Characterization of the Cell Coat of Ehrlich Ascites Tumor Cells†

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ABSTRACT: The spontaneous release of cell surface material from Ehrlich ascites tumor cells was examined by investigating the rate of release of surface-iodinated proteins from lactoperoxidase-labeled cells into isotonic buffer. Greater than 50% of the cell-associated radioactivity, including significant amounts of the major surface-iodinated proteins, was released from cells after 60 min at 4 °C. These experimental conditions allowed for maximal removal of surface proteins with minimal cell damage and were employed to obtain a subcellular fraction which was operationally defined as the cell coat (glycocalyx). The glycocalyx fraction was characterized by the presence of a highly active aminopeptidase (leucyl β-naphthylamidase) and large amounts of glycoproteins and glycosaminoglycans as revealed by the presence of protein, neutral carbohydrate, sulfate ester, uronic acid, and amino sugars. In marked contrast to purified plasma membranes, the glycocalyx fraction contained essentially no (Na^+, K^+)-ATPase activity and little or no sialic acid and cholesterol. 5′-Nucleotidase and alkaline phosphatase were present in low activity in Ehrlich cells and were distributed in both plasma membrane and glycocalyx fractions, although to a lesser extent in the latter material. Polyacrylamide gel electrophoresis of the glycocalyx fraction in the presence of detergent followed by "staining" the gel with radioiodinated concanavalin A revealed the presence of only one heavily stained band (mol/wt 130 000) and multiple lightly stained bands, whereas purified plasma membranes contained numerous heavily stained bands from 60 000 to 130 000 and at 300 000 approximate molecular weights. The data indicate that, under the appropriate conditions, Ehrlich ascites tumor cells rapidly and spontaneously shed a large portion of their cell surface while retaining cell viability. It is concluded that the glycocalyx layer can be routinely fractionated from cells and treated as a biochemically distinct entity from the surface membrane.

The study of plasma membrane fractions of animal cells in recent years has provided considerable biochemical information about the cell surface (Neville, 1975). In part, these studies have been directed at deducing something about the relationship of surface membrane components to cellular functions or properties attributed to the cell surface including various cell surface changes observed in certain disease states. Numerous differences in the cell periphery have been detected between malignant and normal cells including changes in the surface expression of glycoproteins (Hynes & Humphreys, 1974), glycolipids (Hakomori, 1973), and antigenic markers (Baldwin, 1973). Although purified plasma membranes have proven to be valuable in these studies, it has recently become apparent that components which comprise the exterior cell surface layer, often referred to as the cell coat or glycocalyx (Bennett, 1963; Luft, 1976), must be considered as candidates for certain cell surface functions as well as integral surface membrane components. For example, subcellular fractionation studies of NIL-8 cells revealed that a large transformation-sensitive (LETS)[1] glycoprotein which is abundant on the surfaces of untransformed fibrobasts is distributed primarily in a cell coat fraction with enzymic and chemical properties distinct from the plasma membrane (Graham et al., 1975). The location of the LETS glycoprotein external to the plasma membrane is consistent with the release or "shedding" of this surface antigen from chick embryo fibroblasts into the extracellular fluids (Vaheri & Ruoslahti, 1975).

Although the glycocalyx is not a permeability barrier to small molecules, this cell surface layer may act as a barrier toward antibodies, viruses, and other cells. In terms of cell–cell interactions, the components of the cell coat are of primary interest in processes like cell recognition, cell adhesion, and density-dependent inhibition of growth, since these components will first come into contact with each other as cell populations become crowded. Also, tumor-associated antigens, which are released spontaneously into body fluids and consequently may allow tumor cells to avoid immune recognition and destruction (Sjögren et al., 1972), are likely derived from this peripheral layer.

The glycocalyx is a very labile structure, particularly in malignant cells (Kim et al., 1975). It is therefore not surprising that many if not most cell coat components are lost during the isolation of plasma membranes (Luft, 1976; Graham et al., 1975). In addition, mechanical treatment of cells including repeated washings can remove glycocalyx components (Kilarski, 1975). For these reasons, we have undertaken to investigate the optimum conditions for the selective removal of the glycocalyx from viable cells in order to treat the cell periphery as a biochemically defined entity. If the glycocalyx layer can be isolated reproducibly as a fraction with a defined and characteristic composition, biochemical studies of cell surface components will be facilitated.

Materials and Methods

Cells. Ehrlich ascites tumor cells were maintained by intraperitoneal injection of 0.2 mL of the ascites fluid into Swiss male albino mice weighing 25 to 30 g at 8-day intervals. Tumor cells were harvested by collecting the ascites fluid in cold

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[1] Abbreviations used are: LETS, large transformation sensitive; NaCl-*P* buffer, Dulbecco's phosphate-buffered saline, pH 7.4; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; Con A, concanavalin A.

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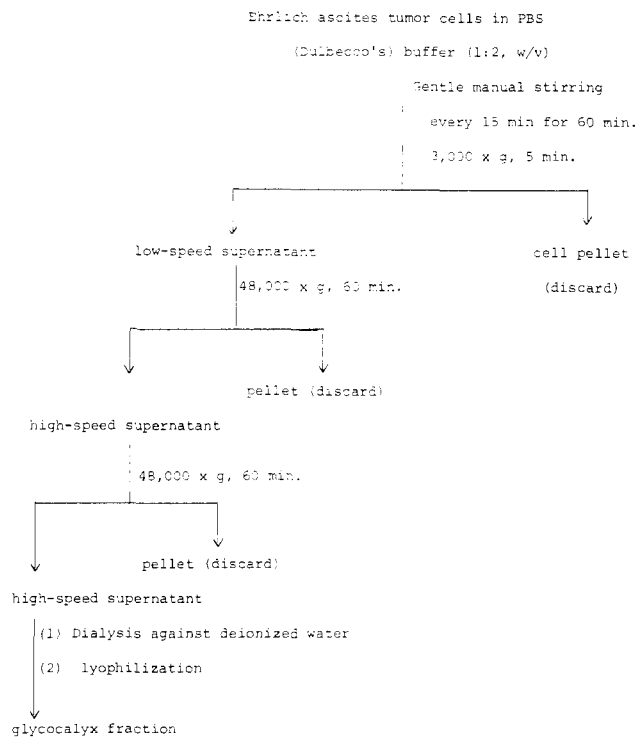

FIGURE 1: Scheme for the isolation of the glycocalyx fraction from Ehrlich ascites tumor cells.

Dulbecco's phosphate-buffered saline (NaCl-*P*, buffer), pH 7.4, and washing the cells five times by centrifugation at 400*g* to remove erythrocytes as previously described (Rittenhouse et al., 1976). The total number of tumor cells in 1 g of a packed, wet-weight pellet obtained by centrifugation at 4300*g* was calculated to be 9×10^8 cells using a hemocytometer chamber and contained 100 mg of protein.

