J. Membrane Biol. 26, 189–204 (1976)


# Cell Size and Mutual Cell Adhesion

## I. Increase in Mutual Adhesiveness of HeLa Cells from Density-Inhibited Suspension Cultures by Hypotonic Treatment

Joris J. Deman, Luc C. Vakaet, and Erik A. Bruyneel

Department of Experimental Cancerology, Clinic for Radiotherapy and Nuclear Medicine, Academic Clinic, State University, Ghent, Belgium, and Department of Anatomy and Embryology, State University Center, Antwerp, Belgium

Received 14 July 1975; revised 3 November 1975

*Summary.* HeLa cells harvested from density-inhibited or fast growing suspension cultures, were incubated in NaCl solutions of different tonicity. Cell size enlargement produced by hypotonicity is accompanied by an increased sedimentation rate of the density-inhibited cells, whereas no appreciable change is observed in the sedimentation rate of fast growing cells. Hypotonicity also has no effect on the sedimentation rate of density-inhibited cells which previously had been treated with neuraminidase or trypsin. It is shown that the effect of hypotonicity on density-inhibited cells cannot be ascribed to release of cell surface sialic acids during hypotonic incubation. Several arguments are presented which indicate that the changes in sedimentation rate, as measured in the rotating suspension system, are not the direct consequence of the alterations in cell size, but rather must be attributed to differences in intercellular adhesiveness resulting from the size alterations. Analogous changes in intercellular adhesiveness and cell size are shown to occur during growth in isotonic suspension culture. The results can be explained by assuming that changes in cell size affect the intercellular adhesiveness by modifying the extent to which cell surface sialic acids counteract adhesion.

In a previous study it was found that removal of sialic acid by neuraminidase considerably enhanced mutual adhesion of HeLa cells harvested from suspension cultures in stationary growth (Deman, Bruyneel & Mareel, 1974). An analogous effect of neuraminidase has been reported for other cell types (Sauter, Lindenmann & Gerber, 1972; Vicker & Edwards, 1972) and for platelets (Seaman & Vassar, 1966). It subsequently was found (Deman & Bruyneel, 1975) that fast growing (FG) HeLa cells, displayed a higher intercellular adhesiveness than cells from suspension cultures in which growth was density-inhibited (DI), and that the adhe-

*Address correspondence to:* Dr. J.J. Deman, Department of Experimental Cancerology, Academic Clinic, Ghent, Belgium.

siveness of DI cells increased towards that of FG cells by treatment with neuraminidase. Remarkably, the cell surface sialic acid content on FG cells, releasable by neuraminidase was found to be approximately equal to that on DI cells. The findings appeared to indicate that the strength of intercellular adhesion during growth is regulated, at least partly, by sialic acids present at the cell periphery.

A weak point in these earlier studies comes from the neglect of possible alterations in mean cell size (A.S.G. Curtis, *personal communication*). The rotating suspension method, used for the measurement of adhesion, is based on the difference in sedimentation velocity according to particle size. Differences in mean cell size, arising during nonsynchronized growth in suspension culture, have been reported (Glinos & Werrlein, 1972), L-929 cells from FG cultures being of larger mean size than DI cells. The same applies for HeLa cells as shown in this paper. It also seems not inconceivable that treatment with neuraminidase might enhance the mean cell size, and therefore would lead to an augmented rate of cell sedimentation.

Therefore, in the present study, experiments were carried out to distinguish between the effects of cell size and intercellular adhesiveness, on the sedimentation rate. It is investigated if there is an increase in mean cell size as a result of neuraminidase treatment. Alterations in mean size of FG and DI cells are produced by incubation of the cells in anisotonic media and the effect of this treatment on the sedimentation rate is measured.

It should be realized that induction of changes in cell size, besides their possible direct effect on the sedimentation rate, also might influence the sedimentation rate indirectly by increasing the tendency for cell aggregation. In that case more adhesive cells will sediment faster because of the formation of larger and more aggregates. To check this, the results of the anisotonic experiments obtained with intact cells are compared with those in which the cells either were trypsinized previously in order to remove adhesive cell surface material, or were desialylated previously by the action of neuraminidase.

## Materials and Methods

### *Cell Culture*

HeLa cells were grown asynchronously in spinner cultures using modified (Miller, 1967) Basal Medium Eagle (BME) supplemented with 10% calf's serum. Unless explicitly

stated otherwise in the text, cell growth was started by dilution of a high density suspension to approximately $0.5 \times 10^6$ cells/ml with fresh culture medium. The spinner vessels had a volume of 2 liters and contained 600 ml of suspension.

In our culture conditions, the prestationary phase of growth was reached at cell densities between $1.8 \times 10^6$ and $2.5 \times 10^6$ cells/ml. At this stage, prolongation of the culture time after renewal of the medium resulted only in a small enhancement of the cell density. Stationary growth was obtained with cell densities between $2.5 \times 10^6$ and $3.0 \times 10^6$ cells/ml (Deman & Bruyneel, 1975). In our terminology, prestationary and stationary cells are called density-inhibited (DI). Cells harvested after 24 hr at densities of approximately $1.0 \times 10^6$ cells/ml, apparently were in exponential growth and are called fast growing (FG). It is emphasized that the term "density inhibition of growth" as used in this and the accompanying paper covers events which may not be identical to those encountered during growth in monolayer culture and for which the same term is used.

