THE EFFECT OF NEURAMINIDASE (3:2:1:18) ON THE AGGREGATION OF CELLS DISSOCIATED FROM EMBRYONIC CHICK MUSCLE TISSUE

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SUMMARY

Embryonic chick muscle cells were used to investigate the effect of removing cell-surface sialic acids on cell aggregation in vitro. Single cell suspensions were prepared by dissociating skeletal muscle tissue of 9-day-old chick embryos with either crystalline or crude trypsin. Cell aggregation was quantitatively estimated by turbidimetric and gyratory shaker methods.

Cells dissociated with crude trypsin and suspended in Hanks's balanced salts solution (BSS) containing 25 u./ml neuraminidase (NANase) only aggregated for 2 h when rotated in an absorptiometer. The inhibitory effect of the enzyme was more pronounced with increasing concentration up to 25 u./ml. Cells dissociated with crystalline trypsin and treated with 100 u./ml NANase immediately exhibited a reduced aggregative competence when gyrated in Eagle's minimum essential medium (MEM) containing 25 u./ml NANase, compared with the controls which were not exposed to NANase. The aggregation rate of muscle cells pretreated with 100 u./ml NANase and suspended in Eagle's MEM was similar to that of the untreated controls. Cell counts showed that under all three experimental conditions cells were not added to aggregates after the 12-h stage. Aggregates formed in Eagle's MEM (the controls) joined together to form larger aggregates after 12 h, but those rotating in the presence of NANase did not display this property.

Lissamine green viability tests showed that cells remained alive throughout the 24-h period in the presence of NANase. Determinations of oxygen uptake, protein synthesis and mitotic index confirmed that general cellular viability was not affected by NANase. Fluorescent-labelled NANase was not taken up by the cells.

Treatment of crystalline trypsin-dissociated muscle cells with 100 u./ml NANase for 30 min at 37 °C significantly reduced their negative electrophoretic mobility. This diminution closely corresponded to the removal of cell-surface sialic acids, as measured by colorimetric tests.

Interpretation of the results in the light of current theories of cell adhesion failed to give support to the concept of adhesion by physical forces. The mechanism by which cellular deformability could influence cellular adhesiveness is modified in the knowledge of the present results.

INTRODUCTION

Recent work has shown that sialic acids form a quantitatively important structural part of the cell surface (Cook, Heard & Seaman, 1962; Gasic & Gasic, 1962; Gasic, Berwick & Sorrentino, 1966; Mayhew, 1966; Rambourg & Leblond, 1967; Simon-Reuss, Cook, Seaman & Heard, 1964). It is surprising, therefore, that our knowledge concerning the role of sialic acids in cell surface membranes extends no further than that they contribute to the charge properties of the cell and form part of the antigenic complex at the cell surface (Cook, 1968).

Several workers have suggested that sialic acids may be involved in cell adhesion...
R. B. Kemp

(Abercrombie & Ambrose, 1962; Ambrose, 1966; Curtis, 1967; Weiss, 1967a; Kemp, 1968). This proposal largely stemmed from attempts to correlate interesting differences in the sialic acid content between several types of normal and malignant or transformed cells with their markedly different adhesive behaviour (Forrester, Ambrose & Macpherson, 1962; Fuhrmann et al. 1962; Forrester, Ambrose & Stoker, 1964).

Weiss (1961) showed that neuraminidase (mucopolysaccharide N-acetyl-neuraminylhydrolase 3:2:1:18) reduced the adhesiveness of rat cells to a glass surface, implying that sialic acids contributed to cellular adhesiveness. In a later paper Weiss (1965) proposed that sialic acids were important in cell adhesion by reason of their role in maintaining the structural rigidity of the cell surface, though he recently conceded that this theory is highly speculative (Weiss, 1968).

Since a suitable terminal inhibitor of the sialic acid biosynthetic pathway is not available, it was decided to remove sialic acid residues from the surface of cells using the specific enzyme neuraminidase (NANase). This enzyme cleaves only the ketosidic linkage joining a terminal acylated neuraminic acid to another sugar or sugar derivative (Gottschalk, 1958). The effect of removing sialic acids on the aggregative competence and electrophoretic mobility of the cells was studied and an attempt was made to correlate these findings with current theories of cell adhesion.

