References

Alfsen, A., Chiancone, E., Wyman, J., and Antonini, E. (1970), *Biochim. Biophys. Acta 200*, 76.

Barnett, D. R., Lee, T. H., and Bowman, B. H. (1972), *Biochemistry 11*, 1189.

Beychok, S., Tyuma, I., Benesch, R. E., and Benesch, R. (1967), *J. Biol. Chem. 242*, 2460.

Black, J. A., Chan, G. F. Q., Hew, C. L., and Dixon, G. H. (1970), *Can. J. Biochem. 48*, 123.

Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., and Finazzi Agro, A. (1968), *J. Mol. Biol. 34*, 347.

Cleve, H., Bowman, B. H., and Gordon, S. (1969), *Humangenetik 7*, 337.

Crumpton, M. J. (1966), *Biochem. J. 100*, 223.

Crumpton, M. J. (1972), *in* Protein–Protein Interactions, Jaenicke, R., and Helmreich, E., Ed., New York, N. Y., Springer-Verlag, p 395.

Geraci, G., Parkhurst, L. J., and Gibson, Q. H. (1969), *J. Biol. Chem. 244*, 4664.

Gibson, Q. H., and Antonini, E. (1963), *J. Biol. Chem. 238*, 1384.

Greer, J. (1971) *J. Mol. Biol. 59*, 107.

Herman-Boussier, G., Moretti, J., and Jayle, M. F. (1960), *Bull. Soc. Chim. Biol. 42*, 837.

Javaherian, K., and Beychok, S. (1968), *J. Mol. Biol. 37*, 1.

Javid, J., Fisher, D. A., and Spaet, T. H. (1959), *Blood 14*, 683.

Kabat, E. A., and Wu, T. T. (1973), *Biopolymers 12*, 751.

Makinen, M. W., Milstein, J. B., and Kon, H. (1972), *Biochemistry 11*, 3851.

Nagel, R. L., and Gibson, Q. H. (1971), *J. Biol. Chem. 246*, 69.

Nagel, R. L., and Gibson, Q. H. (1972), *Biochem. Biophys. Res. Commun. 42*, 959.

Nagel, R. L., Rothman, M. C., Bradley, T. B., Jr., and Ranney, H. M. (1965), *J. Biol. Chem. 240*, PC 4543.

Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219*, 131.

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta 30*, 605.

Waks, M., and Alfsen, A. (1966), *Arch. Biochem. Biophys. 113*, 304.

Waks, M., Alfsen, A., Schwaiger, S., and Mayer, A. (1969), *Arch. Biochem. Biophys. 132*, 268.

Waks, M., Kahn, P. C., and Beychok, S. (1971), *Biochem. Biophys. Res. Commun. 45*, 1232.

Waks, M., Yip, Y. K., and Beychok, S. (1973), *J. Biol. Chem. 248*, 6462.

Yip, Y. K., Waks, M., and Beychok, S. (1972), *J. Biol. Chem. 247*, 7237.

Surface Glycoproteins of Mouse L Cells†

Richard C. Hunt* and Jay C. Brown

ABSTRACT: The surface glycoproteins of mammalian cells may be distinguished from internal glycoproteins by the fact that they are: (a) isolated with the smooth membrane fraction of cell homogenates, (b) degraded when intact cells are treated with proteolytic enzymes, and (c) labeled with reactive chemical groups which do not penetrate the cell membrane. By these criteria two molecular weight classes of glycoproteins, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, have been identified as components of the plasma membrane of mouse L-929 cells. Both are isolated with the smooth membrane fraction of cells lysed by Dounce homogenization. both are digested when cells are exposed to trypsin, and both are labeled by two methods, the pyridoxal phosphate–sodium borotritide system and the galactose oxidase–potassium borotritide method, which are designed to introduce radioactive label specifically into cell surface structures. The two glycoprotein classes have apparent molecular weights of approximately 100,000 and 50,000, respectively, on sodium dodecyl sulfate–polyacrylamide gels and both may contain more than one species of glycopolypeptide chain.

Current research on the surface glycoproteins of mammalian cells has been directed toward answering very basic questions. One wants to know, for instance, how many species of glycoproteins are found on the surface of particular cell types and how different cell types compare in their content and distribution of glycoprotein. How is the carbohydrate portion of these glycoproteins arranged on the polypeptide backbone and how is the whole structure associated with the lipid portion of the plasma membrane?