### *Preparation of the Suspensions and Measurements of the Aggregation and Agglutination Rates*

After harvest, the cells were washed once with physiological saline at 5 °C. Cells not immediately used were stored as pellets at 5 °C with the saline solution covering them.

In some experiments, washed cells were treated with trypsin or neuraminidase. Details for these treatments are described later in the text. Incubation of intact or enzyme-treated cells in NaCl solutions of various tonicity took place at 37 °C for 10 min. The aggregation rate of these cells was measured in NaCl solutions of the same concentration as that used for incubation. HeLa cells which underwent no incubation in a NaCl solution were resuspended in a Tris-Saline Phosphate (TSP) buffer (Deman & Bruyneel, 1974).

In both cases the cells were suspended at cell densities which varied between narrow limits, i.e., between 1.6 and $1.9 \times 10^6$ cells/ml, in order to minimize the influence of collision rate on aggregation (Deman & Bruyneel, 1973).

Before the actual measurement, these suspensions were placed in a water bath at 37 °C for 15 min. They were then made homogeneous by inverting the flasks several times and were transferred to the apparatus for the measurement of aggregation rate.

Aggregation was measured in a Couette-viscosimeter by a method based on the effect of particle size on the rate of sedimentation in a rotating suspension. The ratio between cell concentration in the surface layer after 20 min of rotation at 37 °C, and cell concentration in the homogeneous suspension is called the relative cell concentration (R.C.C.). An increase in aggregation rate observed microscopically is accompanied by a decrease in R.C.C. value. The method is sensitive and reproducible (Deman & Bruyneel, 1973).

### *Pretreatment with Neuraminidase and Trypsin*

For treatment with α-neuraminidase (from Vibrio Cholerae, Serva, Heidelberg, Germany), the HeLa cells were suspended at a concentration of $25 \times 10^6$ cells/ml in Ringer's solution of pH 7.4 (Deman & Bruyneel, 1974). Seven ml of this suspension were shaken for 15 min at 37 °C after addition of 1 ml acetate buffer 0.05 M, pH 5.5, containing 170 units of enzyme. The final pH of the mixture was 7.25. This procedure ensures maximal liberation of neuraminidase-releasable sialic acids from HeLa cells (Deman & Bruyneel, 1974). It has been demonstrated (Barton & Rosenberg, 1973) that the enzyme splits off sialic acid from surface glycoproteins but not from cell surface-bound glycolipids.

Cells were treated with 0.01% trypsin in Ca- and Mg-free Ringer's solution at pH 7.5 (crystalline trypsin, Sigma Chemical Co., St. Louis, Mo.; from bovine pancreas, type I). Incubation took place in a shaker system at 37 °C for 15 min.

Neuraminidase-treated cells were washed twice with physiological saline; trypsinized cells were washed three times.

### *Cell Density and Viability*

The cell concentration in the culture medium after harvest was determined by repeated countings in a Burker hemacytometer.

Viability was assessed by the trypan blue exclusion test. Immediately after harvest, the viability varied from 92% to 96%. After measurement of the aggregation or agglutination rate, the viability varied from 81% to 86%. Pretreatment with neuraminidase or trypsin did not influence viability appreciably.

### *Cell Size*

Cell size was determined microscopically by measurement of cell diameter against a calibrated grid. The mean cell diameter and the standard deviation were calculated from at least 200 measurements on cells selected at random. Telophasic cells were excluded from measurement together with cells which showed damage to the cell membrane. A coverslip was used to avoid drying during the determination. Examination time ranged from 20 to 30 min.

We avoided the electronic method (Coulter technique) for the determination of particle size, because by this technique, cell aggregates are registered as single particles. Thus, differences in mutual adhesiveness between the HeLa cells might induce errors in size determination. Previous treatment of the cells with trypsin, in order to reduce the adhesiveness, was felt to be of limited value because, from microscopic measurements (Table 2), it appeared that incubation with the enzyme, followed by resuspension of the cells in serum-free medium, resulted in a significant increase in mean cell volume.

It should be appreciated that light microscopic measurement of the cell diameter, as used in this study, cannot be regarded as an exact measure of the cell volume. Scanning electron-microscopy reveals that the cell membrane in some instances might form numerous microvilli and other membrane projections. Therefore the finding of cell size alterations during growth by the present method does not necessarily imply the occurrence of cell membrane expansion or contraction. It is equally conceivable, e.g., that an increase in cell size is accompanied by a diminution of the number and size of the surface projections which would leave the cell surface area unchanged.

## Results

### *Variation of Cell Size and Adhesiveness during Growth*

In an initial experiment, HeLa cells from a density-inhibited suspension culture were diluted to low density by the addition of fresh BME