MATERIALS AND METHODS

Cell dissociation

Cells were obtained from skeletal muscle of 9-day-old chick embryos. The tissue was dissociated into separate cells using 0.25% crude trypsin (Difco 1:250) or 0.1% crystalline trypsin (Worthington) in Hanks's BSS at pH 7.6 for 10 min according to the standardized procedure adopted in this laboratory (Cunningham & Hirst, 1967; Kemp, Jones, Cunningham & James, 1967). The dissociated cells were a mixture of fibroblasts and myoblasts and were, for convenience, termed muscle cells. Sterile conditions were employed when preparing cell suspensions for reaggregation studies in the gyratory shaker.

Aggregation techniques

Aggregation of the dissociated cells was quantitatively estimated using the turbidimetric method and the gyratory shaker. Details of the turbidimetric apparatus (Born, 1962; Jones, 1965) have been described by Cunningham & Hirst (1967). Optimum conditions for aggregation by this method were obtained at a cell density of 7 x 10^6/ml. Progress in aggregation was followed by monitoring changes in the amount of light at a wavelength of 600 nm ± 15 (bandpass filter) scattered by the cells in suspension. Neuraminidase (prepared by Behringwerke from Vibrio cholerae), at a final concentration of 25 u./ml, was added to cell suspensions at 0 h.

The aggregation of muscle cells over a 24-h period was investigated using a gyratory shaker (Moscona, 1961). A carefully washed suspension of single cells (1.5 x 10^6/ml) in 3 ml of Eagle's MEM was placed in each 25-ml Ehrlenmeyer flask. The suspension medium contained 20 mg/ml of the fungicide Mycostatin (Paul, 1965). The pH of the medium was maintained at 7.6 throughout the course of the experiment by gassing the flasks with 5% carbon dioxide in air. The cell suspensions, maintained at 37 °C, were swirled around in a sealed flask at 70 rev/min for periods of up to 24 h.

The effect of NANase on the normal pattern of aggregation in the gyratory shaker was studied. Cells were incubated at 37 °C for 30 min in NANase at a concentration of 100 u./ml Hanks's BSS/10^6 cells. A control preparation was similarly incubated in Hanks's BSS. Aliquots of the NANase-treated cell suspension were rotated in Eagle's MEM containing 25 u./ml NANase (pH 7.6). Further aliquots of this suspension were rotated in Eagle's MEM. Un-
Effect of neuraminidase on cell aggregation

753

treated cells suspended in Eagle’s MEM acted as a second control. Heat-inactivated enzyme (56 °C for 15 min) and crystalline NaNase, prepared from a methanolized suspension of *Vibrio cholerae* (Ada, French & Lind, 1961), were employed to test the specificity of the commercial enzyme.

Estimation of cell viability

Viability of the cells was assessed by adding one drop of 2 % aqueous trypan blue (Weiss, 1958) or 2 % aqueous lissamine green (Kemp *et al.* 1967) to 9 drops of a cell suspension. Viable cells exclude these colloidal dyes but they are taken up by dead and dying cells.

Viability was also examined by other techniques. First, aggregates from experiments in which sterile conditions had been maintained, were cultured on a plasma clot (Paul, 1965) for 2 days. The cultures were then fixed and stained using the May-Grünwald-Giemsa technique (Paul, 1965). The stained preparations were examined for mitotic figures. Secondly, the effect of exogenous NaNase on the synthesis of proteins by muscle cells rotated in the gyratory shaker was studied. An aliquot of 0.025 ml of 20 μCi/ml L-[1-14C]leucine was added at 23 h to cells suspended in Eagle’s MEM with or without 25 μ/ml NaNase to give a final concentration of 1 μCi/g × 10^6 cells. The suspensions were further rotated in the gyratory shaker for 60 min. The uptake of radioactive leucine into proteins during this period was determined according to a procedure based on that developed by Humphreys (1966) and modified by Kemp *et al.* (1967). Samples were counted on a Nuclear Chicago model 186A gas-flow radioactive counter to a standard error of 0.5 %. As a third method of estimating cellular viability, the oxygen uptake of cells was assessed using the biological oxygen monitor (Lessler, Malloy & Schwab, 1965).

