SUMMARY

Chick embryo neural retina cells from 7-, 10- and 14-day embryos and muscle cells from 9-day embryos, dissociated mechanically or with trypsin, reaggregate at the same initial rate whether treated before and during aggregation with active or inactive N-acetyl neuraminidase. N-acetyl neuraminidase treatment also has no effect on the size or number of aggregates formed over 24 or 48 h of aggregation. It was also found that the amount of trypsin used to dissociate the tissue into single cells alters the initial rate of aggregation but has no effect on the size or number of aggregates formed over a 24-h period.

INTRODUCTION

It has long been suspected that cell surface molecules play a critical role in many cellular activities. Recently, studies on cell surface glycoproteins and charge density (Cook, 1968), blood group antigens (Watkins, 1966) and cancer cells (Abercrombie & Ambrose, 1962; Buck, Glick & Warren, 1971) have suggested that carbohydrates are functionally important constituents of these surface molecules (see Winzler, 1970, for a general review). One carbohydrate component which appears to be present on most cells thus far examined (Warren, 1963) is sialic acid. This highly charged acid-sugar molecule contributes much of a cell's negative charge (Weiss, 1969) and is thus a determining factor in electrophoretic mobility (Cook, 1968). Investigations of sialic acid density on tumour cells have led to some confusion. Viral-induced tumour cell transformation and loss of contact inhibition have been correlated with both an increase (Defendi & Gasic, 1963) and a decrease (Ohta, Pardee, McAuslan & Burger, 1968; Grimes, 1970) in cell surface sialic acid, while 'malignancy' as assessed by survival time of host animals has been shown not to correlate with sialic acid density (Weiss & Hauschka, 1970).

Enzymic removal of surface sialic acid from embryonic chick neural retina cells has been reported to inhibit the hydrocortisone-induced synthesis of glutamine synthetase (Morris, 1970). Removal of sialic acid also appears to affect some characteristic reactions which occur at the cell surface, for instance, removal may inactivate viral receptor sites (Marcus & Schwartz, 1968), unmask transplantation antigens (Currie, van Doorninck & Bagshawe, 1968), alter the response of tumour cells to a plant agglutinin (Burger & Goldberg, 1967; Burger, 1969) and perhaps reduce the rate of adhesion of chick embryonic muscle cells (Kemp, 1970).

The above suggestions as to the importance of cell surface carbohydrate, and in particular sialic acid, for cell behaviour of various kinds led us to pursue further
investigations of the distribution of sialic acid in chick embryo neural retina and muscle cells and to assess the possible role of sialic acid in the aggregation of cells from these tissues.

MATERIALS AND METHODS

Preparation of cells

Neural retinas were removed from the eyes of 7-, 10- and 14-day White Leghorn chick embryos and used either in 2 pieces or as single cells. Retinas were mechanically dissociated by incubating for 10 min at 37 °C in each of 4 changes of calcium- and magnesium-free Tyrode's solution (CMF), and then pipetting 20 times with a capillary pipette in CMF with 5 μg/ml deoxyribonuclease (DNase; Nutritional Biochemicals Corporation, 90,000 Dornase units per mg, isolated from beef pancreas). The DNase prevents the formation of a viscous material which traps cells (Moscona, 1962; Steinberg, 1963). The suspension was spun at 40 g for 10 min and the cell pellet resuspended in Eagle's Basal Medium (BME; Grand Island Biological Company) containing 5 μg/ml DNase.

Trypsin treatment was by a modification of the method of Moscona (1961). Half retinas were washed with 3 changes of CMF, incubated for 10 min at 37 °C in CMF, washed again in CMF and incubated for 20 min at 37 °C in CMF-trypsin (Armour Tryptar) at pH 6-8. Trypsin concentrations of 6250, 12500, and 18750 NF units in 0-5 ml CMF were used per 7-, 10- and 14-day retina, respectively. The tissues were washed with 3 changes of Tyrode's solution and then dissociated with a capillary pipette in BME containing 5 μg/ml DNase as above.

Nine-day chick leg muscle cells were prepared with trypsin using the above method with the following modifications: the tissue was first minced, all CMF washes were extended to 10-min incubations at 37 °C and the trypsin incubation (12500 NF units in 0-5 ml CMF per leg) was extended to 30 min.

