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ELECTROPHORETIC AND CHEMICAL CHARACTERIZATION OF THE CHARGED GROUPS AT THE SURFACE OF MURINE *CL3* ASCITES LEUKAEMIA CELLS

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SUMMARY

The electrophoretic characteristics of the murine *CL3* ascites tumour were investigated. Treatment of the cells with formaldehyde raised the electrophoretic mobility (E.P.M.) from −1·06 to −1·28 μ/sec/V/cm; subsequent treatment with diazomethane reduced their mobility to zero. The E.P.M. of the diazomethane-treated cells did not alter over the pH range 3·0–8·0. This proved that the only ionic groups at this cell surface were amino and carboxyl groups. The absence of phosphate groups, another possibility, was confirmed by the lack of calcium-ion binding from 10 mM Ca^{2+} solutions.

Neuraminidase treatment reduced the E.P.M. from −1·06 to −0·55 μ/sec/V/cm and free sialic acid was identified in the enzyme supernatant. Subsequent treatment of the cells with formaldehyde raised the mobility to −1·22 μ/sec/V/cm indicating that the change in E.P.M. on neuraminidase treatment was not due solely to the removal of the carboxyl groups of sialic acid but also to a change in the ionic nature of the surface. This change is ascribed to a change in the conformation of the surface protein. The reason for this change and a suggestion for the possible role of sialic acid at the cell surface are mentioned.

Treatment of the cells with trypsin did not affect the viable cells in any way, suggesting that the surface proteins lack the basic amino acids lysine and arginine. Pronase treatment served only to show that much of the sialic acid was bound to protein; the total amount was not determined.

INTRODUCTION

The technique of micro-electrophoresis has been used for a considerable time to measure the zeta (ζ) potential at the surface of many types of mammalian cells and of some bacteria (Heard & Seaman, 1960; Douglas & Parker, 1957). Investigations into the dependence of the electrophoretic mobility (E.P.M.) of cells upon pH, ionic strength, and counter ion concentration in the bulk medium have enabled workers to infer the presence of specific ionic groupings at the cell surface (Cook, Heard & Seaman, 1962).

These identities were all conjectural until the advent of neuraminidase. The action of this enzyme, which as an α-glycosidase cleaves terminal sialic acid residues from other carbohydrate molecules, still remains the only well-defined enzymic reaction for the modification of cell surface components.

Cook (1962) extended the scope of the technique by using specific chemical reactions to modify the charged groups at the surface of the formaldehyde-fixed cell.

This enabled the identity of such groups to be defined within the limits of the reaction used.

The unfixed cell, however, still presents problems as lysis occurs in conditions little removed from physiological. The applicability of results obtained on fixed cells to cells in the native state remains conjectural as do results obtained on native cells when the complexity of what seems a simple reaction is overlooked.

The investigation reported here attempts an analysis of the charged groups at the surface of the *CL3* leukaemia cell.

MATERIALS AND METHODS

Solutions

The following solutions were prepared from 'Analar' grade chemicals and glass-distilled water.

(i) 0·146 M NaCl, (ii) 0·1 M phosphate buffer, pH 7·2, (iii) 0·2 M acetate buffer, pH 5·6, (iv) 0·15 M $CaCl_2$ standardized by a Volhard titration, (v) 0·146 M HCl, (vi) 0·146 M NaOH.

To obtain various pH values, solution (i) was adjusted with either solution (v) or (vi). Solutions containing different calcium ion concentrations were made by mixing the required amount of solution (iv) with solution (i). Solution (ii) added to solution (i) to 20% by volume gave phosphate buffer saline pH 7·2, μ = 0·144 (P.B.S.). Solution (iii) added to solution (i) to a concentration of 20% gave acetate-buffered saline pH 5·6, μ = 0·156 (A.B.S.).

Cells

CL3 cells, derived from a spontaneous mouse lymphoma, were grown intraperitoneally in C-mice. Eight days after injection of 5×10^5 cells the mice were killed with chloroform and the cells collected into 20 vol. of ice cold P.B.S. The supernatant was discarded after centrifuging at 500 **g** for 5 min. After three further washes in P.B.S. these cells are referred to as standard washed cells.

Enzymes

All cell samples were washed twice in the incubation media and made up to 10% by volume before incubation at 37 °C. This will be called a standard suspension.