Membrane Preparations. Plasma membranes were prepared from Ehrlich cell homogenates according to the method of Im et al. (1976). Cells were incubated in a hypotonic medium containing 15 mM sodium phosphate (pH 6.4), 1 mM $MgCl_2$, and 10 mM NaCl for 30 min before breakage of the cells with 25–30 strokes of a tight-fitting Dounce homogenizer. The homogenate was then fractionated by centrifugation at 270*g* for 1.5–2 min and the pellet collected. The pellet was suspended in 3 vol of 7.5 mM sodium phosphate (pH 7.4), containing 5 mM $MgCl_2$, 5 mM NaCl and 18% glycerol (v/v) and incubated briefly at 37 °C followed by filtration through cheesecloth. Centrifugation of the filtrate at 200*g* for 5 min yielded the "crude plasma membrane fraction". This material was suspended by passage through a 27 gauge needle five times. Ultracentrifugation at 25 000 rpm for 1 h of the crude plasma membrane fraction using a discontinuous glycerol density gradient (50, 60, 67, 74, and 83% glycerol) was performed and the 60–67% glycerol interfacial fraction (band 3) was collected as the plasma membrane fraction.

Preparation of Glycocalyx Fraction. The glycocalyx fraction was prepared as shown in Figure 1. All steps were performed at 4 °C to minimize metabolic and proteolytic activity. The cells were harvested from mice 9 days after inoculation and washed at least five times immediately before use. The dialysis and lyophilization steps were omitted for enzyme assays of the glycocalyx fraction since activities of some of the enzymes were significantly reduced by these procedures. Instead, the final supernatant fraction was dialyzed extensively against 50 mM Tris (pH 7.5) and 0.15 M NaCl to remove P_i. The dialyzed glycocalyx material was then concentrated in a minicon-B15 concentrator (Amicon Corporation) and assayed for the enzyme activities listed below.

Enzyme Assays. Adenosine triphosphatase (ATPase) activity was measured at 37 °C in the presence of 10 mM Tris-Cl buffer (pH 8.4) containing 0.05 mM EDTA, 1 mM $MgCl_2$, and 2 mM ATP (Wallach & Ullrey, 1964). The (Na^+,K^+)-dependent Mg^{2+} ATPase activity was calculated from the difference in P_i liberated in the presence and absence of 100 mM NaCl and 10 mM KCl. Inhibition by ouabain was not used in these experiments because at 1 mM it gave incomplete inhibition of the Na^+,K^+-stimulated activity as observed also by Wallach & Ullrey (1964) and Forte et al. (1973). Released P_i was measured as described by Lin and Morales (1977), with the modification that samples were kept on ice after the addition of the molybdate–metavanadate reagent and were read spectrophotometrically within 3 min. This was necessary to minimize hydrolysis of ATP under the acid conditions used in the assay. 5′-Nucleotidase (EC 3.1.3.5) activity was measured with 5′-AMP and β-glycerophosphate as substrates (Sigma Chemical Co.) according to the method of Riemer & Widnell (1975).

The difference between the amount of P_i released using the 5′-AMP substrate and the nonspecific phosphatase activity toward the β-glycerophosphate substrate was used as a measure of specific 5′-nucleotidase activity. Alkaline phosphatase (EC 3.1.3.1) was determined using β-glycerophosphate at pH 8.5 (Riemer & Widnell, 1975). Fresh preparations of the glycocalyx and plasma membrane fractions were used to assay 5′-nucleotidase and Na^+,K^+-ATPase activities since significant loss of enzyme was found after storage at either 4 or −20 °C. Succinate dehydrogenase (EC 1.3.99.1) was assayed by measuring the rate of reduction of 2,6-dichlorophenolindophenol spectrophotometrically at 600 nm as described by Green et al. (1955). Leucyl β-naphthylamidase (EC 3.4.11.1) was determined by incubating samples with L-leucyl-β-naphthylamide in NaCl-P buffer, pH 7.0, at 37 °C and measuring the resulting β-naphthylamine according to the method of Goldbarg & Rutenburg (1958). The reagents for this enzyme assay were obtained from Sigma Chemical Co. in the form of a kit for leucine aminopeptidase determination (Sigma Chemical Co. Technical Bulletin No. 251).

Chemical Determinations. Protein content was measured by the method of Lowry et al. (1951). Neutral carbohydrate was determined by the phenol–sulfuric acid reaction (Dubois et al., 1956). Total sialic acid was measured by the thiobarbituric acid assay after acid hydrolysis of samples (0.1 N H_2SO_4, 1 h at 80 °C) using *N*-acetylneuraminic acid as a standard by the method of Aminoff (1961). Free sialic acid of undialyzed glycocalyx preparations was estimated without prior hydrolysis by reaction with thiobarbituric acid (Aminoff, 1961). Total organic sulfate was released from samples by hydrolysis for 6 h at 100 °C with 1 N HCl. Total sulfate was determined by measuring turbidity of hydrolyzed samples in the presence of gelatin and $BaCl_2$ at 500 nm by the turbidimetric method of Dodgson & Price (1962) as modified by Kawai et al. (1969). Uronic acid was estimated by the carbazole method of Dische (1947) as modified by Bitter & Muir (1962) using glucuronolactone (Sigma Chemical Co.) as a standard. Glucosamine and galactosamine were determined using the long column of the automatic amino acid analyzer after hydrolysis of samples with 4 N HCl at 100 °C for 6 h (Spiro, 1972). Amino acid analysis was performed on the Beckman 120 amino acid analyzer after hydrolysis of samples in sealed ampules under N_2 for 24 h at 110 °C.

Phospholipids and cholesterol were extracted from membrane and glycocalyx preparations with chloroform–methanol

(2:1, v/v) at 22 °C for 24 h (Folch et al., 1957). Phospholipid phosphorus was analyzed by the method of Bartlett (1959) and a factor of 25 was used to convert μg of phosphorus to μg of phospholipid. Cholesterol content was determined by the *o*-phthalaldehyde reaction according to the procedure of Zlatkis & Zak (1969).

RNA and DNA were estimated by phosphate analysis after extraction of lipid from samples according to the Schmidt-Thannhauser procedure (Schmidt, 1957). DNA was also measured fluorometrically using bisbenzimid No. 33258 fluorochrome (American Hoechst, Bridgewater, Conn.) as described by Paigen et al. (1977).