Cells were counted using a Model B Coulter Counter. Appropriate settings were determined for each cell type.

Neuraminidase treatment

Half retinas or cells were treated with neuraminidase (NANase; B grade, Vibrio cholerae 500 units/ml, Cal Biochem; no proteolytic activity could be detected at the concentrations used) as follows: one retina or approximately 80 x 10⁶ cells, 0-71 ml Tyrode's solution without glucose and 0-04 ml (20 u.) NANase (active or inactive) were placed in a 10-ml Erlenmeyer flask. The flask was then gassed with 5% CO₂-air to a pH of 6-8, indicated by phenol red, tightly stoppered and shaken on a rotary shaker at 37 °C for 1.5 h.

NANase was inactivated by heating at 100 °C for 10 min. The activities of 'inactivated' and untreated enzyme were compared by incubating with 0-1 mg/ml neuraminidase (A grade, Cal Biochem) in 2 mM CaCl₂ and 0-125 M sodium acetate in a final volume of 0-4 ml at pH 5.2. The reaction was stopped by addition of sodium periodate for the first step of the TBA assay (see below). Active NANase released N-acetyl neuraminic acid under these circumstances while inactivated NANase did not. Also, inactivated NANase released no sialic acid when incubated with tissue under the standard conditions.

Aggregation of cells

Cells were aggregated in BME containing 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin (Microbiological Associates) and 0-04 ml (20 u.)/ml NANase (active or inactive); 3-ml aliquots of cells in this medium were dispensed to 25-ml Erlenmeyer flasks, gassed with 5% CO₂-air to pH 7.0, indicated by phenol red, and tightly stoppered. The flasks were shaken at 70 rev/min on a gyratory shaker with a 2-75-in. (19-mm) radius of gyration at 37 °C. For 24- and 48-h aggregates, approximately 20 x 10⁶ cells were shaken in each flask. Aggregates were observed and photographed at appropriate times. For studies on the initial rate of aggregation, approximately 5 x 10⁷ cells were shaken in each flask. Flasks were removed from the shaker at
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intervals and diluted with Tyrode's solution to 30 ml; single cells were counted with the Coulter Counter. The number of single cells remaining at each time expressed as a percentage of the single cells present initially was plotted against time. Initial rate data were collected for the first 4 h of aggregation. After 4 h the presence of large aggregates interferes with the counting accuracy.

Sialic acid estimations

Sialic acid was assayed with the 2-thiobarbituric acid method (TBA) of Warren using his corrections for deoxyribose (Warren, 1959), with the Ehrlich assay (Cassidy, Jourdian & Roseman, 1966) or with the periodate-resorcinol method (PR) (Jourdian, Dean & Roseman, 1971). 2-Thiobarbituric acid was obtained from Eastman Kodak and recrystallized from boiling water.

The TBA assay was done directly on the supernatant from NANase-treated tissue or cells to determine the amount of sialic acid released.

To estimate the total sialic acid per retina the tissue was homogenized in distilled water and hydrolysed for 1 h at 80 °C in 0·1 N H₂SO₄. Sialic acid was separated from other tissue constituents by chromatography on Dowex 1X-8 (Biorad) in the acetate form (5 mm x 100 mm column), (Svennerholm, 1963). The sample was applied, the column washed with 2 ml of water, 2 ml of 0·05 M sodium acetate, and the sialic acid eluted with 0·6 M sodium acetate. Fractions were assayed using the Ehrlich procedure or the PR method. The PR method was also applied directly to homogenized tissue.

Protein was estimated by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951) using a crystalline bovine plasma albumin (Armour Pharmaceuticals) standard.

RESULTS

Surface sialic acid

Various concentrations of NANase were tested to determine the optimal concentration for removal of sialic acid. Rate curves for removal of sialic acid from 7-, 10- and 14-day retinas using 0·04 ml (20 u.) NANase per retina (27 u./ml) are shown in

![Graph showing the removal of sialic acid over time](http://example.com/graph.png)

Fig. 1. NANase-removable sialic acid. •—•, 7-day; ▲—▲, 10-day; ○—○, 14-day retina.)
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Fig. 1. Lower concentrations of NANase require more time to reach the same plateau value, while higher concentrations give no increase in the plateau value or rate of removal. Addition of 10 u. of NANase per retina after 2 h of incubation with 20 u. of NANase per retina gives no additional sialic acid in the supernatant at later times. Thus the decrease in rate of removal is not due to inactivation of the NANase originally present. Incubation for 1-5 h with 20 u. of NANase per retina was chosen as the standard procedure.