Fluorescent techniques

Fluorescent NaNase was prepared by conjugation with fluorescein isothiocyanate (FITC) (Riggs *et al.* 1958). The ability of the enzyme conjugate to liberate NaN from α-acid glycoprotein under standard conditions was taken as a measure of its activity (Gottschalk, 1958). The conjugate was then diluted to 25 μ/ml with Eagle’s MEM.

Fluorescence microscopy of FITC-NANase-treated cells was undertaken using a Reichert Zetopan microscope. Fluorimetric analysis of cell-free supernatants was performed on a Unicam SP 800 spectrophotometer with SP 860 spectrofluorimeter accessory.

Estimation of N-acetylneuraminic acid

Sialic acids were assayed as N-acetylneuraminic acid (NAN) by the 2-thiobarbituric acid method (Warren, 1959). In view of the possibility of substantial amounts of 2-deoxyribose in the assay mixture Warren’s correction equation (1959) was employed in the calculation of sialic acid levels.

Electrophoretic mobility studies

The electrophoretic mobility of muscle cells was measured employing a microelectrophoresis apparatus of the type designed by Bangham, Flemans, Heard & Seaman (1958) and modified by Seaman & Heard (1961). The cells were suspended in 0.145 M sodium chloride made 3 x 10^{-4} M with respect to sodium bicarbonate, pH 7.2 ± 0.2 (Heard & Seaman, 1966). All solutions were made up in double-distilled water. Electrophoretic mobilities were determined at 25 ± 0.1 °C, the results, based on the mean of 50-60 measurements, being expressed in μm/s/V/cm.

RESULTS

Aggregation of trypsin-dissociated cells

Cells dissociated with crude trypsin and suspended in Hanks’s BSS were rotated in an absorptiometer in the presence of 25 μ/ml NaNase for 4 h. The treated cells did not aggregate after the first 2 h, as shown by plotting the change in optical density
against time (Fig. 1). It will be seen from Table 1 that increasing concentrations of NANase up to 25 u./ml caused a progressively greater inhibition of aggregation. The optical density figures for aggregation in the presence of 25 and 50 u./ml NANase were virtually identical signifying that the maximum inhibitory effect of NANase was attained at a concentration of 25 u./ml. The optical density figure for cells rotated in the presence of 50 u./ml NANase was not consistent with the general pattern of results and indeed the initial stages of all the optical density curves were erratic owing to the stabilization period required in the turbidimetric method. These results, which were repeated 8 times, were qualitatively reproducible. Both the treated cells and the controls were viable at the end of a 4-h period of rotation.

Cells dissociated with crystalline trypsin and incubated with 100 u./ml NANase (30 min at 37 °C) were rotated in a gyratory shaker in Eagle's MEM containing 25 u./

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Table 1. Optical density of trypsin-dissociated cells rotated by the turbidimetric method in the presence of different concentrations of neuraminidase

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>5 u./ml NANase</th>
<th>12.5 u./ml NANase</th>
<th>25 u./ml NANase</th>
<th>50 u./ml NANase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>-0.116</td>
<td>-0.098</td>
<td>-0.064</td>
<td>-0.052</td>
<td>-0.072</td>
</tr>
<tr>
<td>120</td>
<td>-0.14</td>
<td>-0.137</td>
<td>-0.132</td>
<td>-0.13</td>
<td>-0.134</td>
</tr>
<tr>
<td>180</td>
<td>-0.186</td>
<td>-0.171</td>
<td>-0.162</td>
<td>-0.134</td>
<td>-0.138</td>
</tr>
<tr>
<td>240</td>
<td>-0.21</td>
<td>-0.19</td>
<td>-0.166</td>
<td>-0.134</td>
<td>-0.14</td>
</tr>
</tbody>
</table>
Effect of neuraminidase on cell aggregation

ml NANAse for periods of up to 24 h. These cells possessed a considerably slower rate of aggregation than the controls which had not been subjected to NAnaese treatment (Fig. 2). Cells which had been pretreated with 100 u./ml NAnaese and rotated in Eagle’s MEM did not differ in their aggregation rate from the controls. Counts of the

