J. Cell Sci. 15, 575–590 (1974)
Printed in Great Britain

ON THE MECHANISM OF THE INCREASED AGGREGATION BY NEURAMINIDASE OF 16 C MALIGNANT RAT DERMAL FIBROBLASTS *IN VITRO*

C. W. LLOYD* AND G. M. W. COOK
Strangeways Research Laboratory, Cambridge, U.K.

SUMMARY

The aggregation of briefly trypsinized 16 C malignant rat dermal fibroblasts as measured by gyratory shaker/electronic particle counting technique is increased by neuraminidase. Enzyme treatment also exposes soybean and *Ricinus communis* agglutinin-binding sites as judged by cell agglutination. Desialysed bovine submaxillary mucin and desialysed, agalacto fetuin reversed the neuraminidase-stimulated increase in aggregation, whereas desialysed fetuin and ovalbumin did not. The effective glycoproteins also acted as acceptors in cellular sialyl- and galactosyltransferase reactions, and these activities are detected in plasma membrane fractions.

The possibility that neuraminidase increases cellular aggregation by generating acceptor sites for interaction with cell surface glycosyltransferases, is discussed.

INTRODUCTION

The mechanism by which cells adhere to one another is imprecisely understood but there is gathering evidence to suggest that carbohydrates at the cell surface somehow participate in this process (for review, see Kemp, Lloyd & Cook, 1973). One attraction of the involvement of heterosaccharides in adhesion lies in the possibility that adhesive specificity might be written into the arrangement of the monosaccharide constituents and Roseman (1970) has postulated that in recognizing and binding to such sites, glycosyltransferases on apposing cell surfaces would bring about adhesion. The evidence of Roth and his colleagues (Roth, McGuire & Roseman, 1971*b*; Roth & White, 1972) lends support to this interpretation of the mechanism of intercellular adhesion.

In the present study, the enzyme neuraminidase (NANase) has been used to investigate the role of sialic acid in cell adhesion. Removal of this monosaccharide from the surfaces of 16 C rat dermal fibroblasts brings about an increased adhesiveness which is inhibited by desialysed bovine submaxillary (desiBSM) mucin. The hypothesis that neuraminidase generates new binding sites for an adhesive glycosyltransferase, which also binds to desiBSM, is examined.

* Beit Memorial Research Fellow.

MATERIALS AND METHODS

Cell culture and aggregation

Malignant rat dermal fibroblasts, 16 C (Daniel, Dingle, Webb & Heath, 1963) were grown in 8-oz. (230-ml) glass bottles in Dulbecco's modification of Eagle's medium (kindly donated by the Imperial Cancer Research Fund Laboratories, London) containing 10 % (v/v) heat-treated (56 °C for 30 min) calf serum in a gas phase of 10 % CO_2 in air (v/v). The cells were harvested after 3 d of growth at low density (approx. $1 \times 10^5/cm^2$) since at high density the cells tend to detach from glass. Native serum was found to contain a CMP-*N*-acetylneuraminic acid : desiBSM sialyltransferase which could be completely abolished by the heat treatment.

Cell suspensions were prepared for aggregation assays by a modification of the method described by Edwards & Campbell (1971). Monolayers were washed with 10 ml of warm (37 °C) Tris-saline and then incubated briefly at room temperature with 10 ml of Difco trypsin (0·05 %, w/v)/EDTA (0·11 mM) which was decanted after 20–30 s. At 2 min after the addition of trypsin/EDTA, cells were gently shaken from glass with ice-cold phosphate-buffered saline (PBS) (Dulbecco, see Paul, 1970) containing 5 μg/ml deoxyribonuclease (Sigma Type I, electrophoretically purified). Cells were washed by centrifugation (200 **g**) 3 times in 10 ml of PBS and finally suspended in this solution – each time dispersing the cell pellet with a Pasteur pipette – and dispensed at a known density of approx. 1×10^6 ml, in 3-ml aliquots, into siliconized 25-ml Erlenmeyer flasks. Suspensions were routinely allowed to aggregate at 37 °C on a gyratory shaker at 70 rev/min for 30 min. Aliquots were then removed from the flasks with a disposable 1-ml Oxford pipette tip (the bore of which had been enlarged by cutting off the end 10 mm) and diluted to 10 ml with 0·9 % (w/v) sodium chloride solution. The decline in particle numbers as the cells aggregated over this period was monitored by electronic particle counting using a Celloscope model 401 (supplied by Grant Instruments, Cambridge) with a 100-μm aperture and a lower threshold set at 4·7 μm. The particle count was independent of the temperature (0–37 °C) of the sodium chloride or PBS into which the samples were dispensed. That aggregation was not due to cell loss was checked in some instances by counting the number of cells in aggregated suspensions with a haemocytometer. Viability was determined by dye exclusion tests using 0·1 % (w/v) trypan blue and erythrocin B.

Mucin (bovine submaxillary, Worthington, supplied by Cambrian Chemicals Ltd) was desialysed by weak acid hydrolysis (0·1 N H_2SO_4 at 80 °C for 1 h) neutralized, dialysed and then lyophilized. The property of some preparations of the glycoprotein to form aggregates which interfered with the cell particle count was corrected by subtracting the counts obtained in cell-free glycoprotein blanks from the experimental values.

Desialysed and desialysed, galactose-free fetuin were prepared from fetuin (Gibco) by dilute acid and Smith periodate oxidation (Spiro, 1964) and kindly donated by Miss A. Warley. Ovalbumin was purchased from Sigma Chemical Company and neuraminidase (from *Vibrio cholerae*) as an aqueous solution containing 500 U./ml from Behringwerke, Marburg Lahn, W. Germany. One unit of neuraminidase activity represents the amount of enzyme which liberates 1 μg of NAN (*N*-acetylneuraminic acid) from human α_1 acid glycoprotein in 15 min at 37 °C and at pH 5·5. Under the conditions used here, the enzyme was shown to retain 60 % of its activity (Cook & Jacobson, 1968).

Lectin studies

Cell suspensions of approx. 2×10^6/ml were incubated at 37 °C on a gyratory shaker at 120 rev/min (to limit the extent of aggregation) for 15 min with 10 U. NANase/10^6 cells, washed 3 times in PBS and resuspended to the original density. Controls were pre-incubated in the absence of enzyme. Aliquots of lectin (250 μl) were serially diluted to produce, upon addition of an equal volume of cell suspension, concentrations from 500 to 31·25 μg/ml. After 30 min at room temperature, the contents of each tube were diluted to 10 ml with sodium chloride solution for electronic particle counting. Agglutination was scored as the extent to which the particle count decreased below that of lectin-free controls (in order to correct for spontaneous aggregation) as follows: 5–15 %, +; 15–30 %, + +; 30–60 %, + + +; and > 60 %, + + + +.