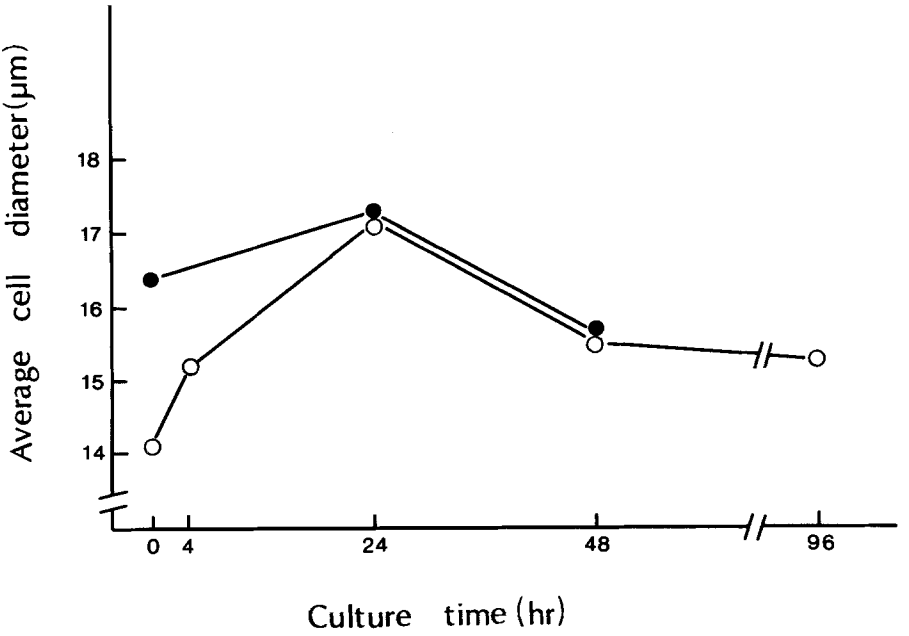


Fig. 1. Increase in mean cell size with time, following dilution of a nonsynchronized HeLa cell suspension. Standard deviations from the mean (average) cell diameter varied from $\pm 1.6$ to $\pm 2.3$. Cell growth: o—o $2.18 \times 10^6$ cells/ml before dilution, $0.60 \times 10^6$ cells/ml at start, $1.24 \times 10^6$ cells/ml after 24 hr, $1.98 \times 10^6$ cells/ml after 48 hr; ●—● $1.42 \times 10^6$ cells/ml before dilution, $0.57 \times 10^6$ cells/ml at start, $1.12 \times 10^6$ cells/ml after 24 hr, $2.01 \times 10^6$ cells/ml after 48 hr

medium. The mean cell diameter of the high density suspension was determined before dilution. Determination was repeated after several culture times at low density. The measurements were always made immediately after harvest of the cells. Fig. 1 demonstrates that, following dilution, the mean cell diameter increased during the first day of culture. After 48 hr, a high cell density was reached and a decline in cell size observed. The Figure also shows the change in mean cell size after dilution of another suspension, which had not yet reached the prestationary phase of growth, and in which the initial mean cell size was larger than that in the preceding experiment. In that case the increase in cell size, after 24 hr of culture, was less considerable.

We previously reported (Deman & Bruyneel, 1975) that the adhesiveness of the HeLa cells was maximal after about 24 hr of culture, when the cell density had doubled from $0.5 \times 10^6$ to approximately $1.0 \times 10^6$ cells per ml. The adhesiveness declined on reaching the prestationary phase of growth. The above results therefore suggest the existence

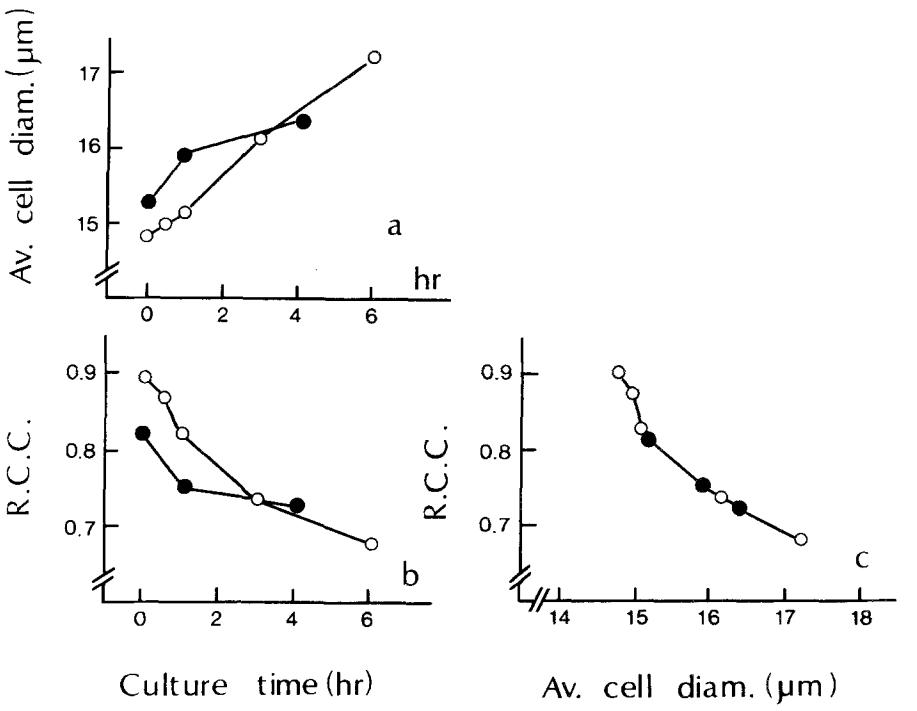


Fig. 2. Increase in mean cell size and sedimentation rate in the first hours following dilution of two density-inhibited HeLa cell suspensions. Standard deviations from the mean cell diameter varied from $\pm 1.8$ to $\pm 2.4$. R.C.C. values are inversely related to sedimentation rate. Cell growth: ●—● $0.60 \times 10^6$ cells/ml start, $0.70 \times 10^6$ cells/ml after 4 hr; ○—○ $0.69 \times 10^6$ cells/ml at start, $0.82 \times 10^6$ cells/ml after 6 hr

of an inverse relationship between cell size and adhesiveness. We emphasize that the term "adhesiveness" when reference is made to earlier work, must be considered as equivalent to "sedimentation rate". The final discussion should show to what extent changes in cell size might be responsible for the differences in sedimentation rate.