Fig. 2. The effect of 25 u./ml NANAse on the progressive reduction in the number of single cells remaining in a suspension of crystalline trypsin-dissociated embryonic chick muscle cells. The cells were pretreated with 100 u./ml NANAse for 30 min at 37 °C and swirled in Eagle’s MEM at 37 °C for a 24-h period. Curves for treated cells (open circles) and controls (dots). Note that the rate of aggregation of NANAse-treated cells was less than that of the controls and that cells in both preparations did not aggregate after 12 h.

numbers of single cells remaining in suspension at set times during the 24-h period revealed that, under all 3 experimental conditions, cells were not added to aggregates after 12 h. Since the 3 cell suspensions were aliquots of the same preparation, the difference in cell counts shown in Fig. 2 could not have been due to variations in the number of red blood cells present. The rate of aggregation of red blood cells in a suspension of muscle cells treated with 100 u./ml NANAse and rotated in the presence
of 25 u./ml NANase was similar to that of the untreated controls (Fig. 3) and could not have caused the differences shown in Fig. 2.

Visual examination of aggregates formed in the presence of NANase from cells pre-treated with 100 u./ml NANase showed that they were consistently smaller than aggregates produced in Eagle's MEM by untreated cells. This difference was already apparent by 4 h (Figs. 4, 5) and persisted at 12 h (Figs. 6, 7) and 24 h (Figs. 8, 9). After 12 h, aggregates formed in Eagle's MEM coalesced to form larger aggregates (Fig. 6). This phenomenon did not occur between aggregates formed in the presence of

![Image](https://example.com/image.png)

Fig. 3. The effect of 25 u./ml NANase on the reduction in numbers of red blood cells in a suspension of crystalline trypsin-dissociated muscle cells swirled in Eagle's MEM at 37 °C for 24 h. Curves for treated cells (open circles) and controls (dots). Note that NANase had little effect on the normal pattern of aggregation.

NANase, the aggregates remaining the same size from 12 h (Fig. 7) until the end of the 24-h experimental period (Fig. 9). They also failed to undergo the increase in cell density characteristic of aggregates formed by the control preparations (Fig. 8). These experiments were repeated, with the same qualitative pattern of results, 11 times.

When crystalline NANase was employed as a substitute for the commercial enzyme, the pattern of results was identical to that described above, using Behringwerke NANase. Heat-inactivated enzyme had no visible effect on the aggregation rate of muscle cells swirled in a gyratory shaker.

**Viability studies**

Dye exclusion tests using lissamine green showed that cells remained alive throughout the 24-h period in the presence of NANase. At 24 h the viability was in the region of 90–95% for both the treated cells and the controls. Aggregates formed in the presence of NANase and cultured for 48 h on a plasma clot were completely healthy. The muscle cells grew out from the aggregates (Fig. 10) and a proportion of them displayed
Effect of neuraminidase on cell aggregation

mitotic figures. As a further viability test, measurements of the incorporation of L-[1-14C]leucine into cellular proteins during the 24th h of the experiment revealed that there was an insignificant difference in protein synthesis of only 1% between cells maintained in Eagle's MEM with NANase and those in Eagle's MEM alone (Table 2). Treated cells also showed little difference in oxygen uptake from the controls (Table 2).

Table 2. Effect of neuraminidase (25 u./ml) on protein synthesis and oxygen uptake of crystalline trypsin-dissociated muscle cells (1·5 x 10⁶/mi) suspended in Eagle's MEM

<table>
<thead>
<tr>
<th>Incorporation of L-[1-14C]leucine into proteins during the 24th h in the gyratory shaker, cpm (corrected for background)</th>
<th>No. of experiments</th>
<th>Eagle's MEM</th>
<th>Eagle's MEM + 25 u./ml NANase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>527·8</td>
<td>521·4</td>
</tr>
</tbody>
</table>

| Oxygen uptake of cells after treatment with 100 u./ml NANase for 30 min at 37 °C (readings taken over 15 min), μlO₂/h/10⁶ cells | 7 | 1·89 | 2·03 |

Table 3. N-acetylneuraminic acid liberated from cells at stages in their preparation and subsequent aggregation