Soybean haemagglutinin, which has a specificity for *N*-acetylgalactosaminyl residues, was

prepared by the method of Lis, Sharon & Katchalski (1966) from soybean meal (British Drug-houses Ltd). *Ricinus communis* agglutinins were isolated by the affinity chromatography technique of Nicolson & Blaustein (1972) from castor beans (Thompson & Morgan Ltd, Ipswich, England). Following affinity chromatography the *Ricinus* agglutinins were fractionated on Sephadex G-100, as described by the authors, into larger (mol. wt. approx. 120000 designated RCA_{120}) and smaller (mol. wt. approx. 60000) fragments. In this work, RCA_{120}, which binds to β-galactosyl residues, was used exclusively. The activity of these lectins was checked by haemagglutination assay using rabbit erythrocytes, together with the appropriate haptenic inhibitors, before use. Samples of fucose-binding protein from *Lotus tetragonolobus* and wheat germ agglutinin, which binds *N*-acetylglucosaminyl residues (prepared by Miles-Yeda Ltd, Rehovoth, Israel) were kindly provided by Dr M. Rashi.

Glycosyltransferase assays

Cells were assayed for glycosyltransferase activity using the following incubation mixture to a final volume of 182 μl: cells, 600 μg desialysed mucin as exogenous acceptor, 0·4 μCi nucleotide sugar (UDP-^{14}C galactose, sp. act. 245 mCi/mmol, or CMP-^{14}C *N*-acetylneuraminic acid sp. act. 262 mCi/mmol) (Radiochemical Centre, Amersham, England) in PBS, pH 7·2 or initially in a Tris-buffered incubation medium (8 mM $MnCl_2$, 0·145 M NaCl, 7 mM KCl, 5 mM Tris, pH 7·2, 10 mM NaN_3). Endogenous controls received no exogenous acceptor. The mixtures were incubated, in triplicate, on a shaking water bath at 37 °C. Reactions were terminated by pelleting the cells at 200 **g** for 3 min and aliquots of supernatant were deproteinized using 1 % (w/v) phosphotungstic acid in 0·5 N HCl after the addition of 50 μl of 3 % (w/v) bovine serum albumin as carrier. Leakage of glycosyltransferase by the cells to the supernatant was measured by performing parallel assays with a cell-free supernatant derived from control cells previously incubated in suspending medium. Precipitates were washed with 5-ml volumes of: 10 % (w/v) trichloroacetic acid; a mixture of chloroform, methanol and diethyl ether, 2:2:1 (v/v/v) and twice in methanol, before drying at 80 °C. Dried samples were dissolved in 0·2 N NaOH at 80 °C, solubilized in a 2-ethoxyethanol/toluene-based scintillant with Beckman Bio-Solv3 and isotope activity determined using a liquid scintillation spectrometer. Counts were corrected to dpm by internal standardization with *n*-[^{14}C]hexadecane (Radiochemical Centre, Amersham).

Plasma membranes were prepared by the method of Warley & Cook (1973) using cells removed from glass with a rubber policeman. In each case, only the first 10 ml of membrane fraction eluted from the glass bead column were retained for study. Plasma membrane fractions were examined by electron microscopy by the procedure outlined by the above authors.

Glycosyltransferase activity of the membranes was assayed using: 0·1 μCi of CMP-^{14}C NAN or UDP-^{14}C gal; an aliquot of membranes; 100 μg exogenous acceptor; 0·03 M $MnCl_2$; 0·03 M Tris HCl, pH 7·0, in 150 μl. The small amount of membrane material isolated in each experiment prohibited protein determinations in each case. Parallel studies exclusively for protein assay (Lowry, Rosebrough, Farr & Randall, 1951) indicated that 5 μg of membrane protein were used for each determination. Where added, Triton X-100 was at a final concentration of 0·1 % (v/v). Pilot experiments indicated both CMP-NAN:desiBSM sialyltransferase and UDP-gal:desiBSM galactosyltransferase to be more active at 25 °C than at 37 °C so that all subsequent incubations were carried out on a shaking water bath at the lower temperature. Product formation was linear for the times of incubation under these conditions. Reactions were terminated and prepared for scintillation counting as described above except that the alkaline digests were dried on to glass-fibre paper (Stoddart & Northcote, 1967) and counted in a toluene-based scintillant.

RESULTS

The effect of neuraminidase on cell aggregation

Neuraminidase (NANase) treatment consistently increased the aggregation of the 16 C cells as judged by a decrease in the electronic particle count when compared to controls. The time course of aggregation is illustrated by Fig. 1 which shows the

rapidity of the effect of NANase (at 5 U./ml) since the particle count reaches a plateau by about 10 min. Untreated cells aggregate to a lesser extent and the rate of aggregation is always reduced in the absence of enzyme. Furthermore, the aggregation of control cells is characterized by the formation of small aggregates. By contrast, the effect of neuraminidase, apart from increasing the number of cells participating in aggregate formation, is to increase the size of the aggregates themselves and this difference is seen in Figs. 2 and 3 which respectively show controls and suspensions incubated with 5 U. NANase/ml after 30 min on the gyratory shaker.

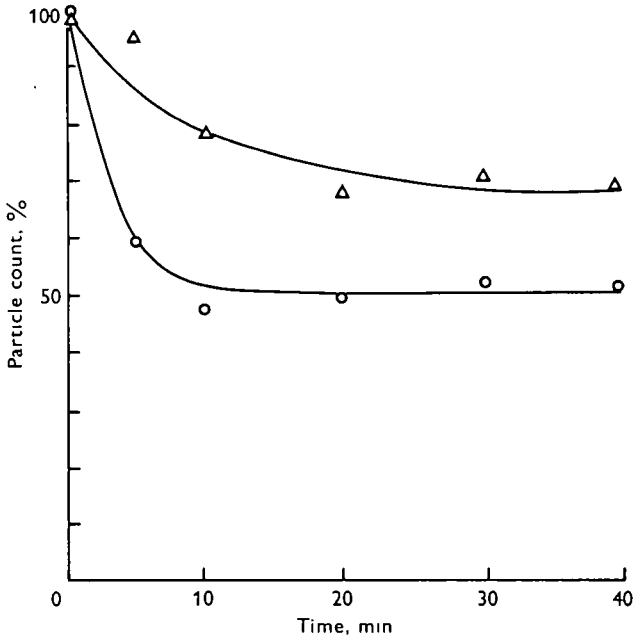


Fig. 1. The effect of neuraminidase at 5 U./10^6 cells, on the aggregation of 16 C fibroblasts with respect to time. △, controls and o, enzyme-treated suspensions. Using the technique of electronic particle counting, a decrease in the particle count is indicative of increased aggregation.