Table 1. Sialic acid values for embryonic chick neural retina

<table>
<thead>
<tr>
<th>Age of embryo, days</th>
<th>NANase-removable sialic acid, μmol/retina</th>
<th>Protein, mg/retina</th>
<th>NANase-removable sialic acid/protein, μmol/mg</th>
<th>Total sialic acid, μmol/retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.0056 ± 0.0002</td>
<td>0.96 ± 0.05</td>
<td>0.0058</td>
<td>0.005</td>
</tr>
<tr>
<td>10</td>
<td>0.0233 ± 0.0002</td>
<td>2.65 ± 0.03</td>
<td>0.0124</td>
<td>0.029</td>
</tr>
<tr>
<td>14</td>
<td>0.0372 ± 0.0002</td>
<td>3.74 ± 0.03</td>
<td>0.0099</td>
<td>0.036</td>
</tr>
</tbody>
</table>

* Ranges over at least 4 experiments.

Table 2. Sialic acid density for embryonic chick neural retina

<table>
<thead>
<tr>
<th>Age of embryo, days</th>
<th>Volume of cell, μm³</th>
<th>Surface area of cell, μm²</th>
<th>Number of cells per retina, x 10⁶</th>
<th>Sialic acid per cell, μmol x 10⁻¹⁵</th>
<th>Sialic acid per unit surface area, μmol x 10⁻¹²/μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>172</td>
<td>150</td>
<td>16</td>
<td>350</td>
<td>2.3</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>118</td>
<td>80</td>
<td>410</td>
<td>3.5</td>
</tr>
<tr>
<td>14</td>
<td>120</td>
<td>118</td>
<td>90</td>
<td>410</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* Estimated with the Coulter counter, calibrated with ragweed pollen.

Table 1 summarizes the data on sialic acid removable by NANase from 7-, 10- and 14-day retinas. Table 2 shows how sialic acid is related to other age-dependent parameters. The sialic acid density (sialic acid per surface area) increases about 50% from 7 to 10 days and then levels off.

Total sialic acid

Total sialic acid per retina proved difficult to determine. The hydrolysis method was originally developed for analysis of relatively purified glycoprotein samples. Differences in concentration of tissue homogenates for hydrolysis (retinas/ml) give variable results: more dilute homogenates yield more free sialic acid per retina. A column, on the other hand, can handle only a limited volume of hydrolysate due to the presence of residual sulphate ion from the acid hydrolysis which elutes sialic acid as it is put through the column. Furthermore, the sample must be equivalent to at least 2 retinas to give enough sialic acid in the appropriate fractions to measure by the relatively insensitive Ehrlich technique. Although the TBA assay is more sensitive than the Ehrlich assay it cannot be used to assay column fractions as it is sensitive to acetate which is used to
elute the column. Other anions that do not interfere in the TBA assay do not separate sialic acid from other tissue components successfully. Total sialic acid reported is based on a hydrolysis time of 1 h; in this time sialic acid is not degraded, and a relatively consistent amount is released. No hydrolysis concentration yielded more sialic acid per retina in 1 h than did the NANase treatment of whole tissue. The highest values obtained from a series of 4 experiments are given in Table 1. The periodate-resorcinol method which detects both free and bound sialic acid when applied to whole tissue homogenates also gave estimates for total sialic acid slightly lower than NANase-removable sialic acid. These results can be understood in 3 ways: either very nearly all of a cell's sialic acid is at the surface, or NANase releases intracellular sialic acid as well as extracellular sialic acid (Nordling & Mayhew, 1966) or a portion of the cell's sialic acid is untouched by either hydrolysis or NANase. These possibilities will be discussed later.

Aggregation

Cells were aggregated in the presence of NANase, so that if sialic acid is replaced during the time of aggregation it would be removed and not affect the behaviour of the cells.