The mean cell size increased very soon after dilution of DI suspensions. The sedimentation rate ("adhesiveness") appeared to increase in a proportional way (Fig. 2). This suggests that, if changes in adhesiveness are responsible for the differences in sedimentation rate, then there is no or little time delay between the changes in cell size and adhesiveness.

Fig. 3 summarizes the results of experiments in which a large number of high density suspensions were diluted to approximately $0.5 \times 10^6$ cells per ml by the addition of fresh BME medium. The suspensions, thus prepared, were harvested after various periods of culture. Those harvested after 24 hr were at densities below $1.2 \times 10^6$ cells per ml, and were fast

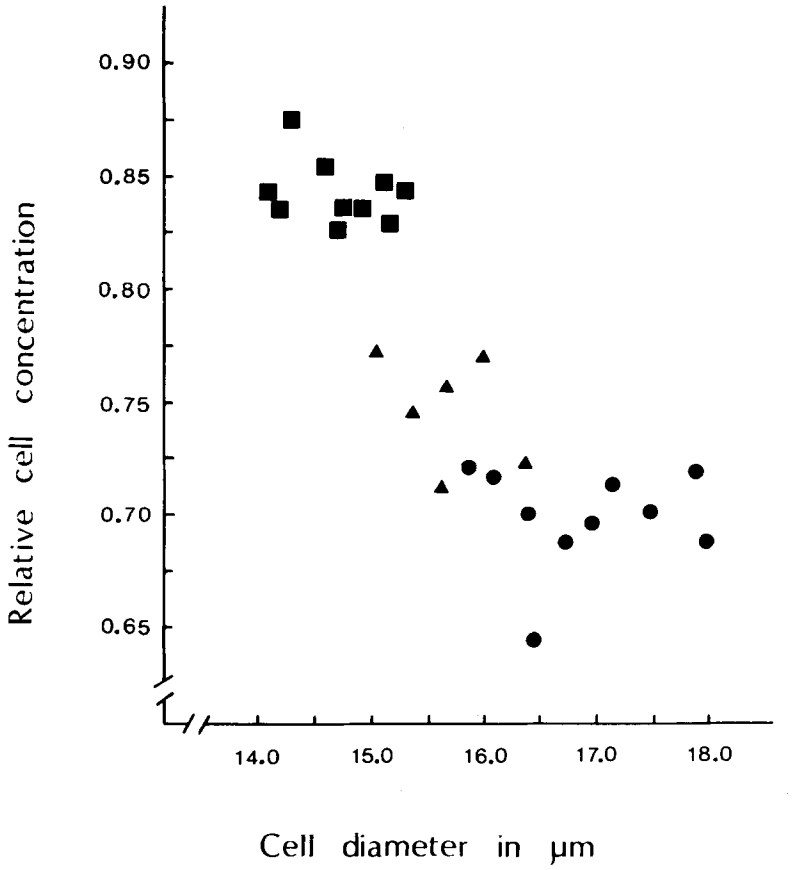


Fig. 3. Mean cell diameters and sedimentation rates of HeLa cells harvested from different cultures. Cell growth was started between 0.5 and $0.6 \times 10^6$ cells/ml by dilution of high density suspensions. Standard deviations from the mean cell diameter varied from $\pm 1.6$ to $\pm 2.5$. ● Harvest below $1.2 \times 10^6$ cells/ml after 24 hr; ▲ Harvest between 1.2 and $1.8 \times 10^6$ cells/ml after 48 hr; ■ Harvest above $1.8 \times 10^6$ cells/ml after 48 or 72 hr

growing. The sedimentation rate of these cells was high and the mean cell volume was large. Cells harvested after 48 hr of culture were at densities between 1.2 and $1.8 \times 10^6$ cells per ml, or above $1.8 \times 10^6$ cells per ml, depending on the growth rate. Suspensions cultured for 72 hr had densities above $1.8 \times 10^6$ cells per ml. Cells from the latter suspensions had a low sedimentation rate and the mean cell size was always found to be smaller than that of cells harvested below $1.2 \times 10^6$ cells per ml. Thus it appears that small size and low sedimentation rate might be characteristic of DI cultures.

Fig. 3 also reveals considerable differences in mean cell size between the populations harvested during fast growth. The same applies for the

cells harvested from DI cultures. It therefore must be clear that, when speaking about growth-induced changes in cell size, we refer to those size variations which occur during the growth of individual populations, as for example in Figs. 1 and 2.

### *Effect of Neuraminidase and Dilysine on Cell Size*

HeLa cells harvested at different densities from suspension culture were treated with neuraminidase. Subsequent measurement of the cell diameter did not reveal the occurrence of a significant change in mean cell size as the result of the enzyme treatment. Also, addition of dilysine (L-lysyl-L-lysine·2HCl; Sigma Chemical Co., St. Louis, Mo.) in various concentrations to HeLa cells from DI and FG cultures had no appreciable effect on cell size.