The actual extent of the neuraminidase-induced increase of aggregation varied between experiments although such variation was not as marked as that between controls. However, regardless of the aggregative ability of controls, neuraminidase invariably increased this value as will be seen in Table 1. Because of variation between experiments, comparisons were restricted to between treatments within an experiment and such comparisons were evaluated using a paired sample 't'-test. Analysis of the 30-min particle counts for 10 separate experiments demonstrated that the difference in the extent of aggregation between enzyme-treated suspensions (5 U./ml) and controls was highly significant ($P < 0{\cdot}001$ for 9 degrees of freedom).

The concentration of enzyme routinely used was 5 U./ml but time-course studies using 1, 5, 10 or 40 U./ml indicated that aggregation was not significantly enhanced by increasing the enzyme concentration within this range and as such, is essentially similar to the findings of Vicker & Edwards (1972) for BHK21C13 cells.

Table 1. *Effect of neuraminidase and glycoproteins on the aggregation* of 16 C cells*

	Experiment no.									
	1	2	3	4	5	6	7	8	9	10
Control	57	62	48	38	28	75	41	17	21	45
NANase	74 (17)†	76 (14)	62 (14)	65 (27)	40 (12)	82 (7)	66 (25)	37 (20)	38 (17)	66 (21)
NANase *plus* desiBSM	61 (4)	63 (1)	46 (−2)	47 (9)	31 (3)	75 (0)	54 (13)			
NANase *plus* ovalbumin	77 (20)	71 (9)	62 (14)	57 (19)	43 (15)					
NANase *plus* desialysed, agalacto fetuin	63 (6)	72 (10)	51 (3)							
NANase *plus* desialysed fetuin	76 (19)	71 (9)		65 (27)						

* Cells were aggregated for 30 min at 37 °C and 70 rev/min using 3 ml of cells at 1×10^6/ml as described in the text. Neuraminidase and glycoproteins were at respective concentrations of 5 U. and 1 mg/ml. Per cent aggregation was calculated as:

$$\left(\frac{\text{particle count at zero time} - \text{particle count at 30 min}}{\text{particle count at zero time}} \times 100\right).$$

† All figures in parentheses refer to the increased aggregation above the controls.

Table 2. *The effects of lectins on the agglutination of NANase-treated 16 C cells*

Concentration of lectin, μg/ml	Wheat germ agglutinin		*Ricinus communis* agglutinin		Soybean agglutinin		Fucose-binding protein	
	Control	NANase	Control	NANase	Control	NANase	Control	NANase
500	+ + +	+ + +	o	+ + + +	o	+ + +	o	+ +
250	+ + +	+ + +	o	+ + + +	o	+ + +	o	o
125	+ + +	+ + +	o	+ + + +	o	+ + +	o	o
62·5	+ + +	+ + +	o	+ + + +	o	+ + +	o	o
31·25	+	+ +	o	+ + + +	o	+ + +	o	o

The extent of agglutination was recorded by electronic particle counting. Agglutination was scored as the decrease in particle count (increased agglutination) beyond the spontaneous aggregation produced in samples incubated without agglutinin as follows: 5–15 %, + ; 15–30 %, + + ; 30–60 %, + + + ; and > 60 %, + + + + .

The effect of neuraminidase on lectin-binding sites

One explanation of the increased aggregation brought about by neuraminidase is that enzyme treatment uncovers adhesive sites previously cryptic in the presence of sialic acid at the cell surface. Such sites are likely to be β-galactosyl or β-*N*-acetylgalactosaminyl occurring subterminally to sialic acid or other residues exposed due to reorientation of the cell surface. To examine this, cells were pre-treated with the NANase and tested for lectin-induced agglutination. Data summarized in Table 2 shows that sites for wheat germ agglutinin, WGA (β-*N*-acetylglucosaminyl) are present upon control cells and are unaffected by neuraminidase treatment of the 16 C cells. Sites for fucose-binding protein are absent upon these cells except after NANase treatment at the highest concentration of that lectin used (500 μg/ml). It was found that RCA_{120} binding sites (β-galactosyl) and those for soybean agglutinin (β-*N*-acetylgalactosaminyl) were only expressed for agglutination upon the surfaces of 16 C cells after neuraminidase treatment. Judging by the agglutination caused by the latter 2 lectins it is clear therefore that in the presence of neuraminidase, increased cellular aggregation is paralleled by the increased agglutinability of β-galactosyl and β-*N*-acetylgalactosaminyl residues.

The addition of glycoproteins and plasma membranes to aggregating cell suspensions

Neuraminidase therefore increases cell aggregation and the adhesive sites generated by this treatment could be β-galactosyl- and β-*N*-acetylgalactosaminyl- residues. If so, exogenous glycoproteins of similar terminal specificities should, by binding to complementary binding sites which would otherwise combine with cell surface receptors, competitively inhibit neuraminidase-stimulated aggregation. Sialylated glycoproteins were not employed because of the presence of neuraminidase, which also precluded the pre-treatment of cells with the enzyme since McQuiddy & Lilien (1973) demonstrate that neuraminidase remains adsorbed to even extensively washed cells. The results of these studies are given in Table 1. In each experiment, where used, desialysed BSM (with terminal β-*N*-acetylgalactosaminyl residues) at 1 mg/ml reversed the effect of neuraminidase. Analysis by paired sample *t*-tests indicates the highly significant difference between particle counts for NANase alone and for NANase plus desiBSM ($P < 0{\cdot}001$ for 8 degrees of freedom). Correspondingly, values for NANase plus desiBSM are not significantly different from controls ($0{\cdot}1 > P > 0{\cdot}05$ for 6 degrees of freedom). That is, the desialysed glycoprotein inhibits the otherwise stimulatory effect of neuraminidase by reversing aggregation towards control levels. The effect of desiBSM on control cells (in the absence of neuraminidase) was not significant.