Lactoperoxidase-Mediated Iodination. Ehrlich cells were iodinated by the lactoperoxidase method of Phillips & Morrison (1971) as previously described (Rittenhouse et al., 1973). All steps were carried out at 4 °C. Cells were harvested from mice 9 days after injection and washed 6× in NaCl-*P* buffer as described above. Lactoperoxidase (Sigma Chemical Co.) was added to a cell suspension containing 3×10^8 cells in 5 mL of NaCl-*P* buffer, pH 7.4, to make the final concentration of the enzyme 10^{-7} M. Carrier-free $Na^{125}I$ (1.0 mCi; Amersham) was added to the reaction mixture and 0.01-mL aliquots of a 10^{-5} M H_2O_2 solution were added at 2-min intervals for 15 min. The reaction was stopped by the addition of 10 vol of cold NaCl-*P* buffer and centrifugation of the cells at 400*g*. The labeled cells were washed three more times. Controls were performed by omitting the addition of H_2O_2 to cell suspensions containing lactoperoxidase and $Na^{125}I$.

Results

Release of Cell Surface Proteins. In order to determine the optimum conditions for "stripping" the cell coat from cells while avoiding cell damage and contamination by cytoplasmic components, the rate of release of surface-iodinated proteins from cells during incubation in NaCl-*P* buffer at 4 °C was studied by taking aliquots from the cell suspension periodically and assaying for the presence of radioactivity in the supernatant fractions. As shown in Figure 2 more than 50% of the radioactive material was released from cells into the supernate during incubation of a labeled cell suspension for 60 min. After a 60-min incubation period, the trypan blue dye exclusion test revealed that 8.0% of the treated cells were permeable to the dye compared to the presence of 6.5–7.0% stained cells in untreated controls indicating that cell viability was not significantly affected by this mild extraction treatment. Longer incubation periods in NaCl-*P* buffer at 4 °C resulted in an increased number of trypan blue stained cells (11.5–12.0% after 3 h) without a significant increase in released cell surface label (Figure 2). Throughout this article, the material released by cells incubated at 4 °C for 60 min in NaCl-*P* buffer as shown in Figure 1 is referred to as "glycocalyx fraction". The average yield of glycocalyx material from 5 separate preparations represented 4.5% by weight of total cell protein with a range of 3.0–5.8%. A typical preparation from 20.6 g of wet-weight packed cells yielded 144 mg of dry weight material with protein accounting for 83% of the weight.

It is possible that a significant amount of the cell-associated radioactivity is comprised by free ^{125}I. In order to assess the macromolecular nature of the components extracted from labeled cells with isotonic buffer, polyacrylamide gel electrophoresis in the presence of detergent (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed on both the cell pellet and supernatant fractions. Radioiodinated cells revealed 10–15 major iodinatable proteins ranging in apparent molecular weights from 30 to 130×10^3 (Figure 3) in general agreement with the surface labeling profile of

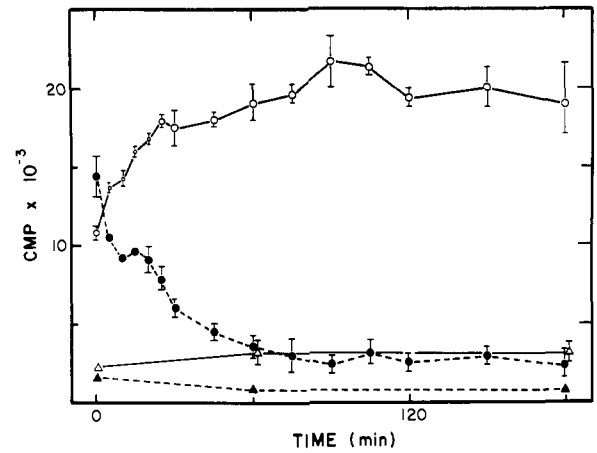

FIGURE 2: Release of ^{125}I-labeled surface components from Ehrlich ascites tumor cells. Cells were radioiodinated with lactoperoxidase as described in Materials and Methods. The radiolabeled cells were washed three times with Dulbecco's buffer (NaCl-*P* buffer) at 4 °C by collecting the cells after centrifugation at 500*g* and resuspending the cells in NaCl-*P* buffer. The cells were then incubated at 4 °C in NaCl-*P* buffer (1:2 wet weight volume). Aliquots (100 μL) were removed periodically and the high-speed supernatant (○—○) and cell pellet (●—●) fractions were measured for radioactivity in a liquid scintillation counter. High-speed supernatant (△···△) and cell pellet (▲···▲) control fractions were obtained from cells treated with lactoperoxidase and $Na^{125}I$ in the *absence* of H_2O_2 and incubated in NaCl-*P* buffer as described above. The error bars represent the range of two determinations.

Ehrlich Cells obtained by Gates et al. (1974). The labeled material near the top of the gel (Figures 3a–c) was not present in all gel runs and may represent highly aggregated proteins or labeled components which were insoluble during gel electrophoresis. Autoradiograms of material eluted from labeled cells by isotonic buffer revealed that significant amounts of the major surface-iodinated proteins were released from cells by mild buffer treatment (Figures 3a–c). However, incubation of labeled cells in NaCl-*P* buffer at 4 °C for 60 min is not sufficient to quantitatively remove any of the major cell surface iodinated proteins (Figures 3d–f). The release of some surface-iodinated proteins at 0 time (Figure 3a) merely reflects the several minutes of lag time required to collect and centrifuge the samples.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the glycocalyx fraction followed by staining with Coomassie blue revealed a disperse group of proteins ranging from 10 000 to greater than 200 000 apparent mol wt (Figure 4d). The major protein bands occurred in the approximate molecular weight range from 45 000 to 85 000 (Figure 4d). A comparison of the sodium dodecyl sulfate-gel profiles of the glycocalyx (Figure 4c) and plasma membrane fractions (Figure 4b) after staining the gels with radioiodinated Con A indicates that there are no major polypeptides unique to the glycocalyx. However, the plasma membrane fraction contained numerous bands heavily stained with radioiodinated Con A, particularly in the molecular weight range from about 60 000 to 130 000 and at 300 000, whereas the glycocalyx fraction contained only one very heavily stained band (mol wt 130 000) and multiple lightly stained bands (Figures 4b and c). Incubation of gels in the presence of 0.1 M α-methyl mannoside completely inhibited Con A labeling of proteins greater than mol wt 20 000 demonstrating the specificity of lectin binding (not shown). The mol wt 130 000 component of the glycocalyx, which is intensely stained with radiolabeled Con A (Figure 4c), is apparently the major Con A "receptor" spontaneously released from the surface of Ehrlich cells. In addition to dramatic differences in the macromolecular profile of plasma membranes and the