It has been reported (Deman *et al.*, 1974; Deman & Bruyneel, 1975) that desialylation of DI cells strongly increased their sedimentation rate in the Couette system used for measurement of the aggregation rate. Addition of dilysine up to a concentration of 8 μg per ml of medium had a similar effect (Deman & Bruyneel, 1974). The present findings demonstrate that the increased sedimentation rate cannot be attributed to the increase in size of single cells, but most probably is due to the increased tendency for aggregation.

### *Cell Size Increase by Anisotonic Treatment*

*Intact Cells.* The interference of cell size with the measurement of cell adhesion can be investigated by previous incubation of the cells in hypotonic NaCl solutions in order to induce swelling. The measurement of the sedimentation rate was performed in NaCl solutions at the same tonicity as those used for swelling. The procedure was expected to register the effect of cell size itself. However, it will fail to give an unambiguous answer on this point, if the adhesive properties of the cell surface are altered by the increase in size. We also cannot exclude the possibility that cell size alterations which occur during growth in isotonic medium might affect intercellular adhesion by the appearance of cell surface modifications other than those resulting from size alterations.

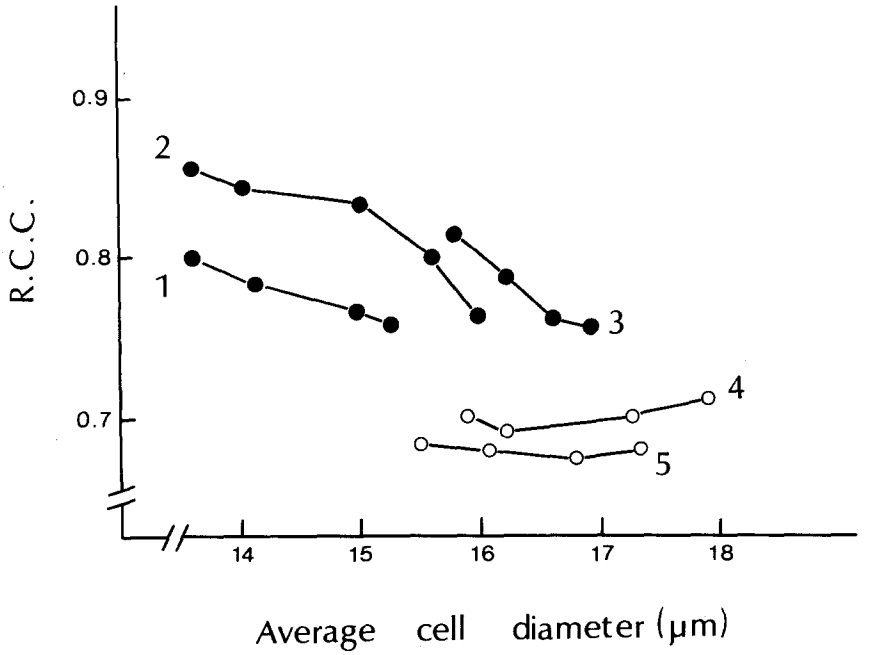


Fig. 4. Changes in sedimentation rate induced by incubation in anisotonic NaCl solutions. NaCl cones: (left), 1.1%, 0.9%, 0.7%, 0.5%, (right). Standard deviations from the mean cell diameter varied from $\pm 1.5$ to $\pm 2.0$ (●) and from $\pm 1.8$ to $\pm 2.6$ (○). ●—● Cells from density-inhibited cultures; (1) harvest at $1.90 \times 10^6$ cells/ml; (2) harvest at $2.48 \times 10^6$ cells/ml; (3) harvest at $1.98 \times 10^6$ cells/ml. ○—○ Cells from low density cultures after 24 hr of growth; (4) harvest at $1.12 \times 10^6$ cells/ml; (5) harvest at $1.03 \times 10^6$ cells/ml

HeLa cells obtained from low density suspensions in fast growth showed no appreciable change in sedimentation rate when placed in solutions of decreasing tonicity. The same treatment induced a significant increase in the sedimentation rate of cells from DI suspensions (Fig. 4). In both cases, hypotonicity caused a significant cell size enlargement. A similar effect of medium tonicity on the sedimentation rate (adhesiveness) of DI cells has been described earlier (Deman & Bruyneel, 1973). As was pointed out there, it seems unlikely that the phenomenon could be explained solely by the minor density differences of the medium ($\Delta p =$ 0.0037 between a 1.1% and a 0.5% NaCl solution), all other changes being ignored.

The result is remarkable because it shows that cell density and/or growth rate, at the time the cells were harvested, are factors which modify the extent to which the effect of hypotonic swelling is exerted. The effect is present on DI cells, i.e. cells of small mean size, and is absent on the larger FG cells.

*Desialylated Cells.* The finding that hypotonicity exerts only an effect on DI cells but not on FG cells, bears some analogy with the effect of neuraminidase. Treatment with the enzyme considerably enhanced the sedimentation rate (adhesiveness) of DI cells, but had no, or only a weak effect on FG cells (Deman & Bruyneel, 1975). Therefore, it seemed worthwhile to examine whether the presence of cell surface sialic acids did influence the effect of hypotonicity.

HeLa cells were treated with neuraminidase previous to their incubation in NaCl solutions of various tonicity. Although the small mean cell diameter of cells from DI cultures significantly was increased by hypotonic treatment, there was no corresponding increase in sedimentation rate. The same applied for desialylated cells from a FG culture (Table 1). Comparison of the sedimentation rates of intact and desialylated cells at 0.9% NaCl, shows that DI cells underwent a strong increase in sedimentation rate, whereas no effect was observed with FG cells, in agreement with the earlier study referred to above.