In contrast to desiBSM, desialysed fetuin (terminal β-galactosyl residues) had no effect on the aggregation of neuraminidase-treated suspensions although the number of experiments was limited by the availability of material.

Removal of the β-galactosyl residues from desialysed fetuin by periodate-oxidation produced an acceptor (possessing terminal β-*N*-acetylglucosaminyl residues) capable of inhibiting the effect of neuraminidase on aggregation, unlike desialysed fetuin itself

(see Table 1). This result suggests, therefore, that the ability of glycoproteins to inhibit the NANase effect depends upon the nature of (at least) the terminal monosaccharide residue.

In 5 separate experiments, ovalbumin (terminal β-*N*-acetylglucosaminyl) was ineffective in reversing the increased aggregation of neuraminidase-treated 16 C cells.

That the glycoproteins did not inhibit the action of neuraminidase at the cell surface by steric hindrance was demonstrated by pre-treating cells with the enzyme (10 U./ml for 15 min at 37 °C and 120 rev/min). By subsequent incubation of the washed cells on a gyratory shaker at 70 rev/min for 30 min it was observed that the glycoproteins inhibited the resultant aggregation in the following order: desiBSM (91 %); desialysed, galactose-free fetuin (70 %); ovalbumin (17 %) and desialysed fetuin (11 %). The order of inhibitory activity noted in this experiment is in qualitative agreement with that achieved by adding the glycoproteins contemporaneously with neuraminidase.

Table 3. *The effect of neuraminidase-treated plasma membrane fractions* on the aggregation† of 16 C fibroblasts*

	Control	NANase	NANase + membranes	Membranes alone
Aggregation‡	100 %	116 %	158 %	114 %
Increased aggregation	—	(16 %)	(58 %)	(14 %)

* 10 μg protein per flask.

† Cells at 1×10^6/ml were aggregated for 30 min at 37 °C as described in Materials and Methods. Where used neuraminidase was added to suspensions at 5 U./10^6 cells.

‡ The figures represent electronic particle counts normalized with respect to a 100 % value given to 30-min controls.

Plasma membrane fractions desialysed by neuraminidase increased the 30-min aggregation value of controls by 14 % (see Table 3). Neuraminidase at 5 U./ml also increased aggregation to a similar extent (16 %). However, addition of neuraminidase plus enzyme-treated plasma membrane to 16 C cells stimulated aggregation by 58 %.

Glycosyltransferase activities of cell suspensions

Desialysed BSM effectively reverses the neuraminidase-induced increase in aggregation and if glycosyltransferases are the adhesive complementary binding sites with which it interacts, then desiBSM:glycosyltransferase activities should be detectable in whole cell preparations.

Initially, an incubation solution similar to that employed by Roth *et al.* (1971*b*) was used (7 mM KCl; 5 mM Tris, pH 7·2; 0·145 M NaCl; 8 mM $MnCl_2$ and 10 mM NaN_3). Results obtained under these conditions (Table 4) confirm that 16 C cells possess a sialyltransferase for which desiBSM is an acceptor. The desialysed mucin also accepts galactose from UDP-^{14}C galactose, although this is difficult to quantitate in view of the high levels of galactosyltransferase activity in the cell-free supernatant of pre-incubated cells. However the result of a separate experiment showed that more isotopic activity was measured in washed cells which had been incubated for 3 h with desiBSM than was determined in the cell pellet in the absence of exogenous acceptor

(2364 ± 47 and 1620 ± 87 dpm/10^6 cells respectively). Therefore the apparent diminution of galactosyltransferase in the presence of cells can be explained by the fact that desiBSM, galactosylated by cell transferases, becomes associated with the cellular fraction which sediments at 200 **g** and is therefore not quantitatively recovered in the supernatant fluid.

In view of the presence of not insignificant levels of glycosyltransferase in the supernatant of cells suspended in Tris-buffered medium, transferase activities were, as a

Table 4. *Cellular desiBSM: sialyl- and galactosyltransferase activities using whole cell suspensions in Tris-buffered medium*

Enzyme source	CMP-^{14}C NAN	desiBSM	dpm
Intact cells	+	+	1082 ± 22
(Endogenous control)	+	−	444 ± 66
Pre-incubated supernatant	+	+	331 ± 1·2
(Endogenous control)	+	−	179 ± 22
	UDP-^{14}C galactose	desiBSM	dpm
Intact cells	+	+	1068 ± 53
(Endogenous control)	+	−	750 ± 128
Pre-incubated supernatant	+	+	7785 ± 379
(Endogenous control)	+	−	502 ± 26

Incubation mixtures were prepared using the Tris-buffered (Ca^{2+}, Mg^{2+} free) medium described in the text and contained 6 × 10^6 fibroblasts per tube. In this experiment, the incubation was for 3 h at 37 °C and the glycosyltransferase activities measured in the acid-precipitable material of supernatants from suspensions centrifuged at 200 **g**. The glycosyltransferases leaked by cells was measured by taking cell-free supernatants from pre-incubated cells (3 h) and the supernatants were subsequently incubated for a further 3 h under similar conditions to experiments containing whole cells. The cells were pre-treated for 15 min at 37 °C with 8 U. of NANase/10^6 cells and then washed. Endogenous controls represent the isotopic activity transferred to material other than the exogenous desiBSM acceptor. Experiments were performed in triplicate and the means, together with the standard deviations, are quoted.

Table 5. *CMP-^{14}C NAN: desiBSM sialyltransferase activity of intact cells incubated in phosphate-buffered saline*

Enzyme source	CMP-^{14}C NAN	desiBSM	dpm
Intact cells	+	+	704 ± 65
(Endogenous control)	+	−	72 ± 43
Pre-incubated supernatant	+	+	0
(Endogenous control)	+	−	0

Cells were incubated in PBS, pH 7·2, at a concentration of 2 × 10^6/tube at 37 °C for 3 h. DesiBSM: galactosyltransferase was not detected under these conditions.

Leakage of sialyltransferase activity to the supernatant was assayed in (cell-free) supernatants of cells pre-incubated for 3 h. This cell-free activity, of which none was detected, is described here as 'pre-incubated supernatant'. Radioactivity was determined in the phosphotungstic acid-precipitable material of supernatants from reaction mixtures centrifuged at 200 **g**. Experiments were performed in triplicate and the figures quoted are the mean values and standard deviations.

consequence, measured in PBS – a solution in which 16 C cells aggregate. Table 5 illustrates that all CMP-^{14}C NAN:desiBSM sialyltransferase is associated exclusively with the cells since no activity could be detected in the supernatant. No galactosyltransferase activity was detected using PBS as the incubation medium and probably reflects the dependence and independence of the galactosyl- and sialyltransferases respectively, to manganous ions which are absent from PBS. Therefore, the relative activities of these 2 enzymes vary according to the composition of the incubation medium though the cells aggregate well in PBS.