Neuraminidase. This was obtained from Beheingwerke Ltd., West Germany, as a solution containing 500 units per ml in A.B.S. The enzyme solution was added to a standard suspension in A.B.S. containing 1 mM Ca^{2+} to give a final concentration of 100 units per ml of cells. After incubation the suspension was cooled to 0 °C and the cells were centrifuged out.

Trypsin. A twice-crystallized salt-free preparation obtained from Gen. Biochem. Inc., Ohio, was used as a 1% solution in P.B.S. containing 1 mM Ca^{2+}. After incubation, enzyme action was stopped by adding to the suspension twice the enzyme concentration of soy-bean trypsin inhibitor (Sigma) and incubating for a further 15 min or simply cooling to 0 °C.

Pronase. A 0·25% solution of 'Grade B' Calbiochem Pronase was used. The standard suspension was made up in medium containing equal volumes of 0·146 M NaCl and 0·005 M $CaCl_2$ adjusted to pH 7·4 (Cook & Eylar, 1965). After incubation the suspension was cooled to 0 °C to retard enzyme action and the cells were removed by centrifugation.

Chemical reagents and methods

Formaldehyde. Cells were treated with formaldehyde according to the method of Heard & Seaman (1961). The 1·5% solution of formaldehyde was prepared thus. Enough solid paraformaldehyde to give a 1·5% solution was added to 0·146 M NaCl pH 8·0 at 65–70 °C. Under these conditions it dissolved with depolymerization. After cooling and adjusting to pH 7·5 with 0·146 M HCl this solution was used as the fixative.

After a minimum period of 30 days fixing at room temperature the cells were washed thrice in P.B.S. and will subsequently be termed standard washed fixed cells.

Diazomethane. This was prepared when needed from 'Diazald' by the method of Gittens & James (1963). Standard washed fixed cells were washed twice in 0·05 M HCl and twice in 95% ethanol before suspending them in excess ethereal solution of diazomethane. After 15 min continuous slow stirring at room temperature they were removed, washed twice in 95% ethanol and then twice in P.B.S. before further use.

Sulphuric acid. To release sialic acid cells were treated for 1 h at 80 °C with 0·4 N H_2SO_4.

Sialic acid was measured by the thiobarbituric acid assay (Warren, 1959). Optical densities were measured on a Unicam SP 500 spectrophotometer using matched silica cells with a 1-cm light path.

Electrophoretic mobilities were measured in an apparatus similar to that described by Bangham, Flemans, Heard & Seaman (1958) using a flatground cylindrical cell. Henry's optical correction was taken into account (Henry, 1938). All measurements were made in the first stationary layer at a temperature of 25 °C.

pH was measured to an accuracy of 0·05 units on a Beckman expanded scale model pH meter using a combined glass/reference electrode.

Standard washed cells were ultrasonically disrupted by passing a 20 KHz signal through a cooled suspension for 5 min.

RESULTS

Microscopic examination of standard washed cells showed them to be of a uniform round shape. Their diameter was reasonably constant at about 8–10 μ. These factors made them a very good system for electrophoretic examination.

Preliminary E.P.M. measurements in P.B.S. showed that they had a negative or anodic mobility of $-1{\cdot}06\ \mu$/sec/V/cm. Measurement of the E.P.M. at differing pH values gave the results shown in Fig. 1. The range over which reversible E.P.M. values could be obtained was restricted to that between pH 4·0 and pH 9·0. The value shown at pH 3·5 was measured less than 30 sec after suspending the cells at this pH; it was irreversible and therefore approximate. In the range in which reversible values could

be obtained the E.P.M. remained constant. Lack of any data above pH 9·5 meant that positive groups which ionized in this region could not be detected.

One anionic group which may be present at cell surfaces is the PO_4''' group of phospholipids. A property which has been taken as indicative of the presence of these

