

Fig. 2. Incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine in the intracellular material of fibroblasts from 8-day-old (a) and 16-day-old (b) embryos. Each point gives the average of values obtained from four separate experiments and the standard error does not exceed 10%. ●——●, [$^{14}$C]glucosamine; ○——○, [$^3$H]leucine.

ity was indicated by trypan blue dye exclusion. In addition, the total lactate dehydrogenase levels were determined in the case of fibroblasts from 8-day-old embryos. In the cells on the dish prior to trypsinization and centrifugation, this level was 0.025 I.U./mg of protein. After the first trypsinization, the

TABLE II

INCORPORATION OF [$^3$H]LEUCINE AND [$^{14}$C]GLUCOSAMINE IN THE TRYPSINATE AND IN THE INTRACELLULAR MATERIAL OF UNTREATED FIBROBLASTS FROM 8- AND 16-DAY-OLD EMBRYOS

The cells were cultured for 24 h before the labelling experiments. Each point gives the average of values obtained from four separate experiments and the standard error does not exceed 10%. Values are expressed in dpm/µg of cellular protein.

| Age of embryo (days) | Time (h) | Trypsinate | | Intracellular material | |
|---|---|---|---|---|---|
| | | Leucine | Glucosamine | Leucine | Glucosamine |
| 8 | 0.5 | 86 | 75 | 108 | 28 |
| | 1 | 102 | 100 | 208 | 54 |
| | 4 | 109 | 126 | 662 | 142 |
| 16 | 0.5 | 123 | 124 | 161 | 60 |
| | 1 | 254 | 225 | 256 | 96 |
| | 4 | 377 | 384 | 766 | 286 |

lactate dehydrogenase level reached 0.030 I.U./mg of proteins in the trypsinized cells, and it was not detectable in the first trypsinate since no variation in absorbance was noted during the time of assay (0—5 min). At 0.5 h of regeneration, in the re-trypsinized cells the lactate dehydrogenase level reached 0.028 I.U./mg of proteins and it was not detectable in the second trypsinate. We could therefore conclude that the substances present in the trypsinates were derived exclusively from the cell membranes. It was then necessary to demonstrate that the large amounts of radioactivity found in the trypsinates were indeed incorporated into macromolecules. To this end, the dialysed trypsinate was analysed by gel filtration on Sephadex G-25 in the presence of SDS or after digestion with pronase.

(a) The [$^3$H]leucine- or [$^{14}$C]glucosamine-labelled trypsinate to which 0.1% SDS had been added was submitted to Sephadex G-25 column chromatography. The elution profiles appear in Fig. 3. In the case of the [$^3$H]leucine-labelled trypsinate, fraction I eluted near the excluded volume and contained 25% of the radioactivity. Fraction II was slightly delayed and represented 64% of the radioactivity; two other fractions, III and IV, eluted later and contained only 6 and 5% of the radioactivity, respectively. Free [$^3$H]leucine eluted with fraction IV.

In the case of the [$^{14}$C]glucosamine-labelled trypsinate, 63% of the radioactivity eluted near the exclusion volume. Fractions II and III eluted later and represented 19 and 11% of the radioactivity, respectively. Fraction IV appeared last and contained 7% of the radioactivity; this fraction eluted at volumes corresponding to those for free [$^{14}$C]glucosamine.

(b) In a second set of experiments, the [$^{14}$C]glucosamine-labelled trypsinate was extensively digested with pronase and the resulting glycopeptides sub-