Glycosyltransferases in isolated plasma membranes

That glycosyltransferase activity towards a high molecular weight acceptor can be detected in cell suspensions, and which is not wholly due to leakage of soluble enzyme, tends to underline the peripheral location of the transferases. In order to test this, enzyme assays were performed on isolated plasma membranes.

Table 6. *Glycoprotein:glycosyltransferase activities of* 16 *C plasma membrane fractions*

Incubation mixture	Sialyltransferase,† dpm	Galactosyltransferase,† dpm
Complete mixture* *minus* Triton X-100	760	1201
Minus desiBSM, *minus* Triton X-100	9	248
Complete mixture	0	1472
Complete mixture *minus* desiBSM	0	0

* Complete mixtures consisted of 0·1 μCi ^{14}C-nucleotide sugar; 0·03 M $MnCl_2$; 5 μg membrane fraction; 100 μg desiBSM; 0·1 % (v/v) Triton X-100 in 0·03 M Tris HCl, pH 7·0 in a final volume of 150 μl and were incubated at 25 °C for 1 h.

† Experiments performed on separate membrane preparations.

The results of subjecting 16 C plasma membrane preparations to sialyltransferase assay are given in Table 6 and show that the membrane fractions possess a sialyltransferase, for which desiBSM is an acceptor, and which is completely inhibited by 0·1 % Triton X-100. Incorporated radioactivity in these incubations was reduced to background levels by addition of neuraminidase. Counts are also transferred from UDP-^{14}C galactose to the desialysed mucin by the membrane preparations but in contradistinction to the sialyltransferase, its activity is not inhibited by the addition of detergent, since results shown in Table 6 indicate Triton X-100 to stimulate the galactosyltransferase (when corrected for endogenous activity) by about 50 %. Desialysed, galactose-free fetuin was found to be approximately 1·7 times more effective than desiBSM as an acceptor for the galactosyltransferase of the isolated plasma membrane fraction.

DISCUSSION

The increased aggregation of 16 C fibroblasts when treated with neuraminidase could be explained in 2 different ways. Firstly, removal of negatively charged sialosyl residues could affect the physical properties of the cell surface such that adhesiveness is enhanced either by increasing deformability (Weiss, 1965) which may be important in probe formation, or by reducing the electrostatic forces of repulsion between cells (Curtis, 1967). Secondly, and this is the interpretation we have chosen to examine, the effect of neuraminidase could be to uncover specific binding sites for adhesion. That binding sites are indeed exposed by neuraminidase is demonstrated particularly well by the enhanced aggregation of 16 C cells (presumably by multivalent ligand binding) in the presence of enzyme-treated plasma membranes (Table 3, p. 581). Vicker & Edwards (1972) have shown that NANase also increases the mutual adhesion of BHK21C13 cells and suggested that a mechanism involving specifically interacting molecules would more easily account for their observations than one depending solely upon surface charge.

Lectin-binding studies indicate that RCA_{120} and soybean agglutinin-binding sites are only available for agglutination following the exposure of 16 C cells to neuraminidase. Since neuraminidase also increases cellular aggregation, these lectin-binding sites could be receptors for cell adhesion and the reversal of the neuraminidase-stimulated increase of aggregation by desiBSM of similar terminal specificity (β-*N*-acetylgalactosaminyl) supports this view. That desialysed fetuin is ineffective in this respect whereas agalacto desialysed fetuin is not, strongly suggests that the identity of the sugar exposed upon the exogenous glycoproteins is an important aspect of their ability to reverse neuraminidase-stimulated aggregation and again, argues against an explanation of this effect in purely physical terms. The nature of terminal carbohydrate residues is critical for other recognition phenomena: Roth, McGuire & Roseman (1971*a*) have shown that the specificity of chick neural retinal aggregates is abolished by treatment with β-galactosidase since more liver retinal cells subsequently adhere to the aggregates and Chipowsky, Lee & Roseman (1973) have reported that 3T3 cells adhere to Sephadex beads to which galactose has been linked whereas they will not adhere to beads derivatized with glucose or *N*-acetylglucosamine. Studies on the attachment of circulatory glycoproteins to rat liver membranes are particularly relevant in the present context. Following the demonstration that hepatocytes recognize and remove from circulation heterosaccharides with β-galactosyl residues exposed by desialysation (Morell *et al.* 1971), Rogers & Kornfeld (1971) showed that treatment of desialysed fetuin with β-galactosidase prevented its clearance by the liver. Not only does the exposure of galactosyl residues trigger uptake but by studying the effectiveness of other glycoproteins, the latter investigators showed that the specificity extended to the configuration of residues deeper within the glycoprotein structure. These findings are in accord with the hypothesis that complementary binding sites at the cell surface bind to specific glycoprotein acceptors present upon neighbouring cells. That desiBSM and desialysed, agalacto fetuin, which inhibit the neuraminidase-stimulated aggregation of 16 C cells, are both glycosylated by these fibroblasts would seem to imply

that surface-located glycosyltransferases are the complementary binding sites as Roseman (1970) has suggested. However, the initial experiments using a Tris-buffered medium were unsatisfactory since higher levels of galactosyl-transferase were detected in the supernatant fluid of cells than of intact cell-suspensions. This, as outlined in the results, may be due to the fact that less isotopically labelled acceptor is assayed in the supernatants of those experiments which use cell suspensions as source of enzyme because the radioactively labelled acceptor becomes attached to cells which are removed by centrifugation at the end of the experiment prior to acid-precipitation of the remaining supernatant. Since cells are removed before the addition of acceptor and isotope in cell-free supernatant control experiments, this difficulty does not arise and may account for the apparently higher extent of transfer in these instances. Also, such discrepancy does not arise with the sialyltransferase assays. Since galactosylated desiBSM attaches to cells but apparently not the sialylated mucin, it is possible that this interaction results from the presence of terminal galactosyl residues which, by analogy with the rat hepatic membrane/desialysed fetuin system mentioned above may cause the association of the newly glycosylated acceptor with the cell pellet.