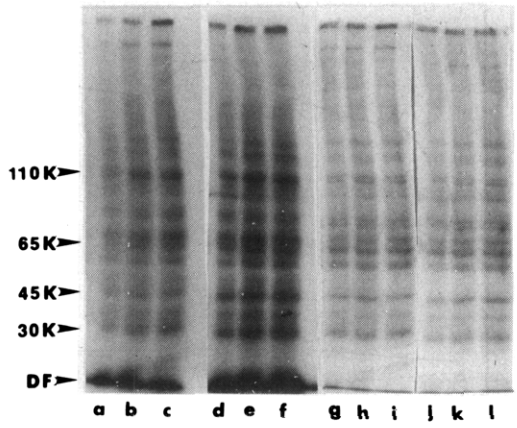

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of ^{125}I-labeled surface components of Ehrlich ascites tumor cells. Samples a, b, and c (autoradiography) and g, h, and i (Coomassie blue stain) represent gels of the total high-speed supernatant fractions from aliquots (100 μL) removed at 0, 30, and 60 min, respectively, from radiolabeled cell suspensions in Dulbecco's buffer (NaCl-*P*) at 4 °C as described in the legend to Figure 2. The high-speed supernates were concentrated tenfold in a Minicon-B15 concentrator (Amicon Corporation) before gel electrophoresis. Samples d, e, and f (autoradiography) and j, k, and l (Coomassie blue stain) represent the total cell pellets obtained after high-speed centrifugation of the aliquots removed at 0-, 30-, and 60-min time points, respectively. Cell pellets were prepared for electrophoresis by suspension of cells in sample buffer containing 0.1% sodium dodecyl sulfate for 1 min followed by centrifugation at 4500*g* for 10 min and the supernatant fraction was collected for electrophoresis. Equal amounts of radioactivity were present in all cell suspension samples before centrifugation and collection of supernatant and pellet fractions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) using a separating gel of 7.5% acrylamide and 0.2% methylenebis(acrylamide) gels containing 0.1% sodium dodecyl sulfate in the Tris-HCl system at pH 8.8. The stacking gels contained 3% acrylamide. The samples were dialyzed against sample buffer containing 0.1% 2-mercaptoethanol and heated at 100 °C for 5 min before electrophoresis. The slab gel was dried and radioactive bands detected with autoradiography by exposure with Kodak No-Screen medical x-ray film for 3 weeks. Molecular weight markers were: β-galactosidase (130 000), bovine serum albumin (68 000), ovalbumin (43 000), pepsin (35 000), trypsin (23 330) and cytochrome *c* (11 700) run in parallel with the samples on the same slab gel.

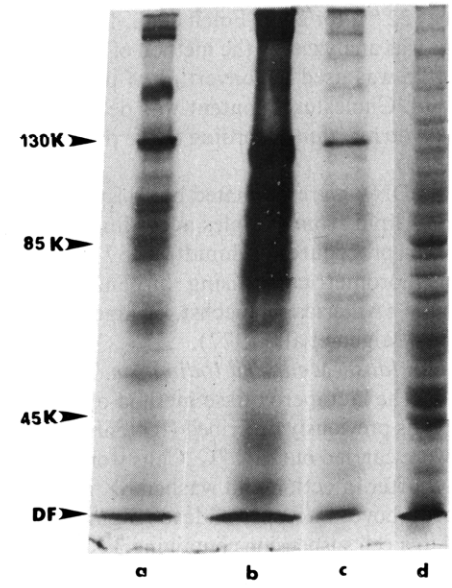

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of cell homogenate, purified plasma membrane, and glycocalyx fractions from Ehrlich ascites tumor cells. Gel electrophoresis was performed on 7.5% gels as described in the legend to Figure 3. The plasma membrane fraction was prepared by the method of Im et al. (1976). The glycocalyx fraction was prepared as described in Figure 1. Molecular weight estimates were made from the known molecular weights of the major proteins of human red blood cell membranes run on the same slab gel. After electrophoresis, the gel was stained with Coomassie blue. The gel was then "stained" with ^{125}I-labeled Con A according to the procedure of Burridge (1976). Con A was prepared from jack bean meal (Sigma Chemical Co.) by the method of Agrawal & Goldstein (1972) and radioiodinated by the chloramine T method (Hunter & Greenwood, 1962) in the presence of D-glucose. The radioiodinated Con A was repurified by affinity chromatography with Sephadex G-50 (Pharmacia). The slab gel was soaked in the presence of radioiodinated Con A (2×10^7 cpm/mg of protein) for 12 h at 22 °C. Human hemoglobin (Sigma Chemical Co.) at 1 mg/mL was included to avoid nonspecific lectin binding to the gel. After extensive washing, the gel was dried and autoradiographed by exposure with Kodak No-Screen Medical x-ray film for 12 h. Autoradiography of: (a) cell homogenate, 30 μg; (b) plasma membrane fraction, 50 μg; (c) glycocalyx fraction, 40 μg; (d) Coomassie blue stained gel of glycocalyx fraction, 40 μg.

glycocalyx fraction, analysis of the "Con A stained" gels of glycocalyx material reveals that many of the macromolecular components in this fraction are glycoproteins (Figure 4c).

A comparison was made of cells which were surface labeled by the lactoperoxidase technique before and after removal of the glycocalyx layer. The cell surface layer was "stripped" from cells by incubation in NaCl-*P* buffer for 60 min at 4 °C prior to radioiodination by lactoperoxidase. As can be seen in Figure 5, prior removal of cell surface material leads to labeled components which are more tightly bound to the cell surface, and although radioactive material is continuously released from these cells, the rate of release is somewhat slower than that from cells which contained intact cell coats before iodination (Figures 2 and 5). Even after a 3-h incubation period more than 50% of the radioactivity originally present in the cell pellet remained bound to cells (Figure 5).

Removal of the glycocalyx from cells does not appear to unmask a completely new class of surface proteins. However, several minor differences in the profiles of the iodinated proteins were apparent (Figures 3 and 6). Heavily labeled proteins at 110, 75, 65, and 45 $\times 10^3$ approximate molecular weights were revealed in the surface labeling profile of cells depleted of glycocalyx before iodination (Figures 6c and 6d), whereas iodinated cells with intact cell coats exhibited a more uniform surface labeling pattern (Figures 3d–f).