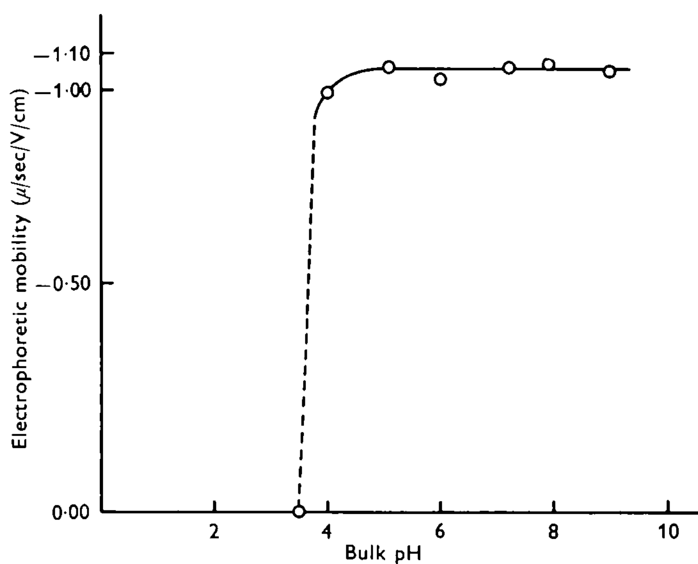

Fig. 1. The relationship between the pH of the suspending medium and the E.P.M. of *CL* 3 cells. In all cases the ionic strength was 0·146 and all values were reversible to neutrality.

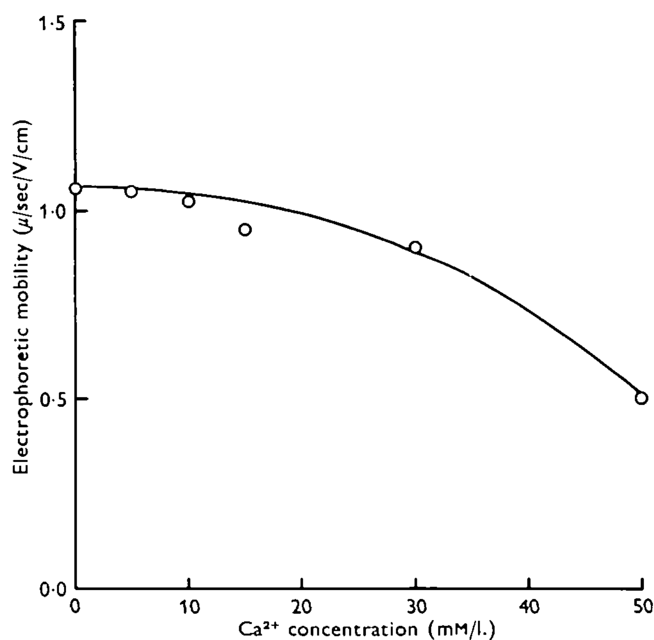

Fig. 2. The variation in the E.P.M. of *CL 3* cells brought about by increasing concentrations of Ca^{2+} in the suspending medium at constant ionic strength.

groups at cell surfaces is a considerable binding of Ca^{2+} ions from solutions of comparatively low Ca^{2+} concentration (Forrester, 1964).

The extent of binding was judged by the decrease in E.P.M. obtained on electrophoresing the cells in Ca^{2+} containing media. As can be seen from Fig. 2 no significant decrease occurred until the solution contained 35 mM Ca^{2+}. If a significant decrease in E.P.M. at 10 mM Ca^{2+} is taken as indicative of the presence of PO_4''' groups then this experiment indicates otherwise.

Enzyme treatment

Neuraminidase. The effect of neuraminidase on the E.P.M. of these cells was investigated in conjunction with assays of the amount of sialic acid released into the supernatant. The results are shown in Fig. 3.