In view of the levels of supernatant glycosyltransferase in the Tris-buffered system, assays were consequently performed in phosphate-buffered saline in which the cells routinely aggregate. With PBS as a suspending medium, CMP-NAN:desiBSM sialyltransferase was detected in cells without leakage of the enzyme to the supernatant but the galactosyltransferase could not be detected presumably because of the absence of Mn^{2+} from this solution. In this medium, between 29 and 39 % of cells examined at 3 h were stained with erythrocin B or trypan blue and since homogenates possessed about 5 times as much sialyltransferase activity as 'intact' cells it is possible that the stained cells were responsible for the cellular activity. The fact that no soluble sialyltransferase was detected in the supernatant militates against this and as Roth *et al.* (1971*b*) have discussed, it is difficult to conceive of a mechanism by which large glycoproteins enter a cell, are glycosylated and then released. The activity of an homogenate is perhaps not the best model for the sialyltransferase activity of cells which, although stained with dye, still maintain their morphological integrity. Cell debris was frequently seen under phase in 3-h incubation mixtures and membrane-bound sialyltransferase activity in this component could be responsible for some of the total activity. Because of the extensive cellular aggregation, the loss of cells over the period of incubation was difficult to quantitate microscopically and for this reason glycosyltransferase activities were estimated in plasma membrane preparations which also confirm the peripheral location of these enzymes. Using the method of Warley & Cook (1973) plasma membranes are isolated in the form of sheets, not vesicles, devoid of contamination by cytoplasmic organelles and when applied to 16 C cells, CMP-NAN:desiBSM sialyltransferase and UDP-galactose:desiBSM/desialysed agalacto fetuin galactosyltransferases of high specific activities were identified in these fractions. This supports the findings of Roth *et al.* (1971*b*) and Roth & White (1972) which indicate glycosyltransferases to be borne upon the surfaces of intact cells and others have also identified glycosyltransferases in plasma membrane fractions (Warren, Fuhrer & Buck, 1972; Aronson, Tan & Peters, 1973; Barber & Jamieson, 1971*a*, *b*;

Bosmann, 1971, 1972*a*, *b*). In the present studies the adhesions induced by neuraminidase are likely to involve a glycoprotein:sialyl- and/or galactosyltransferase.

If plasma membranes are produced by fusion of Golgi vesicles with the cell surface (Hirano *et al.* 1972) it follows that in so doing membrane-bound glycosyltransferases and their glycoprotein substrates would also become externalized at the plasma membrane, but whether or not the enzymes retain their function of glycosylation would, in the absence of an experimentally administered donor, depend upon the availability of cellular nucleotide sugar to the surface membrane. Glycosyltransferases might also remain inactive with limiting levels of divalent cations and this is underlined by our inability to detect a galactosyltransferase in 16 C cells incubated in PBS whereas addition of Mn^{2+} to whole cells in Tris-buffered medium and to isolated plasma membrane fractions revealed its presence. In view of this, it could be considered that peripheral glycosyltransferases participate in adhesion by forming enzyme/substrate complexes and that glycosylation is not a necessary consequence of intercellular adhesion. Similarly, Aronson *et al.* (1973) conclude that desialysed fetuin (cf. Morell *et al.* 1971; Rogers & Kornfeld, 1971) with terminal galactosyl residues, attaches to a liver plasma membrane galactosyltransferase by enzyme/product binding as distinct to enzyme/substrate binding involving a sialyltransferase.

Alternatively, Roseman (1970) has proposed that adhesions are dissociated upon completion of the glycosylation reaction between cells which thereby modify each other's surfaces. In this case, adhesion could be recycled by the enzymic removal of the terminal glycosyl residue (Pricer & Ashwell, 1971; Bosmann, 1972*b*) or stabilized by a secondary mechanism, e.g. the regulated release of hyaluronic acid (Toole, Jackson & Gross, 1972). In accord with other investigations already discussed, the present results suggest that some specific membrane asialoglycoproteins, unlike sialylated glycoproteins, are receptor sites for intercellular adhesion. The presence of sialic acid, as McQuiddy & Lilien (1971) observe, would seem to be inessential to specific adhesion because of its ubiquity and negative charge properties but since desialysed glycoproteins may be specific receptors for adhesion it is proposed that a special property of some sialyltransferases, whether at the cell surface or by completing precursor membrane within the cell, is to limit net adhesiveness by 'protecting' sub-adjacent heterosaccharide sequences from surveillance. The process of sialylation may therefore exert a modulatory effect upon cellular interaction. It is of especial interest therefore that Warren *et al.* (1972) (see also Bosmann, 1972*a*) have found a specific growth-dependent glycoprotein: sialyltransferase – mainly in the plasma membrane, the activity of which is increased in dividing as opposed to non-dividing cells and in polyoma-transformed BHK21C13 fibroblasts as compared against non-transformed cells. Polyoma-transformed BHK21C13 cells aggregate much less than the 'normal' fibroblasts (Edwards, Campbell & Williams, 1971) and although numerical, topological and qualitative variations in surface receptors may all contribute to diminished adhesiveness the possibility that sialylation of specific sites could also represent an important control, merits consideration.

We are grateful to Dr Mary Daniel for her constant help and advice and for the original inoculum of 16 C cells; to Dr G. D. Clarke for the generous provision of growth media and to

Mr M. Abercrombie, F.R.S., for his interest in this work. We also thank Dr Audrey Glauert and Mr R. A. Parker for the electron-microscopic examination of membrane fractions; Mr M. F. Applin for producing the figures and Mrs E. Broad for typing the manuscript.

G. M. W. Cook is a member of the External Scientific Staff of the Medical Research Council.

REFERENCES

ARONSON, N., TAN, L. Y. & PETERS, B. P. (1973). Galactosyl transferase – the liver plasma membrane binding-site for asialo-glycoproteins. *Biochem. biophys. Res. Commun.* **53**, 112–118.

BARBER, A. J. & JAMIESON, G. A. (1971*a*). Platelet collagen adhesion: characterization of collagen glucosyltransferase of plasma membranes of human blood platelets. *Biochim. biophys. Acta* **252**, 533–545.

BARBER, A. J. & JAMIESON, G. A. (1971*b*). Characterization of membrane-bound collagen galactosyltransferase of human blood platelets. *Biochim. biophys. Acta* **252**, 546–552.

BOSMANN, H. B. (1971). Platelet adhesiveness and aggregation: the collagen:glycosyl, polypeptide:*N*-acetylgalactosaminyl and glycoprotein:galactosyl transferases of human platelets. *Biochem. biophys. Res. Commun.* **43**, 1118–1124.