<table>
<thead>
<tr>
<th>Stage of treatment</th>
<th>μmol NAN/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>In supernatant after dissociation with 0·1% crystalline trypsin</td>
<td>2·8 x 10⁻³</td>
</tr>
<tr>
<td>In supernatant after pretreatment with 100 u./ml NANase</td>
<td>1·3 x 10⁻⁶</td>
</tr>
<tr>
<td>In supernatant after aggregation in the presence of 25 u./ml NANase</td>
<td>4·6 x 10⁻⁴</td>
</tr>
<tr>
<td>In cells after crystalline trypsin dissociation</td>
<td>9·0 x 10⁻⁵</td>
</tr>
<tr>
<td>In crystalline trypsin-dissociated cells after NANase pretreatment</td>
<td>6·3 x 10⁻⁶</td>
</tr>
<tr>
<td>In supernatant after trypsinization of aggregates formed in Eagle's MEM after 24 h</td>
<td>3·4 x 10⁻⁵</td>
</tr>
<tr>
<td>In supernatant after trypsinization of aggregates formed in Eagle's MEM + 25 u./ml NANase after 24 h</td>
<td>1·4 x 10⁻⁵</td>
</tr>
</tbody>
</table>

During the 24-h period in which cells were rotated in Eagle's MEM containing 25 u./ml NANase, 4·6 x 10⁻⁴ μmol NAN/10⁶ cells was released into the medium (Table 3). NAN found in the supernatant after trypsinization of aggregates produced in the presence of 25 u./ml NANase was 1·4 x 10⁻⁵ μmol/10⁶ cells, only 40% of that found in the supernatant after trypsin dissociation (10 min at 37 °C) of aggregates formed from cells not exposed to NANase (Table 3).

Fluorescence microscopy of aggregates formed after 24 h in Eagle's MEM con-
R. B. Kemp

Taining an FITC-NANase conjugate equivalent to 25 u./ml NANase did not reveal intracellular localization of the enzyme. The fluorescence was too faint for meaningful photography. Spectrophotometric measurements (Unicam SP 800) of the exogenous FITC-NANase remaining in Eagle's MEM after 24 h showed that the presence of cells for that period of time caused only a 22-7% loss of conjugate from the medium.

Electrophoretic mobility studies

The negative mobility of muscle cells dissociated with crystalline trypsin was significantly reduced (P < 0.001) by treatment with 100 u./ml NANase in Hanks's BSS for 30 min at 37 °C (Table 4). This decrease in negative mobility was expressed in terms of a diminution in surface charge density which in turn was represented as loss of electrons per 10¹⁰ cells. The charge of an electron is 4.8 x 10⁻¹⁰ e.s.u. and the surface area of an average muscle cell was estimated as 530 μm². Assuming that each NAN possesses one free electron, it is seen that the loss of NAN from the electrokinetic slip plane of the cells closely corresponds to the amount of NAN in a free form found in the supernatant after incubation of the cells with 100 u./ml NANase (Table 3).

Table 4. Effect of neuraminidase treatment (100 u./ml for 30 min at 37 °C) after crystalline trypsin dissociation on the electrokinetic properties of chick embryo muscle cells suspended in 0.145 m NaCl: 3 x 10⁻⁴ m NaHCO₃, pH 7.2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No. of readings</th>
<th>Mobility, μm/s/V/cm</th>
<th>Surface charge density (e.s.u. cm⁻²)</th>
<th>No. of electrons lost/10¹⁰ muscle cells</th>
<th>Theoretical yield of NAN/10¹⁰ muscle cells, μg</th>
<th>Experimental yield of NAN/10¹⁰ muscle cells (from Table 3), μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline</td>
<td>50</td>
<td>-0.99 ± 0.04</td>
<td>2767</td>
<td>—</td>
<td>—</td>
<td>8.7 x 10³</td>
</tr>
<tr>
<td>NANase</td>
<td>60</td>
<td>-0.76 ± 0.04</td>
<td>2104</td>
<td>663</td>
<td>7.3 x 10¹⁶</td>
<td>37.0</td>
</tr>
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Discussion

The results of this investigation showed that NANase inhibits the aggregation of embryonic chick muscle cells (Figs. 1, 2). This effect was probably due to the removal by the specific enzyme NANase of sialic acids shown to occur at the muscle cell surface (Tables 3, 4).