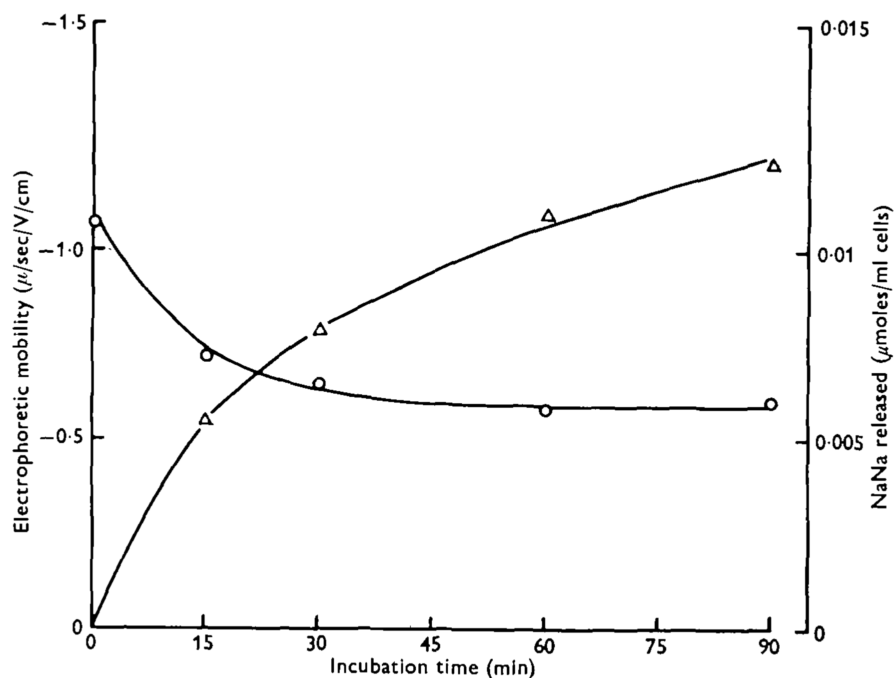

Fig. 3. The effect of neuraminidase treatment on the E.P.M. of *CL3* cells (—○—) and the amount of free sialic acid released per ml of packed cells (—△—). At all times the amount of sialic acid released is more than that necessary to account for the E.P.M. changes.

The decrease in E.P.M. from $-1·06$ to $-0·58\,\mu$/sec/V/cm due to removal of the charged carboxyl groups of the sialic acid molecules was completed after 45 min incubation. The release of sialic acid continued long after this and the amount released was always in excess of that needed to account for the change in surface charge density as calculated from the E.P.M.

Trypsin. Difficulties were encountered when using trypsin as any cell suspension to which trypsin was added gelled very quickly. It was possible to disperse the gel by

adding a small amount of DNase and incubating for a short time. The formation of this gel was ascribed to the release, by trypsin, of DNA from some of the cells in the original suspension.

After 90 min incubation with trypsin the E.P.M. of the cells had decreased by approximately 10%. The amount of neuraminidase-labile cell-bound sialic acid did not change appreciably and as none was identified in the hydrolysed trypsin supernatant it was concluded that trypsin had little effect on this cell surface.

Pronase. The changes in E.P.M. of standard washed cells on incubation with Pronase are shown in Table 1.

Table 1. *Effect of Pronase treatment on electrical mobility*

Time of Pronase treatment (min)	Electrophoretic mobility (μ/sec/V/cm)
0	−1·02
5	−0·45
30	−0·63
60	−0·91
90	Not measurable

Table 2. *Release of N-acetyl neuraminic acid by neuraminidase and by acid hydrolysis*

Method of hydrolysis	μmoles of NaNa per ml cells	
	Whole cells	Disrupted cells
1. H_2SO_4	$7{\cdot}4 \times 10^{-1}$	$6{\cdot}8 \times 10^{-1}$
2. Nanase	0·0	0·0
1. Nanase	$5{\cdot}9 \times 10^{-1}$	$7{\cdot}8 \times 10^{-1}$
2. H_2SO_4	Small amount	0·0

NaNa, *N*-acetyl neuraminic acid; nanase, neuraminidase.

After an initial large fall in E.P.M. it begins to increase and is still rising after 90 min. The suspension did not gel (cf. trypsin) but after incubation for 90 min the cells were unable to withstand washing, and on suspending them in saline they lysed. No whole cells could be obtained from the ensuing gel. Cells treated for 60 min showed an increased Ca^{2+} binding capacity possibly associated with exposed phospholipid phosphate groups.

Sialic acid assays on the acid-hydrolysed enzyme supernatant revealed the presence of considerable amounts of sialic acid. The total concentration however varied greatly between experiments.

Chemical treatment

Sulphuric acid. A comparison of the total acid-hydrolysable sialic acid with the total neuraminidase-labile sialic acid was performed on both intact and ultrasonically

disrupted cells (Table 2). Acid treatment released in addition to sialic acid a large number of substances which interfered with the Warren assay for sialic acids. For this reason all values for acid labile sialic acid in cells (Table 2) should be taken as minimum values.

These results show that all the enzyme labile sialic acid is also acid labile but that in intact cells all the acid labile molecules were not enzyme labile. Release of similar amounts of sialic acid by acid or enzyme from ultrasonically disrupted cells suggests that all the sialic acid is linked in the same way and that the difference noted using intact cells was due possibly to a steric effect.