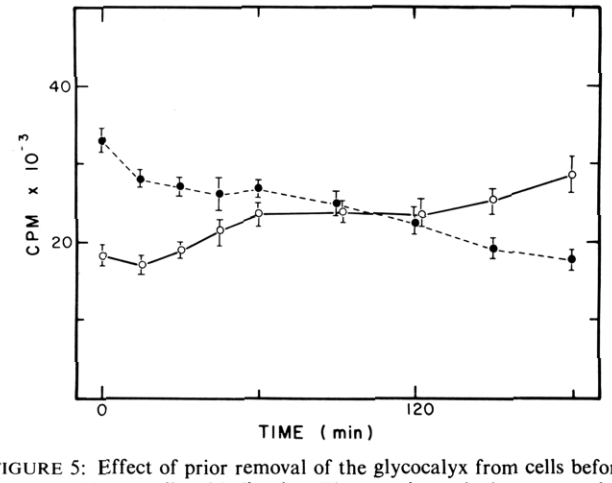

FIGURE 5: Effect of prior removal of the glycocalyx from cells before lactoperoxidase-mediated iodination. The experiment is the same as that described in the legend to Figure 2 except that the cells were incubated for 60 min in Dulbecco's buffer (NaCl-*P* buffer) at 4 °C immediately *before* the lactoperoxidase labeling procedure described in Methods and Materials. The radiolabeled cells were washed three times in cold NaCl-*P* buffer by centrifugation at 500*g* and resuspension in NaCl-*P* buffer. The cells were then incubated in NaCl-*P* buffer at 4 °C and aliquots were removed periodically in order to assay the high-speed supernatant (○) and cell pellet (●) fractions for radioactivity in a liquid scintillation counter. The error bars represent the range of two determinations.

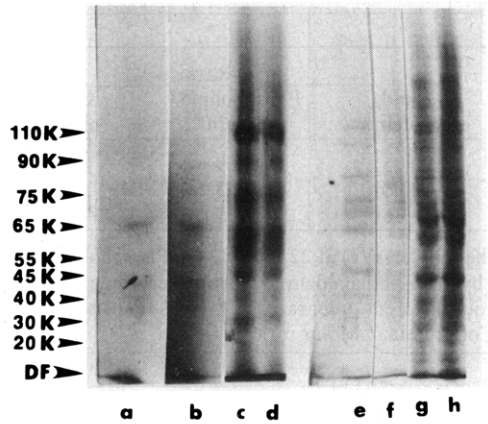

FIGURE 6: Surface radioiodination profiles of Ehrlich ascites tumor cells labeled with lactoperoxidase after removal of the glycocalyx from cells. Lactoperoxidase-mediated iodination, extraction of Ehrlich cell-surface components with Dulbecco's buffer (NaCl-*P*), sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and autoradiography were performed as described in Methods and Materials and in the legend to Figure 3. Cells were incubated for 60 min at 4 °C in Dulbecco's buffer (NaCl-*P*) *before* iodination with lactoperoxidase. Radiolabeled cells were washed three times in cold NaCl-*P* buffer by centrifugation at 500*g* and resuspension in NaCl-*P* buffer. Samples a and b (autoradiography) and e and f (Coomassie blue stain) represent gels of the total high-speed supernatant fractions (100 μL) obtained from radiolabeled cells incubated for 0 and 60 min, respectively in NaCl-*P* buffer at 4 °C. The high-speed supernatant fractions were concentrated tenfold in a Minicon-B15 concentrator (Amicon Corporation) before gel electrophoresis. Samples c and d (autoradiography) and g and h (Coomassie blue stain) represent the total cell pellets obtained after high-speed centrifugation of the aliquots removed at 0- and 60-min incubation periods, respectively, in NaCl-*P* buffer. Equal amounts of radioactivity were present in all cell suspension samples before centrifugation and collection of supernatant and pellet fractions.

TABLE I: Enzyme Analysis of Plasma Membrane and Glycocalyx Fractions.[a]

	Fraction		
	Homogenate[c]	Plasma membrane	Glycocalyx
(Na^+,K^+)-ATPase	0.28	6.44	<0.15
5′-Nucleotidase[b]	0.12	0.18	0.10
Alkaline phosphatase	0.08	0.28	0.11
Leucyl β-naphthylamidase	1.2	1.5	1.5
Succinate dehydrogenase	0.151	<0.007	<0.007

[a] The specific activities of the various enzymes are expressed in terms of μmol of substrate utilized h^{-1} (mg of protein)$^{-1}$ at 37 °C. At least two determinations were made for each enzyme activity and fraction. [b] The activity of 5′-nucleotidase was corrected for liberation of phosphorus by nonspecific phosphatase by determining the hydrolysis of β-glycerophosphate under the same assay conditions used for 5′-AMP as described in Materials and Methods. [c] The cell homogenate was prepared by incubating cells in a hypotonic medium containing 15 mM sodium phosphate (pH 6.4), 1 mM $MgCl_2$, and 10 mM NaCl for 30 min. The cells were broken with 25–30 strokes of a tight-fitting Dounce homogenizer.

Marker Enzymes. Several enzymes which have been used as marker enzymes for surface membranes are less concentrated or completely absent in the glycocalyx fraction (Table I). The most dramatic difference was the finding that the (Na^+,K^+)-stimulated ATPase activity was highly enriched in plasma membranes with little or no specific activity detected in glycocalyx preparations (Table I). In contrast, both 5′-nucleotidase and alkaline phosphatase were present in both plasma membranes and glycocalyx material although with significantly lower specific activities in the latter fraction (Table I). In agreement with the work of Molnar et al. (1969), the overall cellular 5′-nucleotidase activity of Ehrlich cells was quite low and in some experiments little or no activity could be detected. Leucyl β-naphthylamidase was present in high activity in both glycocalyx and plasma membrane fractions (Table I). The absence of significant activity of succinate dehydrogenase in the glycocalyx fraction indicates that cell breakage or leakage of internal membrane enzymes were not major sources of enzymic activities found in the glycocalyx fraction (Table I).

TABLE II: Amino Acid Composition[a] of Plasma Membrane and Glycocalyx Fractions of Ehrlich Ascites Tumor Cells.

	Fraction	
Amino acid	Plasma membrane[b]	Glycocalyx[b]
Lys	10.9	8.3
His	7.2	2.2
Arg	4.7	5.3
Asp	7.4	10.9
Thr	5.8	5.4
Ser	8.0	5.5
Glu	5.6	12.9
Pro	1.3	5.9
Gly	9.1	7.8
Ala	8.2	7.5
Val	8.2	6.7
Met	1.5	1.2
Ile	5.6	5.1
Leu	10.0	9.2
Thr	2.2	2.6
Phe	4.4	3.7

[a] Molar ratio per 100 amino acid residues. [b] Samples (1–2 mg) were suspended in 6 M HCl and hydrolyzed for 24 h at 110 °C.

TABLE III: Chemical Composition of Plasma Membrane and Glycocalyx Fractions.