We next investigated if the effect of hypotonicity on intact DI cells could possibly be ascribed to release of sialic acids during incubation in hypotonic NaCl medium. 200 ml of a HeLa cell suspension harvested from a culture at $1.86 \times 10^6$ cells per ml, were divided into two portions

Table 1. Effect of medium tonicity on cell volume and sedimentation rate following pretreatment of HeLa cells with neuraminidase[a]

| Cell density at harvest (cells per ml) | Incubation, % NaCl | Intact cells | Cells pretreated with neuraminidase | | | |
|---|---|---|---|---|---|---|
| | | 0.9% | 1.1% | 0.9% | 0.7% | 0.5% |
| $2.1 \times 10^6$ | Mean cell diameter (μm ± SD) | 14.4 ± 1.8 | 13.1 ± 1.6 | 13.9 ± 1.6 | 14.7 ± 2.0 | 14.9 ± 1.9 |
| | Sedimentation rate (R.C.C.) | 0.879 | 0.749 | 0.749 | 0.748 | 0.743 |
| $2.4 \times 10^6$ | Mean cell diameter (μm ± SD) | 14.5 ± 1.6 | 14.2 ± 2.0 | 14.7 ± 1.9 | 15.2 ± 1.8 | 15.9 ± 2.1 |
| | Sedimentation rate (R.C.C.) | 0.812 | 0.671 | 0.671 | 0.664 | 0.672 |
| $0.9 \times 10^6$ | Mean cell diameter (μm ± SD) | 17.8 ± 2.5 | 16.4 ± 2.3 | 17.1 ± 2.3 | 17.5 ± 2.6 | 19.3 ± 2.6 |
| | Sedimentation rate (R.C.C.) | 0.722 | 0.738 | 0.722 | 0.718 | 0.725 |

[a] Neuraminidase-treated cells were washed twice with NaCl 0.9% and then were incubated during 10 min at 37 °C in NaCl solutions of various salt concentration. Measurements were performed on these suspensions. SD: standard deviation; R.C.C.: relative cell concentrations.

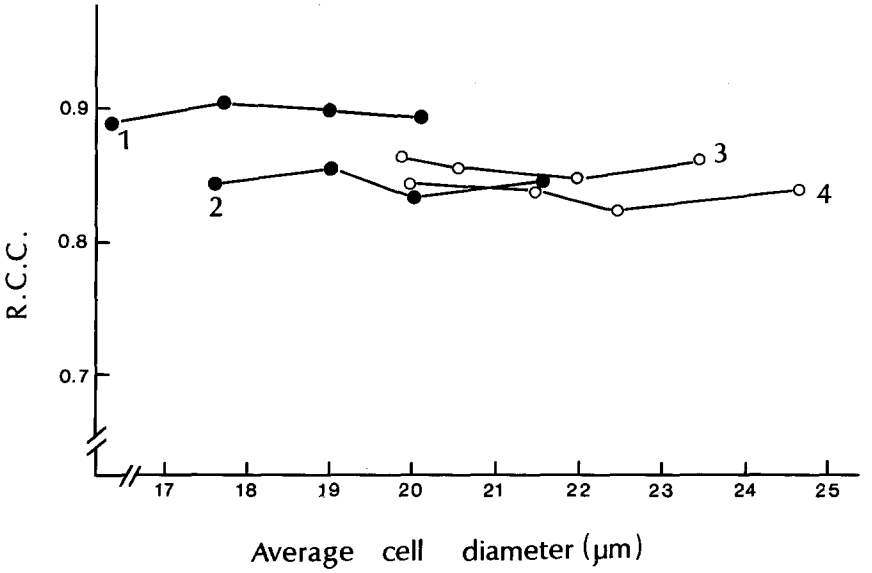


Fig. 5. Sedimentation rate following incubation of trypsinized cells in anisotonic NaCl solutions. NaCl cones: (left), 1.1%, 0.9%, 0.7%, 0.5%, (right). Standard deviations from the mean cell diameter varied from ± 2.0 to ± 2.7 (●), and from ± 2.6 to ± 3.1 (○). ●—● Cells from density-inhibited cultures, trypsinized after harvest; (1) harvest at $2.59 \times 10^6$ cells/ml; (2) harvest at $3.04 \times 10^6$ cells/ml. ○—○ Cells from low density cultures harvested after 24 hr and subsequently trypsinized; (3) harvest at $0.97 \times 10^6$ cells/ml; (4) harvest at $1.16 \times 10^6$ cells/ml

and incubated respectively in 0.9% and 0.5% NaCl solution for 30 min at 37 °C. After centrifugation, the supernatants were assayed for sialic acid according to the Warren method (Warren, 1959). No detectable amounts were present. The supernatants also were examined for released (glyco)proteins according to the method of Lowry, Rosebrough, Farr and Randall (1951), and the amounts were found to be almost identical, i.e. 1.2 μg protein released in 0.9% NaCl and 1.3 μg protein in 0.5% NaCl (protein was expressed as albumin per $10^6$ cells).

The pellets after centrifugation were treated with neuraminidase. The amounts of sialic acid split off by the enzyme were 0.68 and 0.69 mM per $10^6$ cells, respectively, for cells preincubated in 0.9% and 0.5% NaCl. The results demonstrate that hypotonicity does not promote the release of sialic acid and of (glyco)protein to an appreciable extent.