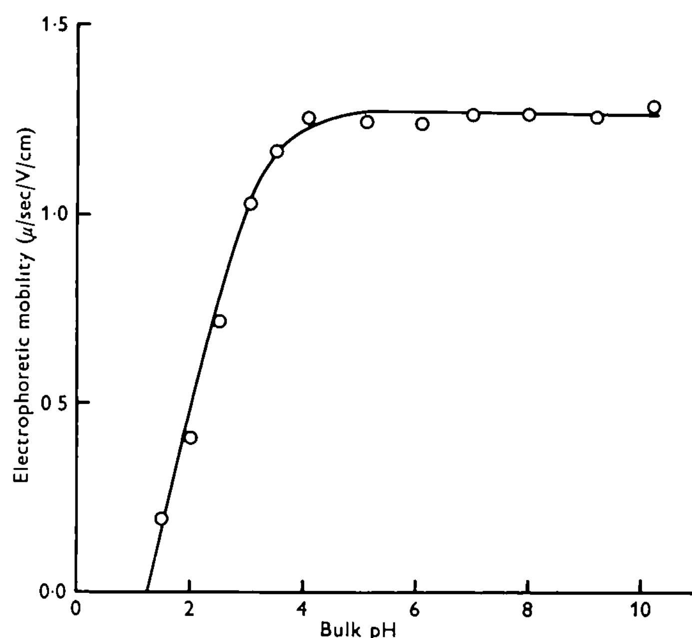

Fig. 4. The relationship between the pH of the suspending medium and the E.P.M. of *CL 3* cells fixed in 1·5 % formaldehyde for 30 days.

Formaldehyde. Formaldehyde was used both to stabilize the cells for further treatment and to obtain an estimate of the contribution of charged amino groups to the E.P.M.

After 30 days fixation the E.P.M. of these cells rose from $-1{\cdot}06$ to $-1{\cdot}28$ μ/sec/V/cm. Such cells showed exceptional pH stability as is shown in Fig. 4. The indicated isoelectric point of 1·3 is difficult to explain in terms of the ionic groups identified at the surface. It is possibly due to the modification of the pK values of some of the ionic species present by interaction with other charged groups.

That formaldehyde treatment produces a change in the E.P.M. of the cells indicated that the suface contained both cationic and anionic groups; the former were responsible for an E.P.M. of $+0{\cdot}22$ μ/sec/V/cm and the latter for the final E.P.M. of $-1{\cdot}28$ μ/sec/V/cm.

Formaldehyde was also applied to neuraminidase-treated standard washed cells.

These cells, with an original E.P.M. of $-0.58\ \mu$/sec/V/cm, had a final E.P.M. of $-1.22\ \mu$/sec/V/cm. Thus the anionic contribution to the E.P.M. of neuraminidase-treated cells ($-1.22\ \mu$/sec/V/cm) is little changed from that of standard washed cells whilst the contribution of the cationic amino groups has increased from $+0.22$ to $+0.64\ \mu$/sec/V/cm. The conclusion to be drawn from this seems to be that removal of sialic acid from these cells is accompanied by other changes in the surface at the same time.

Diazomethane. Standard washed fixed cells treated with diazomethane had a resultant mobility of $0.00\ \mu$/sec/V/cm. This remained constant between pH 3·0 and 8·0 indicating that it was a true zero E.P.M. and that all the anodic groups present on the surface of standard washed fixed cells were carboxyl groups.

DISCUSSION

In agreement with all other reported measurements on mammalian cells, *CL3* cells bore a net negative surface charge. This gave them an anodic mobility of $-1.5\ \mu$/sec/V/cm at neutral pH (Fig. 1). It was not possible to obtain any unequivocal information on the nature of these surface groups by a study of this mobility as the cells became unstable at the relevant pH values, i.e. outside the range pH 4·0–9·0.

Measurement of the cellular E.P.M. after treatment with formaldehyde showed that this initial mobility was the algebraic sum of both cationic and anionic contributions. A study of the chemistry of the reaction of formaldehyde with proteins by French & Edsall (1945) has shown that the only charged group with which it will react at neutral pH is the charged amino group NH_3^+; removal of this as an uncharged derivative must therefore account for the change in E.P.M. from -1.06 to $-1.28\ \mu$/sec/V/cm.

The existence of positively charged NH_3^+ groups at this cell surface indicates a difference between these cells and human erythrocytes, the only other cell studied in such detail (Cook, 1962; Haydon & Seaman, 1967). These latter cells showed no difference in E.P.M. after formaldehyde treatment and thus bore no amino groups.