	Fraction		
Component (μg/mg of protein)	Homogenate	Plasma membrane	Glycocalyx
Neutral carbohydrate	34.8[b]	62.3[b]	53.4[c]
Total carbohydrate	43.9	91.4	60.4
Hexosamines	4.3[b]	16.1[b]	6.2[b]
Galactosamine[a]	24%	<3%	42%
Glucosamine[a]	76%	>97%	58%
Sialic acid			
Total	4.8[b]	13.0[b]	0.8[c]
Free			<0.2
Total sulfate	3.8[b]	17.8[b]	9.1[c]
Uronic acid	5.3[b]	24.7[b]	8.0[c]
RNA	157[b]	61[b]	12[c]
DNA	78[b]	8[b]	<3[c]

[a] Calculated as percent of total amino sugar. [b] Average of two different preparations. [c] Average of four different preparations.

Chemical Composition. Protein represented most of the material in the glycocalyx fraction accounting for from 80 to 90% by weight. The amino acid compositions of the glycocalyx and plasma membrane fractions were compared and significant differences were found (Table II).

The total carbohydrate content was decreased in the glycocalyx material with a calculated ratio of carbohydrate to protein by weight in the glycocalyx fraction of 0.06 compared to a ratio of 0.09 found in plasma membranes (Table III). A more dramatic difference in carbohydrate composition of these two fractions was the finding that the amino sugar content of purified plasma membranes is represented solely by glucosamine, whereas the glycocalyx contained both galactosamine

TABLE IV: Lipid Composition of Plasma Membranes and Glycocalyx Fractions.[a]

Fraction	Phospholipid (mg/mg of protein)	Cholesterol (mg/mg of protein)	Ratios		
			Lipid/protein[d]	Phospholipid/protein[e]	Cholesterol/phospholipid[f]
Homogenate	0.143[b]	0.025[b]	0.25	0.19	0.35
Plasma membrane	0.411[b]	0.141[b]	0.90	0.53	0.69
Glycocalyx	0.27[c]	0.005[c]	0.36	0.35	0.04

[a] Lipids were extracted from Ehrlich ascites cell fractions with chloroform-methanol (2:1, v/v) at 22 °C for 24 h. Phospholipid phosphorus was analyzed by the method of Bartlett (1959). Cholesterol content was determined by the *o*-phthalaldehyde reaction according to the procedure of Zlatkis & Zak (1969). [b] Average of two different preparations. [c] Average of four different preparations. [d] The sum of phospholipid and cholesterol; μmol of lipid per mg of protein. [e] Micromoles of phospholipid per mg of protein. [f] Molar ratio.

(42%) and glucosamine (58%) as shown in Table III.

The glycocalyx fraction contained appreciable quantities of uronic acid, sulfate ester, and amino sugars although these components were more concentrated in plasma membranes (Table III). A more striking difference in distribution of carbohydrate between the two fractions was observed for sialic acid content. As expected, plasma membranes contained relatively large quantities of bound sialic acid with an enrichment of almost threefold compared to the cell homogenate (Table III). In marked contrast bound sialic acid was almost completely absent in the glycocalyx fraction (Table III). In addition, no free sialic acid was detected in the glycocalyx after analysis of the fraction before dialysis (Table III).

Measurements of phospholipid and cholesterol content showed large differences in lipid distribution between the glycocalyx and plasma membrane fractions (Table IV). The phospholipid content of plasma membranes was almost twice the value found in the glycocalyx fraction (Table IV). An even more dramatic difference in lipid distribution between the two fractions was the almost complete absence of cholesterol in the glycocalyx (Table IV). The weight ratio of phospholipid to protein and molar ratio of cholesterol to phospholipid were calculated to be 0.53 and 0.69, respectively, for the plasma membrane fraction and were in the expected ranges for plasma membranes for Ehrlich ascites tumor cells reported in previous investigations (Molnar et al., 1969; Nachbar et al., 1976). In contrast, the phospholipid/protein (0.35) and cholesterol/phospholipid (0.04) ratios of the glycocalyx fraction varied significantly from reported values for plasma membranes (Table IV).

The very low levels of DNA detected in either the glycocalyx or plasma membrane fractions indicate the absence of significant contamination of these preparations by internal cellular components (Table III).

The plasma membrane fraction contained significant quantities of RNA, although the amount was much reduced compared to the cell homogenate (Table III). The RNA content of the plasma membrane fraction is consistent with the association of RNA with Ehrlich cell plasma membranes as shown by Juliano et al. (1972). The RNA present in the glycocalyx fraction (less than 5% of the RNA content of the cell homogenate; Table III) may represent the release of intracellular RNA during the extraction of the glycocalyx from cells in vitro. Alternatively, RNA from the extracellular fluids from damaged cells may have become attached to the surface of Ehrlich cells growing in vivo.

Discussion

In these studies, we have determined the optimal conditions for removal of surface-iodinated proteins with minimal cell damage in order to provide an operational definition of the cell coat or glycocalyx fraction of Ehrlich ascites tumor cells. An operational definition is necessary since the exact boundaries of the cell surface and plasma membrane have proven difficult to define in the past either morphologically or biochemically (Luft, 1976).

It is apparent from the present data that many of the surface glycoproteins of Ehrlich ascites tumor cells, which are labeled by the lactoperoxidase-iodination procedure, can be partially released from viable cells by mild elution with cold isotonic buffer (Figure 3). It should be mentioned that significant labeling of internal proteins is possible when dead cells are present during lactoperoxidase-iodination experiments (Gates et al., 1974). In the present study, dead cells were not removed after iodination, for example with Ficoll-Hypaque gradients, since this procedure would likely lead to the loss of iodinated peripheral proteins. However, several observations are consistent with the cell-surface localization of the major iodinated proteins of Ehrlich cells shown in Figure 3. The majority of the incorporated label was found to reside in approximately six proteins ranging in molecular weight from 30 000 to 110 000 (Figure 3). In contrast, the random iodination of intracellular proteins would have been expected to result in heavy labeling of a larger number and wider molecular weight range of proteins. Also, it can be seen in Figure 3 that each of the six major iodinated proteins can be partly removed from whole cells at very short time intervals in NaCl-*P* buffer at 4 °C. In addition, recent immunofluorescent studies of viable Ehrlich tumor cells using antisera raised in rabbits against antigens in the Ehrlich cell glycocalyx fraction have confirmed the cell surface localization of the major proteins in this fraction (H. G. Rittenhouse, manuscript in preparation).

The release of cell-surface glycoproteins into culture media or isotonic buffer has been reported by several investigators for a number of different animal cell types. Tumor-associated antigens from both mouse B-16 melanoma (Poskitt et al., 1976) and human melanoma cells (Stuhlmiller & Seigler, 1977) have been found to be released spontaneously into tissue culture medium. Truding et al. (1975) have found that three major molecular weight classes of glycoproteins at 87 000, 66 000, and 55 000 were released from murine neuroblastoma cells after a 2-h incubation in culture medium. At least one of these released glycoproteins had previously been shown to be available for lactoperoxidase-catalyzed iodination (Truding et al., 1974).