BOSMANN, H. B. (1972*a*). Sialyltransferase activity in normal and RNA- and DNA-virus transformed cells utilizing desialysed, trypsinized cell plasma membrane external surface glycoproteins as exogenous acceptors. *Biochem. biophys. Res. Commun.* **49**, 1256–1262.

BOSMANN, H. B. (1972*b*). Platelet adhesiveness and aggregation. II. Surface sialic acid, glycoprotein:*N*-acetylneuraminic acid transferase, and neuraminidase of human blood platelets. *Biochim. biophys. Acta* **279**, 456–474.

CHIPOWSKY, S., LEE, Y. C. & ROSEMAN, S. (1973). Adhesion of cultured fibroblasts to insoluble analogues of cell-surface carbohydrates. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2309–2312.

COOK, G. M. W. & JACOBSON, W. (1968). The electrophoretic mobility of normal and leukaemic cells of mice. *Biochem. J.* **107**, 549–557.

CURTIS, A. S. G. (1967). *The Cell Surface: Its Molecular Role in Morphogenesis.* London: Academic Press.

DANIEL, M., DINGLE, J. T., WEBB, M. & HEATH, J. C. (1963). The biological action of cobalt and other metals. I. The effect of cobalt on the morphology and metabolism of rat fibroblasts *in vitro*. *Br. J. exp. Path.* **44**, 165–176.

EDWARDS, J. G. & CAMPBELL, J. A. (1971). The aggregation of trypsinized BHK21 cells. *J. Cell Sci.* **8**, 53–72.

EDWARDS, J. G., CAMPBELL, J. A. & WILLIAMS, J. F. (1971). Transformation by polyoma virus affects adhesion of fibroblasts. *Nature, New Biol.* **231**, 147–148.

HIRANO, H., PARKHOUSE, B., NICOLSON, G. L., LENNOX, E. S. & SINGER, S. J. (1972). Distribution of saccharide residues on membrane fragments from a myeloma-cell homogenate: its implications for membrane biogenesis. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2945–2949.

KEMP, R. B., LLOYD, C. W. & COOK, G. M. W. (1973). Glycoproteins in cell adhesion. In *Progress in Surface and Membrane Science*, vol. 7, pp. 271–318. London and New York: Academic Press.

LIS, H., SHARON, N. & KATCHALSKI, E. (1966). Soybean haemagglutinin, a plant glycoprotein. I. Isolation of a glycopeptide. *J. biol. Chem.* **241**, 684–689.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MCQUIDDY, P. & LILIEN, J. E. (1971). Sialic acid and cell aggregation. *J. Cell Sci.* **9**, 823–833.

MCQUIDDY, P. & LILIEN, J. E. (1973). The binding of exogenously added neuraminidase to cells and tissues in culture. *Biochim. biophys. Acta* **291**, 774–779.

MORELL, A. G., GREGORIADIS, G., SCHEINBERG, I. H., HICKMAN, J. & ASHWELL, G. (1971). The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. biol. Chem.* **246**, 1461–1467.

NICOLSON, G. L. & BLAUSTEIN, J. (1972). The interaction of *Ricinus communis* agglutinin with normal and tumor cell surfaces. *Biochim. biophys. Acta* **266**, 543–547.

PAUL, J. (1970). *Cell and Tissue Culture.* Edinburgh & London: Livingstone.

PRICER, W. E. & ASHWELL, G. (1971). The binding of desialylated glycoproteins by plasma membranes of rat liver. *J. biol. Chem.* **246**, 4825–4833.

ROGERS, J. C. & KORNFELD, S. (1971). Hepatic uptake of proteins coupled to fetuin glycopeptide. *Biochem. biophys. Res. Commun.* **45**, 622–629.

ROSEMAN, S. (1970). The synthesis of complex carbohydrates by multiglycosyl transferase systems and their potential function in intercellular adhesion. *Chem. Physics Lipids* **5**, 270–297.

ROTH, S., MCGUIRE, E. J. & ROSEMAN, S. (1971*a*). An assay for intercellular adhesive specificity. *J. Cell Biol.* **51**, 525–535.

ROTH, S., MCGUIRE, E. J. & ROSEMAN, S. (1971*b*). Evidence for cell surface glycosyltransferases. Their potential role in cellular recognition. *J. Cell Biol.* **51**, 536–547.

ROTH, S. & WHITE, D. (1972). Intercellular contact and cell surface galactosyl transferase activity. *Proc. natn. Acad. Sci. U.S.A.* **69**, 485–489.

SPIRO, R. G. (1964). Periodate oxidation of the glycoprotein fetuin. *J. biol. Chem.* **239**, 567–573.

STODDART, R. W. & NORTHCOTE, D. H. (1967). Separation and measurement of microgram amounts of radioactive polysaccharides in metabolic experiments. *Biochem. J.* **105**, 61–63.

TOOLE, B. P., JACKSON, G. & GROSS, J. (1972). Hyaluronate in morphogenesis. Inhibition of chondrogenesis *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1384–1386.

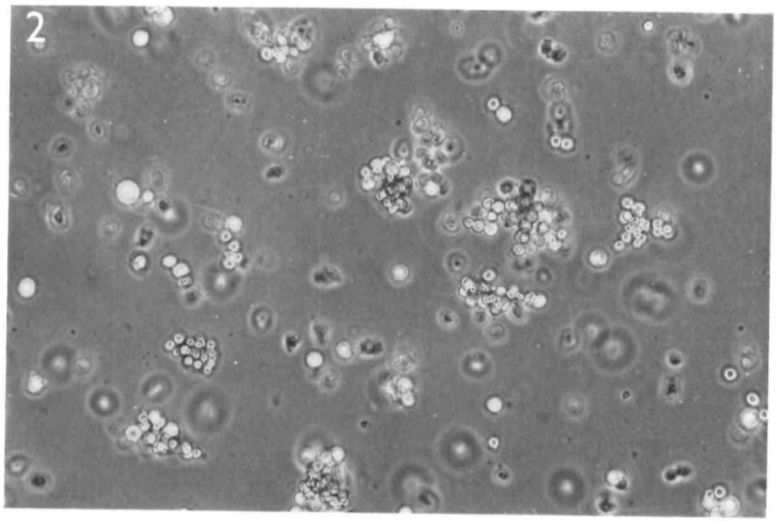
VICKER, M. G. & EDWARDS, J. G. (1972). The effect of neuraminidase on the aggregation of BHK21 cells and BHK21 cells transformed by polyoma virus. *J. Cell Sci.* **10**, 759–768.