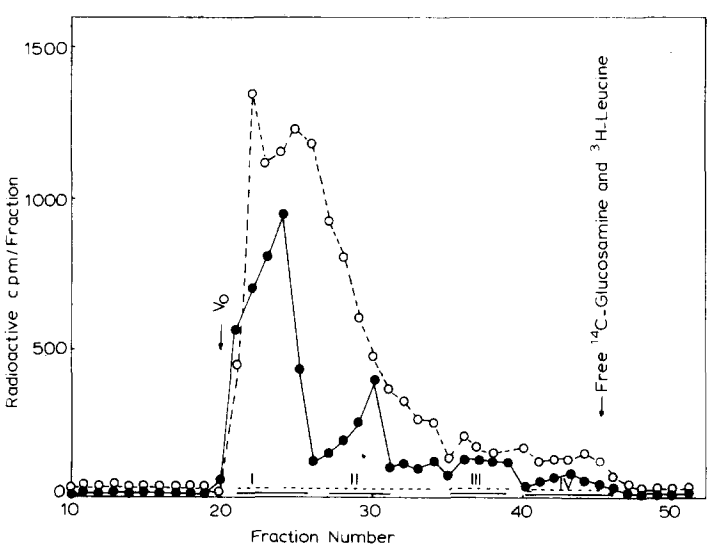



Fig. 3. SDS Sephadex G-25 column chromatography of [$^{14}$C]glucosamine- and [$^3$H]leucine-labelled trypsinates of fibroblasts from 8-day-old embryos. Samples were applied to a column (1.2 × 53 cm) of Sephadex G-25 fine and eluted with 0.1% SDS in 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1. 1 ml fractions were collected. Blue dextran and free labelled precursors were eluted in fractions 20 and 45, respectively.

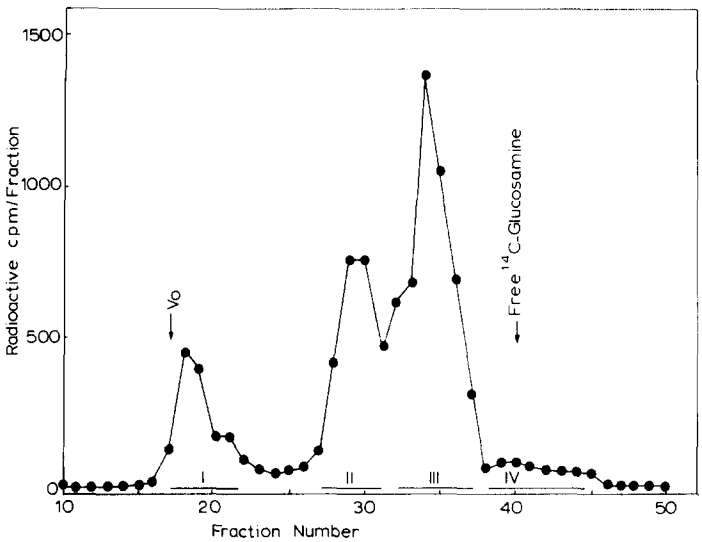



Fig. 4. Sephadex G-25 column chromatography of the [$^{14}$C]glucosamine-labelled trypsinate of fibroblasts from 8-day-old embryos. After extensive digestion with pronase as described in Materials and Methods, the sample was applied to a column (1 × 63 cm) of Sephadex G-25 and eluted with 0.01 M $NaHCO_3$/0.15 M NaCl, pH 8.1. 1 ml fractions were collected. Blue dextran and free labelled precursor were eluted in fractions 17 and 40, respectively.

mitted to Sephadex G-25 column chromatography. The elution profiles appear in Fig. 4. Fraction I eluted near the excluded volume. It contained 15% of the radioactivity and represented the non-hydrolysed glycoproteins. Fractions II and III eluted later and represented 27 and 50% of the radioactivity, respectively. Fraction IV appeared last and contained 8% of the radioactivity. This fraction eluted with free [$^{14}$C]glucosamine. The results show that, after dialysis of the trypsinate, at least 92% of the radioactivity was incorporated into macromolecular constituents. The radioactivity remaining adsorbed and attributable to non-incorporated precursors was less than 10%.

The [$^{14}$C]glucosamine-labelled trypsinate was submitted to paper chromatography after hydrolysis and more than 95% of the radioactivity was associated with glucosamine and only traces of sialic acid were detected.

(3) Regeneration of cell surface glycoproteins was confirmed by direct determination of the amounts of sialic acid and total hexosamines in the trypsinate of replated fibroblasts from 8-day-old embryos after replating for a 1 h and a 4 h culture. The amount of sialic acid was 1.75 $\mu g/10^8$ cells after a 1 h culture and 2.35 $\mu g/10^8$ cells after a 4 h culture. These amounts represented 58 and 78%, respectively, in comparison with the amount measured in the trypsinate obtained from the control culture (3 $\mu g/10^8$ cells) (Table III). The amount of hexosamines was 8.4 and 23 $\mu g/10^8$ cells after a 1 h and a 4 h culture, respectively, and represented 22 and 61% as compared to the control (37.5 $\mu g/10^8$ cells).