*Trypsinized Cells.* Incubation of trypsinized HeLa cells in NaCl solutions of various tonicity did not result in a significant alteration in sedimentation rate (Fig. 5). Cells were taken both from DI and FG cultures. Comparison with Fig. 4 shows that the result with DI cells

Table 2. Changes in mean cell size and sedimentation rate following trypsinization in absence of $Ca^{2+}$ and $Mg^{2+}$

| Cell density at harvest (cells per ml) | Mean cell diameter (μm ± SD) | | Sedimentation rate (R.C.C.) | |
|---|---|---|---|---|
| | Intact cells | Trypsinized cells | Intact cells | Trypsinized cells |
| $0.8 \times 10^6$ | 18.8 ± 2.8 | 24.3 ± 4.1 | 0.681 | 0.827 |
| $1.0 \times 10^6$ | 16.4 ± 2.0 | 20.6 ± 2.7 | 0.637 | 0.852 |
| $1.1 \times 10^6$ | 18.0 ± 2.7 | 21.5 ± 3.0 | 0.683 | 0.842 |
| $2.6 \times 10^6$ | 14.7 ± 2.0 | 17.5 ± 1.9 | 0.825 | 0.906 |
| $2.7 \times 10^6$ | 15.1 ± 1.9 | 19.5 ± 2.6 | 0.800 | 0.855 |

After treatment with 0.01% trypsin, the cells were washed three times with NaCl 0.9%. Cell diameters were measured in NaCl 0.9%.

Table 3. A comparison between the effects on cell size and sedimentation rate

| | Mean cell diameter | Sedimentation rate |
|---|---|---|
| FG cells compared to DI[a] cells after growth of the same suspension culture | Increase | *Increase* |
| Neuraminidase treatment of DI cells | Unchanged[b] | *Increase* |
| Neuraminidase treatment of FG cells | Unchanged | Unchanged |
| Dilysine added to DI cells | Unchanged | *Increase* |
| Dilysine added to FG cells | Unchanged | Unchanged |
| Hypotonic swelling | | |
| of DI cells | Increase | *Increase* |
| of neuraminidase-treated DI cells | Increase | Unchanged |
| of trypsin-treated DI cells | Increase | Unchanged |
| of FG cells | Increase | Unchanged |
| of neuraminidase-treated FG cells | Increase | Unchanged |
| of trypsin-treated FG cells | Increase | Unchanged |
| Trypsin treatment of DI cells | Increase | *Decrease* |
| Trypsin treatment of FG cells | Increase | *Decrease* |

[a] FG and DI cells: fast growing and density-inhibited cells, respectively, in suspension culture.

[b] Unchanged: no change of statistical significance detected.

is in contrast with that obtained with intact DI cells, and also that trypsinized cells are of larger mean size in isotonic medium than intact cells. Moreover, identical differences in tonicity produce a larger size

increase in trypsinized cells than in intact cells. The results are not contrary to the assumption that trypsin might augment membrane deformability. The earlier studies of Weiss and Clement (1966) demonstrated that trypsin-releasable material plays a role in maintaining cell surface rigidity.

From Table 2 it is seen that trypsinization by itself, while decreasing the sedimentation rate, produces a considerable enhancement of cell size. We recall that incubation with trypsin occurred in isotonic Ringer's solution devoid of $Ca^{2+}$ and $Mg^{2+}$, and that no serum was added to the cells after trypsinization. A similar effect of trypsin in serum-free medium has been reported by Mallucci, Wells and Young (1972). They found that cell size enlargement occurred even when the stimulating effect of trypsin on DNA synthesis was inhibited, indicating that trypsin directly affected the cell surface.

## Discussion

Measurement of the cell diameter during growth of HeLa cells in suspension reveals that during rapid growth at low density, the mean cell size is considerably larger than that of DI populations. These results confirm previous findings on other cell types. Glinos and Werrlein (1972) reported that, following dilution of a DI fibroblast culture, there was a marked, progressive increase in mean cell volume, which reached its maximum after 20 hr and then declined. In lymphocytes, a close relationship was found between mitosis and cell volume, the latter being regulated by the $(Na^+ + K^+)$-ATPase ($Mg^{2+}$-dependent) activity (Jung & Rothstein, 1967; Quastel & Kaplan, 1970).

As pointed out in the Introduction, it could not be excluded beforehand that the enhancement of the sedimentation rate of FG cells might be due to the increase in mean size of single cells, and not to the increase in number and particle size of the aggregates. The finding that hypotonic swelling of intact DI cells enhances their sedimentation rate, can be interpreted in favor of a direct relationship between cell size and sedimentation rate.

Arguments against such direct relationship come from the following observations:

(1) Hypotonic swelling does not significantly increase the sedimentation rate of (a) trypsinized DI cells, (b) desialylated DI cells, or (c) intact FG cells (Table 3).

(2) Trypsinization results in a considerable increase in size of the HeLa cells. When compared to intact cells, trypsinized cells display a lower sedimentation rate (Table 3).

(3) Comparison of different cell populations harvested at similar growth rates (or at comparable cell densities), reveals the existence of a considerable variation in mean cell size, without a parallel change in sedimentation rate (Fig. 3).