WARLEY, A. & COOK, G. M. W. (1973). The isolation and characterization of plasma membranes from normal and leukaemic cells of mice. *Biochim. biophys. Acta* **323**, 55–68.

WARREN, L., FUHRER, J. P. & BUCK, C. A. (1972). Surface glycoproteins of normal and transformed cells: A difference determined by sialic acid and a growth-dependent sialyltransferase. *Proc. natn. Acad. Sci., U.S.A.* **69**, 1838–1842.

WEISS, L. (1965). Studies on cell deformability. I. Effect of surface charge. *J. Cell Biol.* **26**, 735–739.

(*Received* 4 *January* 1974)



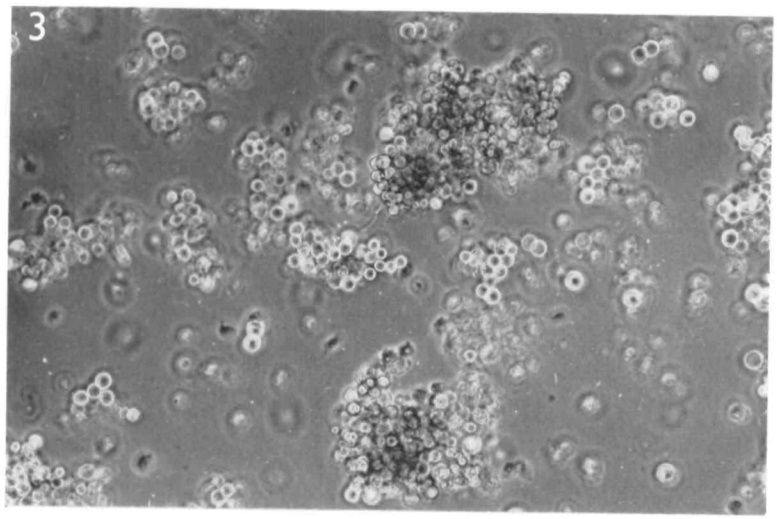


Fig. 2. Aggregates formed in controls after rotation at 70 rev/min in PBS and 37 °C for 30 min. × 125.

Fig. 3. Larger aggregates formed by 16 C cells in the presence of neuraminidase at a concentration of 5 U./10^6 cells after 30 min on the gyratory shaker at 70 rev/min at 37 °C. × 125.

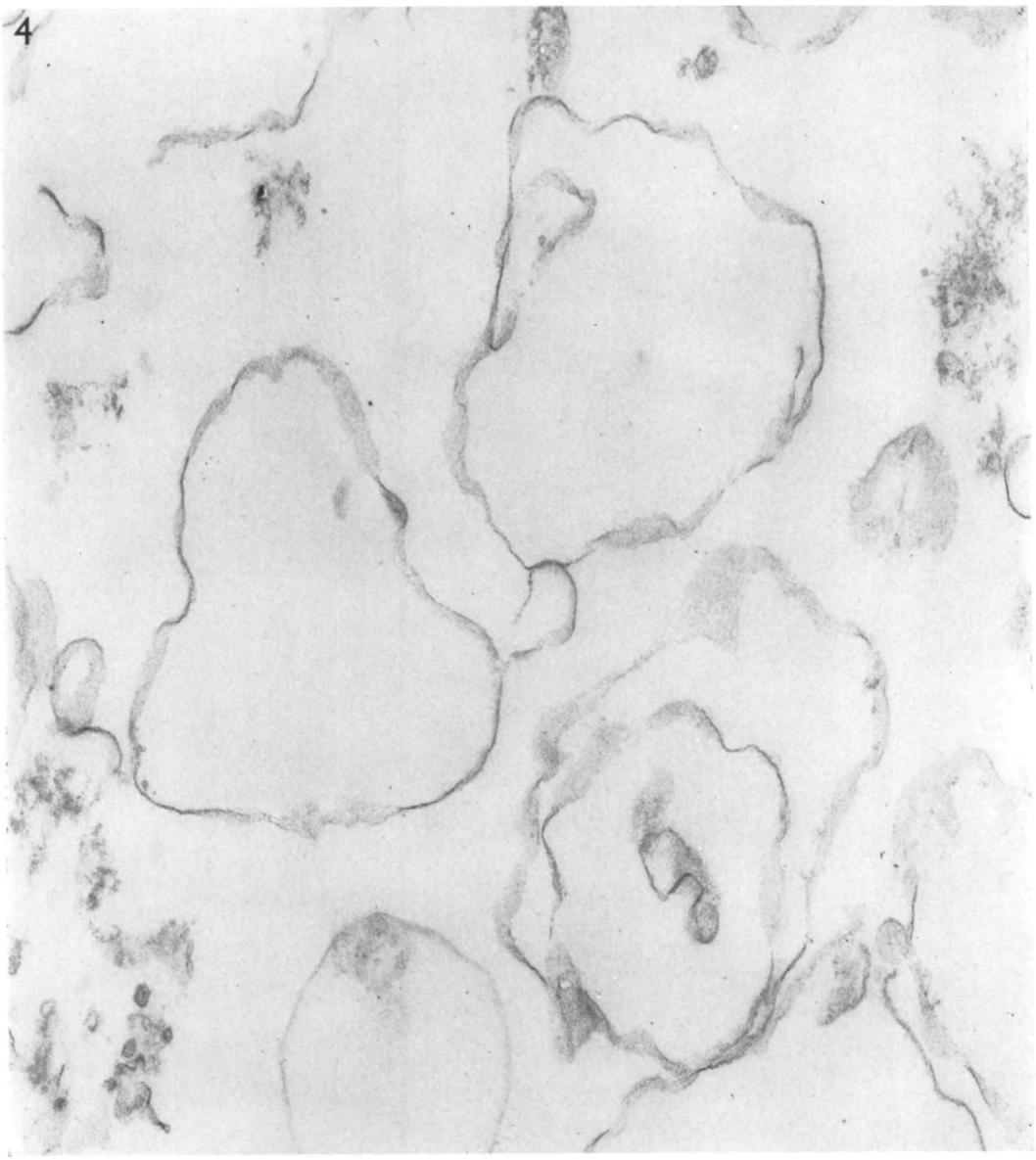


Fig. 4. A typical field of a thin section obtained from the plasma membrane fraction of 16 C malignant rat dermal fibroblasts. The fraction is seen to consist of membrane sheets devoid of contaminating material. × 55000.