However, the release of lactoperoxidase-iodinated proteins from mouse L cells into culture medium has been shown to be highly temperature dependent and is completely inhibited by reducing the temperature to 4 °C (Hubbard & Cohn, 1975). In agreement with the work of Hubbard & Cohn (1975), we have found that incubation of mouse LM cells in NaCl-*P* buffer for 60 min at 4 °C releases less than 5% of the protein eluted from Ehrlich ascites tumor cells under the same conditions (Dr. L. Takemoto, unpublished work). Therefore, ef-

fective extraction of glycocalyx material from cells with isotonic buffer at low temperature may be restricted to a few special cell types, while higher incubation temperatures may be necessary in most cases. In this regard, tumor cells grown in the ascites form may be particularly suitable for the facile removal of the cell periphery since these cells contain very labile surface coats and have been shown to readily release glycoproteins into culture media (Molnar et al., 1965; Dorval et al., 1976).

Comparisons of enzymic activities of the glycocalyx and plasma membrane fractions clearly reveal both qualitative and quantitative differences between these two fractions (Table I). In particular, the (Na^+,K^+)-Mg^{2+}-ATPase, an integral membrane protein, is present only in the plasma membrane fraction with little or no activity in the glycocalyx material. 5′-Nucleotidase and alkaline phosphatase were present in the glycocalyx fraction although in low activities. At this time, the only enzyme that has been found in high specific activity in the glycocalyx fraction of these cells is an aminopeptidase (leucyl β-naphthylamidase).

Work in other laboratories indicates that cell-surface aminopeptidase (Vannier et al., 1976) and 5′-nucleotidase (Riemer & Widnell, 1975) activities are associated with integral plasma membrane proteins in normal tissues. The partial removal of these two enzymes from viable Ehrlich cells by mild isotonic buffer treatment in this study may reflect a more labile surface coat in malignant cells as compared to normal cells. The lability of cell-surface coats has been correlated in some instances with malignancy (Luft, 1976) and Kim et al. (1975) have reported that several plasma membrane marker enzymes including 5′-nucleotidase seem to dissociate from the membrane into the blood of metastasizing tumor-bearing rats. One mechanism for the facile release of these surface enzymes may be the selective release of surface proteins by endogenous proteases during the incubation of cells in isotonic buffer. Proteolytic cleavage at susceptible bonds near the "footpad" regions where surface glycoproteins are partially embedded in the lipid matrix (Figure 7) could result in the release of glycoproteins in a relatively intact and functional state. Proteolytic cleavage of cell-surface proteins could also explain the relatively large number of Coomassie blue stained proteins in the glycocalyx fraction after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 4). In this regard, Vannier et al. (1976) have shown that an enzymatically active, hydrophilic part of an aminopeptidase can be released from intestinal brush border membranes by papain treatment leaving a small hydrophobic peptide of 8000 to 10 000. The loss of relatively small hydrophobic peptide fragments would not necessarily be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. However, extensive and random proteolytic degradation of the cell-surface fraction of Ehrlich cells would seem to be ruled out by the presence of major high molecular weight proteins, which are detected either by Coomassie blue or radioiodinated Con A after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 4).

An asymmetrical distribution of a variety of surface membrane markers has been found in several investigations of plasma membrane subfractions. These include the surface antigen and (Na^+,K^+)-ATPase of Ehrlich ascites tumor cells (Wallach, 1967) and 5′-nucleotidase, leucine aminopeptidase, and (Na^+,K^+)-ATPase in rat liver (Evans, 1970) and NIL-8 fibroblasts (Graham et al., 1975). With regard to the striking differences in sialic acid and cholesterol content of the glycocalyx and plasma membrane fractions found in the present study (Tables III and IV), it is interesting to note that little or no sialic acid or cholesterol was also found in a cell coat fraction

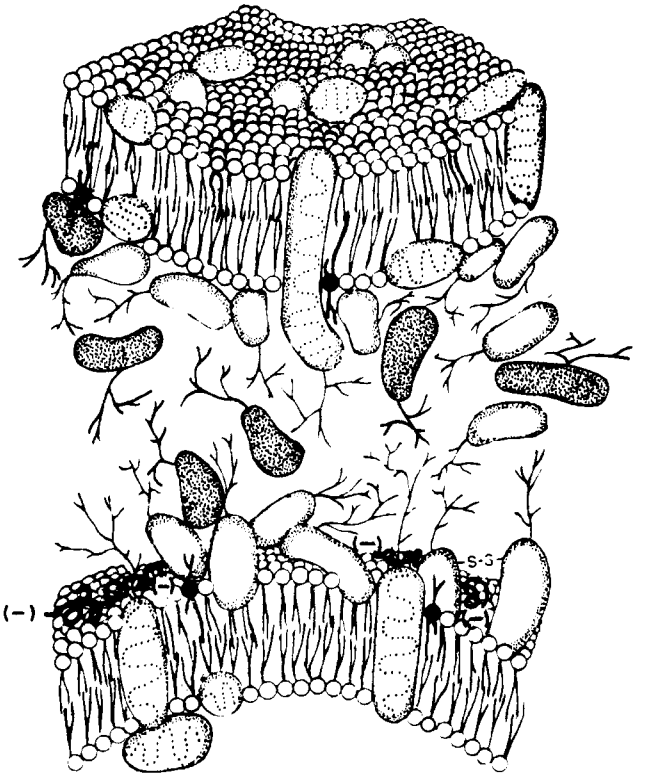

FIGURE 7: Schematic representation of the surface membranes and glycocalyx layer of two nucleated animal cells. In this scheme, three main regions of surface membrane can be delineated: (i) peripheral membrane proteins on the inner side of the membrane; (ii) integral membrane proteins which are embedded deeply in the lipid bilayer or span the bilayer; and (iii) glycoproteins and glycosaminoglycans located either external to the membrane or which contain a small "footpad" region embedded in the exterior part of the membrane. The peripheral glycoproteins and negatively charged glycosaminoglycans on the cell surface (glycocalyx layer) which are depicted as bound to integral membrane proteins and/or embedded partially into the lipid bilayer are thought to represent cell-surface components which can be removed from cells by mild extraction with isotonic buffer. A few serum glycoproteins (shaded moieties) are shown either bound directly to the plasma membrane proper or indirectly to the membrane by attachment to outer peripheral membrane glycoproteins. Cell-surface glycoproteins, which have been "shed" from the glycocalyx layer, are shown in the extracellular fluid. Glycolipids are shown as part of the lipid matrix of the plasma membrane.

from NIL-8 fibroblast membranes (Graham et al., 1975). Also, Glick (1974) has reported that glycoproteins, which are loosely associated with hamster fibroblast surface membranes and released into the culture media in large amounts, contain less sialic acid than more tightly cell-bound glycoproteins.