There were small differences in the way in which the inhibition took effect depending on the type of apparatus employed in the study. The inhibition by NANase of cells aggregating in the turbidimeter did not become evident until the cells had been rotating for 2 h. These cells were not incubated with NANase before being rotated. It is possible therefore that NANase took 2 h to remove completely the cell-surface sialic acids.
In contrast, NANase quickly inhibited the aggregation of cells agitated in the gyratory shaker. These cells were pretreated with 100 u./ml NANase for 30 min at 37 °C, which removed the sialic acids remaining after trypsin dissociation. When suspended in Eagle’s MEM containing 25 u./ml NANase the pretreated cells began aggregating immediately, but their rate of aggregation was slower than that of the cells in the control preparation. The inhibitory effect of NANase did not, however, result in a longer first phase of aggregation (accumulation of cells into aggregates). The cells remaining free at the 12-h stage did not aggregate after this time nor did the already-formed aggregates join up as they did in the controls. Pretreating cells with 100 u./ml NANase did not on its own have a discernible effect on the rate of aggregation probably owing to the rapid regeneration of cell surface sialic acids (Marcus, Salb & Schwatz, 1965).

NANase may have affected the aggregative competence of muscle cells by altering the metabolic balance of the cell, but in general terms this would imply that the enzyme was taken up by the cells. Most of the evidence on the uptake of NANase by cells infers that this is not the case (Wallach & Eylar, 1961; Eylar, Madoff, Brody & Oncley, 1962; Kraemer, 1966, 1967; Marcus et al. 1965). However, Nordling & Mayhew (1966) showed that fluorescent-labelled NANase was taken up by RPMI No. 41 cells. The application of similar fluorescent techniques to muscle cells did not confirm this result.

The ability of NANase-treated muscle cells to exclude the lissamine green dye indicated that these cells were viable. Protein synthesis, oxygen uptake and mitosis of the muscle cells were not affected by the presence of exogenous NANase. These three sensitive tests for cellular metabolism gave support to the evidence from lissamine green viability checks. The results shown in Table 4 demonstrating a 1:1 ratio between the cell surface sialic acids as calculated from the drop in negative electrophoretic mobility and the acids found in the supernatant after NANase treatment, indicate that sialic acids were not liberated from within the cells. This series of results, which confirm the findings of Ruhenstroth-Bauer et al. (1962) for the in vitro and in vivo viability of NANase-treated HeLa cells and ascites cells, strongly suggest that exogenous NANase does not enter muscle cells and that it does not upset the overall metabolic balance of these cells by its action at the cell surface.

Kraemer (1968) recently found that cytotoxic impurities were present in some commercial preparations of NANase. However, Weiss (1965) showed that commercial NANase prepared by Behringwerke does not contain enzyme impurities. Other possible impurities in Behringwerke NANase could not have caused the observed effect on the aggregation of muscle cells because a purified crystalline sample of NANase gave the same result as the commercial preparation. Moreover, the inhibitory effect could not have been caused by the enzyme acting as an inhibitory protein because heat-inactivated NANase did not exhibit any effect on aggregation.

The possible mechanism by which sialic acids could influence the process of aggregation is dependent upon which, if any, of the current theories of cell adhesion is correct (Curtis, 1966, 1967; Weiss, 1967a). This topic cannot be discussed in this paper but it is relevant to mention that the present results do not appear to support the theory of cell adhesion based on the interaction of physical forces (Curtis, 1960,
1962). The implication behind this theory (1962) is that the higher the negative charge of the cell surface the greater the potential energy barrier and the less the ability of the cells to adhere to one another. In the present studies the converse appears to be true because NANase-treated muscle cells possessed a small surface charge when compared with the controls but aggregated at a slower rate than normal. Therefore, the behaviour of muscle cells suspended in a medium containing NANase did not conform to the predictions implicit in the theory of adhesion by physical forces.