*Effect of cycloheximide*

In order to investigate whether or not new protein synthesis was required during cell re-adhesion and regeneration of cell surface glycoproteins, the

TABLE III

EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF [$^3$H]LEUCINE and [$^{14}$C]GLUCOSAMINE IN THE TRYPSINATE AND THE INTRACELLULAR MATERIAL FROM REPLATED FIBROBLASTS OF 8- AND 16-DAY-OLD EMBRYOS

Each point gives the average of values obtained from four separate experiments and the standard error does not exceed 10%. In these experiments cycloheximide was used at a final concentration of 3 μg/ml and added at time 0 of the assay. Results are expressed in dpm/μg of cellular protein.

| Age of embryo (days) | Time after replating (h) | Cyclo heximide added | Trypsinate | | Intracellular material | |
|---|---|---|---|---|---|---|
| | | | Leucine | Glucosamine | Leucine | Glucosamine |
| 8 | 1 | — | 65 | 60 | 38 | 8.5 |
| | | + | 67 | 59 | 16 | 5.2 |
| | 4 | — | 102 | 120 | 220 | 40 |
| | | + | 56 | 77 | 37 | 22 |
| 16 | 1 | — | 81 | 75 | 40 | 15 |
| | | + | 82 | 78 | 10 | 6.8 |
| | 4 | — | 258 | 282 | 320 | 85 |
| | | + | 136 | 197 | 38 | 24 |

fibroblasts from 8- and 16-day-old embryos were treated with cycloheximide, a potent inhibitor of protein synthesis.

(1) Neither the attachment nor the spreading of fibroblasts from 8- or 16-day-old embryos was modified in the presence of cycloheximide (0.1-10 μg/ml) at any of the experimental times (Table I). In contrast, when the cell cultures were pre-treated for 4 h with cycloheximide (3 μg/ml) before the re-adhesion assay, none of the fibroblasts (from 8- or 16-day-old embryos) re-adhered to the substratum (Table I).

(2) The effect of cycloheximide was studied on the incorporation of [$^3$H]-leucine and [$^{14}$C]glucosamine in the trypsinates of the different fibroblasts. After a 1 h incubation, cycloheximide at a final concentration of 3 μg/ml had no effect on the regeneration of cell surface glycoproteins (Table III), whereas after a 4 h culture, there was significant inhibition in the trypsinates of all the fibroblasts (Table III).

(3) In all the fibroblasts, the intracellular incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine was inhibited 60% by cycloheximide (3 μg/ml) as from 1 h of culture (Table III).

## Discussion

The data presented here clearly show that in fibroblasts from chick embryos at different stages of development and subjected to trypsin treatment, restoration of cell adhesion, cell morphology and cell surface glycoproteins occurs very nearly in parallel as, irrespective of age, all needed similar recovery times. As expected, fibroblast morphology appeared to be closely related to cell readhesion to the substratum [2,3]. In our experiments using primary cultures, regeneration of all three parameters was rapid and it seemed reasonable that these biological phenomena might well be governed by the replacement of cell membrane constituents, the turnover of which is rapid. Based on results

obtained either on cell-adhesion [5–7,25]or the re-synthesis of cell surface constituents [26–28], it has been suggested that the trypsin-sensitive cell surface glycoproteins are involved in this process. To our knowledge, the present report is the first study of both re-adhesion and cell surface regeneration together with a view to finding a direct correlation between these factors in the case of normal cells. Nevertheless, other cell models, such as fibroblast cell lines, 3T3 cells and their mutant counterparts deficient in cell surface glycoprotein synthesis [29–33], have been used to establish such a correlation.