The heterogeneity of plasma membrane subfractions may represent, in some cases, different specialized regions of plasma membrane as seen in the morphologically different regions of the liver cell membrane (Neville, 1974). In addition, it has been shown for some cells that the outer and inner monolayer portions of the plasma membrane are biochemically distinct (Rothman & Lenard, 1977). A third possible explanation for the isolation of heterogeneous subcellular fractions derived from the cell surface is the presence of two separable biological layers at the cell surface, the plasma membrane proper and the glycocalyx. The distinguishing biochemical and enzymatic properties of the glycocalyx fraction strongly suggest that this material is not derived from plasma membrane fragments. Although vesicles from a very specialized portion of the surface membrane cannot be ruled out as the source of the material removed by isotonic buffer, we feel that a more attractive working hypothesis is that this mild extraction procedure re-

moves a layer of cell-surface glycoproteins which bind with various looseness of attachment to the external portion of the plasma membrane. The extraction of glycocalyx components by isotonic buffer corresponds to properties of peripheral membrane proteins as defined by Singer & Nicolson (1972) in the fluid mosaic model of biological membranes. Accordingly, the model in Figure 7 depicts a glycocalyx layer composed of external glycoproteins, some of which may be partially embedded into the lipid bilayer.

The finding that glucosamine is the only amino sugar in purified plasma membranes in our studies agrees with the work of Molnar (1967), who reported the presence of glucosamine and absence of galactosamine in microsomal fractions of Ehrlich cells. By contrast, Cook et al. (1965) have reported the presence of galactosamine in a microsomal fraction of Ehrlich cells. The presence of galactosamine in the glycocalyx fraction (Table III) is consistent with the data of Langley and Ambrose (1967) who reported galactosamine-containing mucopeptides released from Ehrlich cells by trypsin. The absence of galactosamine in purified plasma membranes, in spite of the presence of this amino sugar in the glycocalyx of Ehrlich cells, is further evidence that some cell coat components are quantitatively lost during the isolation of surface membranes.

The presence of relatively large amounts of amino sugars, uronic acids, and organic sulfate in the glycocalyx fraction indicates that a significant portion of the glycosaminoglycans of Ehrlich cells is located at the cell periphery. Although the evidence on this point is still rather limited, it would therefore seem that, in Ehrlich ascites tumor cells, negative charge in the form of bound sialic acid is associated primarily with integral membrane components, while a large portion of the negatively charged glycosaminoglycans are associated in a more peripheral manner to the membrane. Recent studies of glycosaminoglycans have shown these substances to be present on the cell surfaces of a wide variety of cultured animal cells (Kraemer, 1971) and they have been implicated in cellular adhesion processes (Culp, 1976). It is quite possible that the glycocalyx fraction obtained from Ehrlich cells grown in vivo will prove to be similar in some respects to the substrate-attached material remaining on tissue culture substrates after dissociation of cells in vitro from the substrate by treatment with EGTA (Culp, 1976). The presence of glycosaminoglycans and surface glycoproteins in the Ehrlich cell glycocalyx fraction is consistent with this idea.

The external location and lability of the glycocalyx make this cell surface layer a likely source of antigens which are "shed" during growth of tumor cells in vivo, thereby allowing tumor survival by interfering with the host's immune system (Sjögren et al., 1972). In accord with this possibility, we have found that specific antisera raised in rabbits against antigens in the glycocalyx fraction of Ehrlich ascites tumor cells recognize circulating antigens in ascites fluid from tumor-bearing mice (H. G. Rittenhouse, manuscript in preparation). Also, recent immunochemical studies have revealed that specific serum components remain tightly bound to the surface of Ehrlich ascites tumor cells grown in vivo even after extensive washing (H. G. Rittenhouse, manuscript in preparation). Therefore the glycocalyx layer is perhaps best thought of as a dynamic structure with no sharp boundaries between it and the lipid matrix of the plasma membrane on the one side and the external environment on the other side.

The facile isolation of a biochemically characterized fraction from the cell periphery which is distinct from the plasma membrane should allow systematic studies regarding the biochemical structure and functional significance of antigens which are released from cells during tumor growth in vivo. In addition, the macromolecular profiles and biochemical compositions of glycocalyx fractions from transformed cells and their untransformed counterparts may provide information about cell-surface alterations in malignant cells, which are not readily revealed by studies of plasma membrane preparations.

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Surface Areas of Lipid Membranes†

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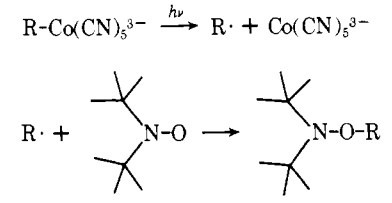
ABSTRACT: Upon photolysis, alkyl pentacyanocobaltate complexes generate alkyl radicals which react rapidly and specifically with nitroxide radicals, and which do not penetrate phospholipid bilayers. By measuring the loss of paramagnetic resonance signal intensity when multilamellar liposomes containing a small amount of spin-labeled lipid are exposed to these radicals, we have measured the proportion of lipid on the external surface of liposomes. We have shown that liposomes prepared under specified conditions from dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and binary mixtures of dipalmitoylphosphatidylcholine and cholesterol all have the same proportion of external lipid.

In recent work we have studied antibody binding to lipid hapten-sensitized liposomal membranes as a function of host lipid composition (Brûlet et al., 1977; Brûlet & McConnell, 1976, 1977; Humphries & McConnell, 1977). Analogous studies were made of antibody-dependent complement fixation as a function of host lipid composition (Humphries & McConnell, 1975, 1977; Brûlet & McConnell, 1976, 1977). The quantitative interpretation of these experiments has been hampered by uncertainties concerning the number of haptens exposed to antibody on the external surface of these liposomal structures. This type of problem is in fact encountered in a wide variety of quantitative biophysical and biochemical studies of liposomes and lipid vesicles.

In recent work it has been shown that photo-chemical fragments of alkyl pentacyanocobaltates react rapidly and essentially specifically with the free radical nitroxide group of nitroxide spin labels according to (Sheats & McConnell, 1977):



As shown in the present paper, this affords an accurate, sensitive, and rapid spectroscopic method for determining the number of spin-label haptens on the external surfaces of liposomes. The number of externally exposed haptens is a measure of the external surface area of the liposomes.

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