The role of sialic acids in cellular aggregation may lie in their maintaining the structural rigidity of the cell surface as proposed by Weiss (1961, 1965). He showed that NANase (Weiss, 1965) and proteolytic enzymes (Weiss, 1966) increased the deformability of Sarcoma 37 and Ehrlich murine ascites cells whilst liberating cell-surface sialic acids. It has also been proved that phagocytes exhibit a higher degree of association with other particles when their deformability has been increased (Weiss, Mayhew & Ulrich, 1966). Weiss (1965, 1967b) postulated that increasing cellular deformability would facilitate the formation of the low radius of curvature probes thought to be important in cell-to-cell adhesions (Bangham & Pethica, 1960). Cellular adhesiveness would thus be improved because the ability of the cells to penetrate the potential energy barrier would be enhanced (Weiss, 1964).

According to Weiss's theory (1965, 1967b) the surface deformability of muscle cells suspended in a medium containing NANase would be increased. These cells should therefore adhere to one another more readily than the cells in the controls particularly as they possess a reduced surface charge. Contrary to these expectations, the aggregative competence of NANase-treated cells was less than that of the controls. A mechanism based on cellular deformability and embracing the present result can be envisaged by postulating a range of deformability over which cells can be adhesive. Cells with a surface deformability outside this range would be non-adhesive. Thus, increasing the deformability of non-adhesive cells could render the cells adhesive possibly owing to the increased ability to form low radius of curvature probes (Weiss, 1965). However, if the surface of a normal cell were made more deformable by incubating with NANase, the resulting cellular deformability might be outside the 'adhesive range'. The cells would thus be rendered non-adhesive because the radius of curvature of the probes was too high.

Even assuming that cells must penetrate a potential-energy barrier before adhering (Weiss, 1964) an adhesive mechanism must still be brought into play (Steinberg, 1964; Jones & Morrison, 1969). As sialic acids possibly can form cell-to-cell bonds (Weiss & Mayhew, 1967) either directly (Weiss, 1967a) or through the mediation of calcium bridges (Coman, 1961; Bangham & Pethica, 1960), as well as being an agent in the maintenance of cellular deformability (Weiss, 1965), it is extremely difficult to be certain of the mechanism by which NANase reduces the adhesiveness of embryonic chick muscle cells.

I am grateful to Professor Bryn M. Jones for his advice in preparing this manuscript. I also thank Miss Isobel Cunningham for helpful suggestions on tissue culture techniques, Mrs Barbara Jones for measurements of oxygen uptake, Mrs Alise Howse and Miss Barbara Morris for technical assistance and Mr R. A. Moore for the photomicrographs. This investigation was supported by grants from the Science Research Council.
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Effect of neuraminidase on cell aggregation

Fig. 4. Relatively large aggregates produced by crystalline trypsin-dissociated cells after rotation for 4 h in Eagle's MEM at 37 °C. x 500.

Fig. 5. Shows the inhibitory effect of NANase on the size of aggregates produced by crystalline trypsin-dissociated cells after a 4-h period in Eagle's MEM containing 25 u./ml NANase at 37 °C. Compare with Fig. 4. × 575.
Fig. 6. Aggregates formed from crystalline trypsin-dissociated muscle cells joining together to form larger aggregates after a 12-h period of rotation in Eagle's MEM at 37 °C. × 500.

Fig. 7. NANase at a concentration of 25 u./ml allowed the formation of small aggregates after a period of 12 h in Eagle's MEM at 37 °C but prevented the coalescence of aggregates, typical of the controls (see Fig. 6). × 700.
Effect of neuraminidase on cell aggregation

Fig. 8. Aggregates formed by crystalline trypsin-dissociated cells over a 24-h period of rotation in Eagle's MEM at 37 °C were large and compacted. × 500.

Fig. 9. Aggregates formed by crystalline trypsin-dissociated cells over a 24-h period of rotation in Eagle's MEM containing 25 u./ml NANase at 37 °C were not significantly larger than at 12 h. There were no signs of aggregate coalescence or consolidation. × 500.
Fig. 10. Fibroblast-like cells growing out from aggregates cultured for 48 h on a plasma clot. The aggregates were formed during a 24-h period in Eagle's MEM containing 25 u./ml NANase from crystalline trypsin-dissociated muscle cells. Note the mitotic figures. × 1500.