The above findings can be interpreted by making two basic assumptions:

(1) *Alterations in cell size have no appreciable influence on the sedimentation rate of single cells in our measuring system.* The possibility must be considered that the system is not sensitive enough to measure the increased sedimentation rate, which is expected to occur when the cell volume increases, and the buoyant cellular density remains unchanged. Another possibility is that the volume increase might be accompanied by a diminution in buoyant cellular density, which counteracts the effect of the increase in volume.

(2) *Cell size alterations mainly affect the action of cell surface sialic acids on mutual cell adhesion.* By this assumption, it can be explained why anisotonicity has no effect on desialylated DI cells, nor on FG cells for it is known that sialic acids present on the latter cells do not counteract adhesion. It has been demonstrated previously that the role of sialic acid essentially consists in the counteraction of the adhesiveness which is conferred to the cells by trypsin-releasable material (Deman *et al.*, 1974). Therefore, it is clear that in absence of this material, i.e. on trypsinized cells, sialic acids cannot modulate adhesiveness, nor can cell size alterations affect the adhesiveness by the mediation of the sialic acid residues. In this connection we mention that about two-thirds of the original sialic acid content on HeLa cells is still present after trypsinization (Deman *et al.*, 1974). The reduced sedimentation rate of the large, trypsinized HeLa cells, indicates that our measuring system essentially records the decrease in aggregation rate caused by trypsin.

It should be appreciated that the differences in adhesiveness between FG and DI were not found to be caused by loss of sialic acids from the cell periphery (Deman & Bruyneel, 1975), nor is the adhesive difference observed after hypotonic swelling of DI cells described in the present paper. The present study therefore strongly suggests that cell size increase, either occurring during growth in culture, or induced artificially by incubation in anisotonic media, does not affect the sedimentation rate in a

direct way, but rather does so by decreasing the capability of cell surface sialic acids to counteract adhesion.

Above considerations imply a direct cause and effect relationship between size changes and adhesiveness. However, the experiments do not exclude the possibility that the changes in size and adhesiveness occurring during growth, both might be the result of some third factor which is dependent on growth. The occurrence of this third factor becomes less probable if additional evidence could be presented for the mechanism by which changes in cell size and adhesiveness are assumed to be connected. Sialic acids have been interpreted (Deman & Bruyneel, 1974) to be responsible for the generation of long-range repulsion forces on HeLa cells. In the accompanying paper (Deman, Vakaet & Bruyneel, 1976) it is examined to what extent changes in cell size have an influence on repulsion.

The authors are much indebted to Mrs. R. Van Cauwenberghe for technical assistance. This study was supported by a grant from the Fund for Medical Research (FGWO), Belgium.

## References

Barton, N.W., Rosenberg, A. 1973. Action of Vibrio cholerae neuraminidase (sialidase) upon the surface of intact cells and their isolated sialolipid components. *J. Biol. Chem.* **248**:7353

Deman, J., Bruyneel, E. 1973. A method for the quantitative measurement of cell aggregation. *Exp. Cell Res.* **81**:351

Deman, J.J., Bruyneel, E.A. 1974. Evidence for long-range electrostatic repulsion between HeLa cells. *Exp. Cell Res.* **89**:206

Deman, J.J., Bruyneel, E.A. 1975. Intercellular adhesiveness and neuraminidase effect following release from density inhibition of cell growth. *Biochem. Biophys. Res. Commun.* **62**:895

Deman, J.J., Bruyneel, E.A., Mareel, M.M. 1974. A study on the mechanism of intercellular adhesion. Effects of neuraminidase, calcium and trypsin on the aggregation of suspended HeLa cells. *J. Cell Biol.* **60**:641

Deman, J.J., Vakaet, L.C., Bruyneel, E.A. 1976. Cell size and mutual cell adhesion. II. Evidence for a relation between cell size, long-range electrostatic repulsion and intercellular adhesiveness during density-regulated growth in suspension. *J. Membrane Biol.* **26**:205

Glinos, A.D., Werrlein, R.J. 1972. Density dependent regulation of growth in suspension cultures of L-929 cells. *J. Cell. Physiol.* **79**:79

Jung, C., Rothstein, A. 1967. Cation metabolism in relation to cell size in synchronously grown tissue culture cell. *J. Gen. Physiol.* **50**:917

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265

Mallucci, L., Wells, V., Young, M.R. 1972. Effect of trypsin on cell volume and mass. *Nature, New Biol.* **239**:53

Miller, A.O.A. 1967. Characterization of messenger ribonucleic acid in partially synchronized HeLa cells. *Arch. Biochem. Biophys.* **122**:270

Quastel, M.R., Kaplan, J.G. 1970. Early stimulation of potassium uptake in lymphocytes treated with PHA. *Exp. Cell Res.* **63**:230

Sauter, C., Lindenmann, J., Gerber, A. 1972. Agglutination of leukemic myeloblasts by neuraminidase. *Eur. J. Cancer* **8**:451

Seaman, G.V.F., Vassar, P.S. 1966. Changes in the electrokinetic properties of platelets during their aggregation. *Arch. Biochem. Biophys.* **117**:10

Vicker, M.G., Edwards, J.G. 1972. The effect of neuraminidase on the aggregation of BHK 21 cells transformed by polyoma virus. *J. Cell Sci.* **10**:759

Warren, L. 1959. The thiobarbituric acid assay of sialic acid. *J. Biol. Chem.* **234**:1971

Weiss, L., Clement, K. 1966. Deformability of murine ascites sarcoma 37 cells. *Ann. Med. Exp. Fenn.* **44**:155