The rate of aggregation was determined for cells which had been dissociated with trypsin. It was of interest to know how much sialic acid was removed by trypsin: is there enough sialic acid left after trypsinization to ensure that the cells treated with inactive NANase differ significantly in their surface sialic acid content from cells treated with active NANase? The amount of sialic acid released by NANase from cells previously dissociated with trypsin was therefore compared with the amount of sialic acid released by NANase from untrypsinized retina. Trypsin-removable sialic acid is 53% of the NANase-removable sialic acid. Thus trypsinized cells treated with inactive NANase (controls) have approximately half (0.016 μmol/retina) of the 'normal' amount of sialic acid. This residual sialic acid from trypsinized cells is not necessarily at the surface. Trypsin removes surface material, therefore trypsin-removable sialic acid is probably surface sialic acid. But the remaining sialic acid could come from inside the cell and/or from the cell surface.

The initial rate of aggregation of cells from 7-, 10- or 14-day retina or from 9-day muscle dissociated with trypsin was the same whether the cells were pretreated with active or inactive NANase (Fig. 2). This means that either sialic acid is not important in aggregation, or that trypsinization has removed the relevant sialic acid from both kinds of cells. If the latter were true, the fact that trypsinized cells do aggregate, even in the presence of active NANase, supports the view that this sialic acid is not a necessity for aggregation. Nevertheless, to ensure further that the decrease in surface sialic acid due to trypsin treatment did not lead to the similarity of aggregation rates, aggregation kinetics were determined using mechanically dissociated cells. Mechanical dissociation removes less than 10% of the NANase-removable sialic acid. Therefore, mechanically dissociated cells must retain at least 80% of the trypsin-removable sialic acid on the surface. After NANase treatment, no significant difference in aggregation kinetics was seen between active- and inactive-NANase
treated mechanically dissociated cells (Fig. 3). Thus, the presence of sialic acid at the cell surface does not seem to be a factor influencing the initial rate of aggregation of chick embryo retina or muscle cells at the stages used.

Fig. 2. Initial rate of aggregation. •—•, treated with inactive NANase; ○—○, treated with active NANase, before and during aggregation. A, 7-day; B, 10-day; C, 14-day retina. D, 9-day muscle, dissociated with trypsin.

Longer-range effects on aggregation were investigated by aggregating cells previously treated with NANase in the presence of NANase for 24 and 48 h. Aggregates formed in the presence of active NANase contain less than half as much sialic acid as aggregates formed in the presence of inactive NANase. Although there is a significant difference in sialic acid content, no difference in number or size of aggregates can be seen between the 2 groups (Fig. 5). However, even the control aggregates have less sialic acid after 24 h in culture than the cells had at the beginning of incubation. We do not understand why sialic acid decreases during this time, but the fact that aggregation proceeds and the aggregates formed are maintained while sialic acid is decreasing argues again that aggregation and adhesion in this case do not depend on the presence of high levels of sialic acid.
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Fig. 3. Initial rate of aggregation. •—•, treated with inactive NANase; O—O, treated with active NANase, before and during aggregation. 10-day retina, mechanically dissociated.

Fig. 4. Initial rate of aggregation of 10-day retina cells dissociated with different concentrations of trypsin. O—O, 1000 u.; •—•, 2500 u.; △—△, 4000 u./retina.

Trypsin concentration

The concentration of trypsin used for the preparation of cells affects the initial rate at which cells aggregate. Cells aggregate faster if less trypsin has been used (Fig. 4 and compare Fig. 2h, 10-day retina dissociated with trypsin with Fig. 3, 10-day retina mechanically dissociated). Nevertheless, final aggregate size (after 24 or 48 h) is the same for cells from one age of embryo regardless of the trypsin concentration used. This indicates that trypsinization conditions can influence behaviour of cells soon after their preparation. Also, the initial rate of aggregation of cells does not necessarily correlate with final aggregate size; thus there are probably 2 separable processes involved. This conclusion was also drawn by Ball (1965) in studies concerned with the effect of embryo extract on aggregation and is discussed at length by Lilien (1969) in relation to results obtained by a number of investigators.
The results of these experiments show that sialic acid is probably not involved in the initial rate of aggregation or the final size of the aggregates of neural retina cells from 7-, 10- or 14-day chick embryos or muscle cells from 9-day chick embryos. The distribution of sialic acid in chick embryo retina cells remains unclear. Either all of the sialic acid is on the surface, or some proportion is inside the cell. If most of the sialic acid is on the surface, then after trypsinization, half is still there, and can be removed by NANase. The fact that cells treated with trypsin and NANase aggregate similarly whether the NANase was active or inactive, in this case would indicate that sialic acid is not important in aggregation. If, on the other hand, close to half of the cell's sialic acid is inside, then trypsin treatment removes essentially all of the surface sialic acid and further treatment with NANase, active or inactive, causes no additional decrease of surface sialic acid. However, the fact that trypsinized cells do aggregate would indicate that for this case, the presence of sialic acid on the cell surface is not required for aggregation. Also, mechanically dissociated cells, retaining 80% of the trypsin-removable (surface) sialic acid do not aggregate differently after treatment with active or inactive NANase.