The negatively charged groups on both the cell types mentioned in the previous paragraph were recognized by their capacity to react with diazomethane. In both cases the cells had an E.P.M. of zero after diazomethane treatment which did not change over a wide pH range indicating that all the anionic groups on the surface of standard washed fixed cells were carboxyl.

Trypsin was used by Cook, Heard & Seaman (1960) to isolate a sialo-mucopeptide from the surface of human erythrocytes; the failure of this enzyme to produce any change in the E.P.M. of the *CL3* cells pin-points a further distinction between these two cell types.

The production of a gel on incubation of these cells with trypsin together with the apparently unreconcilable constancy of the E.P.M. suggests the presence of two cell types in the original suspension. The most likely division which can be made here is between 'viable' and 'non-viable' cells as judged by dye uptake (Holmberg, 1961). The non-viable cells which would be permeable to the enzyme molecules would be digested, releasing DNA, whilst those with a permeability barrier, i.e. viable, would resist any damage. This seems probable in light of the observation that the apparent

viability of a suspension sometimes increased on treatment with trypsin, in accord with the complete removal of some non-viable cells by tryptic digestion.

From their lack of any reactivity with trypsin it can also be inferred that the surface proteins lack any appreciable content of the basic amino acids lysine and arginine as these are necessary to satisfy the specificity requirements of this enzyme.

Pronase provided little new information about the surface except to show that sialic acid was bound in part at least, to protein and that in the standard washed cell phosphate groups were a dominant ionic species after removal of the surface protein.

The action of neuraminidase in reducing the E.P.M. from $-1{\cdot}06$ to $-0{\cdot}58\ \mu$/sec/V/cm suggests sialic acid as a primary surface charge determinant. This was not unexpected and was confirmed by analysis of the enzyme supernatant. The amount of sialic acid assayed was always in excess of that needed to account for the change in surface charge density as calculated from E.P.M. changes. This is in agreement with many other observations (Wallach & Ullrey, 1962; G. F. Seaman, personal communication).

Further treatment of neuraminidase-treated cells with formaldehyde produced surprising results. The increase in E.P.M. from $-0{\cdot}58$ to $-1{\cdot}22\ \mu$/sec/V/cm indicated an increased concentration of amino groups on the surface after neuraminidase treatment. Concomitant with this the contribution of negatively charged groups was changed very little from that found in standard washed cells even though the carboxyl groups of sialic acid have been removed by the enzyme. As the removal of sialic acid itself produces no new charged groups the extra ones measured must have been present before treatment. The large increase in the contributions of both amino and carboxyl groups therefore most likely arises from a surface re-orientation brought about by the removal of sialic acid.

The re-orientation can be explained if one assigns to sialic acid the function of a primary determinant of membrane configuration. Removal of the highly acidic carboxyl groups of sialic acid ($pK_a = 2{\cdot}6$) by neuraminidase could necessitate the reformation of the tertiary structure of the residual surface protein to present a more hydrophilic surface to the external aqueous environment. This would account for the increased surface charge density noted.

Extension of this idea to the cell *in vivo* where exposure of sialic acid to the external environment could produce changes in the local pH and thus possibly cause the cell membrane to change its configuration would give sialic acids a vital role in many cellular processes in which the membrane participates. Such a role would account for the biological ubiquity of this molecule.

In the preceding experiments the charged surface of *CL3* cells has been shown to be a mosaic of positively charged amino groups and negatively charged carboxyl groups There remains the question of the positions they occupy.

The amino groups may arise from unsubstituted amino sugars or from proteins as (i) 'N' terminal chain ends or (ii) as the amino groups of lysine or arginine. Lack of reaction with trypsin removes the last alternative and as most amino sugars at cell surfaces have been found in an N-acylated form the most probable position of the amino groups is at 'N' terminal protein chain ends.

Sialic acids are certainly the source of many of the carboxyl groups to be found at this cell surface. Others may be present in various forms of proteins as (i) 'C' terminal chain ends, (ii) glutamic or aspartic acids within protein chains or (iii) as these acidic amino acids at 'N' terminal chain ends. This latter suggestion would account for both the amino and carboxyl groups. Without accurate amino acid analysis of purified membrane fragments differentiation between these alternatives remains impossible.

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