Although the cell surface regeneration occurred unusually rapidly in our model, other authors have also reported such fast regeneration [27,28,34–36]. Courtois and Hughes [28] noted that regenerated cell surface glycoproteins contained more [$^{14}$C]glucosamine than trypsinized cells. It has been reported that in chick embryo fibroblasts in secondary culture, 80% of the glucosamine-labelled macromolecules were incorporated into the trypsin-sensitive cell surface glycoproteins in less than 3 h [35]. Molnar et al. [36] found that in Ehrlich tumor cells, 70% of their labelled fucose was incorporated into the plasma membrane fraction within 30 min. We have had similar finding using precursors other than glucosamine, namely [$^{14}$C]fucose which cannot be metabolized (Vernay, M., unpublishes results). It is not possible that some of our observations were artefacts, such as adsorbed as opposed to incorporated radioactivity, since gel filtration of the native and pronase-digested trypsinates clearly demonstrated that 80–90% of the radioactivity was incorporated into the trypsin-sensitive cell surface glycoproteins. Moreover, [$^{14}$C]glucosamine was not metabolized within 4 h.

It is difficult to explain the unusual phenomenon of obvious and well-documented rapid replacement of membrane constituents.

(i) Excessive activity of glycosyltransferases may play a key role in the process of repair in situ [27], but this would not explain the rapid rate of leucine incorporation. (ii) Trypsin treatment itself could be at least partly responsible. It is known to induce the stimulation of the processes of synthesis and cell growth in the case of normal cells [37]. Trypsin should thus stimulate the regeneration of cell surface glycoproteins in older cells exhibiting behavior similar to normal cells [11,13]. Our results did not support this possibility. Trypsin is also known to enhance the normal process of shedding in chick embryo fibroblasts in secondary culture and in 3T3 cells [25]. This, however, would not totally explain our results. (iii) Coupled with the second possibility, the most likely explanation for our results would seem to be one involving the deposition of micro-exudates rather than the in situ regeneration of membrane glycoproteins. It has been observed that cells deposit micro-exudates on the substratum [25]. The micro-exudates appear to contain shed cell surface components or secreted substances [37] and also appear to be derived from cell fragments such as the bulbous ends of filipodia or other cell micro-extensions [25]. Metabolic experiments have indicated the presence of protein and carbohydrate in the micro-exudate [38–40].

A further question was whether or not de novo protein synthesis was taking place in view of the high rate of leucine incorporation. Experiments with cycloheximide, a potent inhibitor of protein synthesis, were therefore under-

taken. The results show that de novo protein synthesis was indeed involved in the regeneration of the cell surface glycoproteins and cell re-adhesion. However, in the short-term experiments, the presence of cycloheximide did not significantly alter either cell re-adhesion or regeneration. This was probably due to the fact that though cell re-adhesion and regeneration occurred rapidly, the cycloheximide-induced inhibition of protein synthesis needed a definite time to occur sine it was only partial within 2 h and total within 4 h. Similarly, other cell types have been found to attach to the substratum and to be unaffected by cycloheximide treatment for short periods of time [26,41–44]. This is consistent with the finding that partial regeneration of cell surface glycoproteins was sufficient to allow marked cell re-adhesion in chick embryo fibroblasts.

The relationship was also age-dependent. Fibroblasts from young embryos (8-day-old) re-adhered more and more rapidly than fibroblasts from older embryos (16-day-old). Our results are to be compared with those of Moscona [12] on the spontaneous aggregation of embryo cells, which was more marked in the cells from young embryos. Similarly, the regeneration of cell surface glycoproteins occurred more rapidly in fibroblasts from young embryos than in those from older embryos. The speed of glycoprotein regeneration may be associated with the proliferative capacity of the cells. In contrast with the older embryo fibroblasts which have a low proliferative capacity [11], both young embryo fibroblasts and Zajdela's hepatoma cells [33], which possess a high proliferative capacity, exhibit a rapid regeneration of cell surface glycoproteins.

It is therefore evident that the re-adhesion of chick embryo fibroblasts and the restoration of their morphology involved trypsin-sensitive cell surface glycoproteins. Moreover, the regeneration and/or shedding of the cell surface glycoproteins occurred rapidly and was probably facilitated by the trypsin treatment which promoted the deposition of micro-exudates containing glycoproteins.

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