Another possibility is that a small fraction of the surface sialic acid, not susceptible to either NANase or trypsin, is active in aggregation. If this is the case, these are not the only molecules involved in aggregation, as the change in aggregation rates after trypsinization with different concentrations show.

Cells were pretreated with NANase to remove sialic acid from the surface and aggregated in the presence of NANase. Therefore replacement of surface sialic acid, which may occur fairly rapidly (Kraemer, 1966), cannot account for the similar patterns of aggregation of cells treated with active and inactive NANase.

The rate of aggregation of cells is sensitive to trypsinization conditions. The surfaces of cells from embryos of different ages with unknown variations in distribution of surface molecules may be affected in different ways by tryptic digestion. We do not think that any surface property of trypsinized cells can be legitimately compared unless the cells were identical before the trypsin treatment. For instance, differences in electrophoretic mobilities of trypsinized cells from embryos of different ages (Gershman, 1970) may relate more to differences in trypsin sensitivity than to inherent differences in electrophoretic mobility or 'normal' surface charge.

The loss of sialic acid from aggregates during culture is being investigated further. Aggregation of dissociated cells may lead to cell interactions which are not like the interactions maintained in vivo. If response to trypsin damage, or culture under particular in vitro conditions leads to surface structures different in some way from in vivo structures, can the cells pursue normal activities, especially those which seem to involve cell interactions? (Morris & Moscona, 1970, for instance, have investigated the relationship between cell arrangement and the onset of glutamine synthetase activity.)

Sialic acid does not seem to be involved in the aggregation of dissociated retina cells, a result not altogether surprising. Although it is a major component of the cell surface, the fact that it carries a large negative charge makes it an unlikely candidate.
for a role in bringing cells together, and also its very ubiquity argues against a role in a
process which often involves specificity. The fact that 7-day retina, with lower
sialic acid density than 10-day retina, aggregates better as measured by the size of the
aggregates (Moscona, 1962) also argues against sialic acid being important for aggre-
gation. Sialic acid therefore does not seem to be a positive factor in either initial, non-
specific aggregation which is insensitive to inhibitors of protein synthesis or in long-
term specific aggregation requiring protein synthesis (Lilien, 1969). It may have a
negative role in the sense that the negative charge may work against cells approaching
each other close enough to stick. (Cells treated with active NANase tend to aggregate
slightly faster than those treated with inactive NANase.) Nevertheless, studies on
sialic acid are valuable, as it can serve as a marker for cell surface glycoproteins. In
that capacity we are continuing work with sialic acid, studying replacement rates and
turnover of cell surface materials.

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REFERENCES
Cancer Res. 22, 525-548.
Chick Neural Retina Cells; The Effects of Age and Culture Conditions. Ph.D. Dissertation,
The University of Chicago, Illinois.
BUCK, C. A., Glick, M. C., & WARREN, L. (1971). Glycoproteins from the cell surface of
control and virus transformed cells. Science, N.Y. 172, 169-172.
BURGER, M. M. (1969). A difference in the architecture of the surface membrane of normal and
GRIMES, W. J. (1970). Sialic acid transferases and sialic acid levels in normal and transformed
cells. Biochem. 9, 5083-5092.
246, 430-435.
dissociated from embryonic chick muscle tissue. J. Cell Sci. 6, 751-756.
KRAEMER, P. M. (1966). Regeneration of sialic acid on the surface of Chinese hamster cells in
LOWRY, O. H., ROSEBROUGH, N. J., FARR, D. L. & RANDALL, R. J. (1951). Protein measure-


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Fig. 5. Twenty-four-hour aggregates of 10-day retina cells. Pretreated and aggregated with A, active NANAse, B, inactive NANAse. × 60.