Studies of this type have been most thorough in the case of human erythrocytes which are found to have a single major glycoprotein species associated with the plasma membrane. This glycoprotein has been isolated in pure form and found to contain M and N human blood group specificities (Winzler, 1969; Marchesi *et al.*, 1972). The N-terminal portion of this glycopolypeptide contains all the carbohydrate and is located on the external surface of the cell while the C-terminal portion is devoid of carbohydrate and is thought to extend through the lipid bilayer to the interior surface of the cell (Segrest *et al.*, 1973; Marchesi *et al.*, 1972; Bretscher, 1971). Similar, but less extensive, studies have been carried out on other cell types. For example, glycoprotein components have now been characterized from the plasma membranes of human platelet (Phillips, 1972; Nachman *et al.*, 1973), rat liver (Glossmann and

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Neville, 1971), rat kidney brush border (Glossmann and Neville 1971), mouse liver (Evans, 1970), and HeLa cells (Atkinson and Summers, 1971).

We have examined the surface glycoproteins of cultured mouse L cells with the intention of answering the above basic questions for this cell type. Here we show how cellular glycoproteins can be resolved into classes by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. Criteria are then adopted for distinguishing between cell surface or plasma membrane associated glycoproteins and intracellular non-surface glycoproteins. By these criteria L cells are found to contain two molecular weight classes of glycoproteins which qualify as cell surface components.

Materials and Methods

Cell Growth. Monolayer cultures of mouse L-929 cells were grown in 100-mm Falcon plastic tissue culture plates in Eagle's minimal essential medium containing 10% calf serum and antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of fungizone) at 37° in a humidified atmosphere of 95% air, 5% CO_2. The cell doublng time was approximately 23 hr under these conditions and cells were subcultured by trypsinization every 2–3 days. Cellular proteins were radioactively labeled by including 2 μCi/ml of a ^{14}C-labeled amino acid mixture (Calatomic Chlorella hydrolysate, 2 mCi/mg) in the growth medium. Glycoproteins were labeled selectively by growing cells in the presence of either 4 μCi/ml of D-[^3H]-glucosamine (Amersham, 1.9 Ci/mmol) or 1 μCi/ml of D-[^{14}C]-glucosamine (New England Nuclear, 56 mCi/mmol). Glucosamine was found to be superior to mannose, mannosamine, or fucose as a metabolic source of radioactivity for glycoproteins. Cells were grown for 48 hr in the presence of radioactive precursors to a final density of approximately 3×10^6 cells per plate and harvested by scraping the monolayer with a rubber policeman. Harvested cells were ordinarily washed twice with Dulbecco's phosphate-buffered saline (PBS) before further operations were performed.

Sodium Dodecyl Sulfate–Polyacrylamide Gels. Whole cells and cell fractions to be analyzed by polyacrylamide gel electrophoresis were first dissolved in 100 μl of a solution of 1% sodium dodecyl sulfate containing 1% 2-mercaptoethanol and placed in a boiling water bath for 2 min. Glycerol (20 μl) was added to the cooled sample to increase its density. Between 2×10^6 and 5×10^6 cells solubilized in this way could be analyzed on a single gel. Polyacrylamide gels (7%) were prepared according to the method of Davis (1964) except that the stacking and separating gels contained 0.1% sodium dodecyl sulfate. The electrode buffer contained 3.0 g of tris(hydroxymethyl)aminomethane and 14.4 g of glycine per liter of 0.2% sodium dodecyl sulfate. The gels were 80 mm long by 5 mm in diameter and were run at 0.5 mA/gel until the tracking dye (Bromophenol Blue) was approximately 20 mm from the bottom (anode) end of the gel. This took approximately 18 hr. Gels were calibrated for molecular weight using the following standard proteins: thyroglobulin (mol wt 167,000), catalase (mol wt 57,000), pepsin (mol wt 35,000), hemoglobin (mol wt 17,000), and lysozyme (mol wt 14,000).

Gels were frozen after electrophoresis and sliced into 1-mm thick disks with a Mickle gel slicer; 0.5 ml of a 9:1 (v/v) mixture of NCS tissue solubilizer (Amersham) and distilled water was added to each slice in a counting vial. Vials were then heated in a water bath at 60° for 2 hr, cooled, and diluted with 10 ml of toluene-based scintillation fluid. These were counted in a Packard 3320 liquid scintillation spectrometer. When samples contained both ^{14}C and ^3H the results were corrected for 10% "spillover" of ^{14}C counts in the ^3H channel.

Preparation of Smooth Membranes. Smooth membranes and other subcellular fractions were prepared as described for L cells by Wagner *et al.* (1972). Between 5×10^6 and 10^7 labeled cells were harvested, suspended in 4 ml of reticulocyte standard buffer (RSB) consisting of 0.01 M Tris-HCl (pH 7.5), 0.01 M NaCl, and 0.0015 M $MgCl_2$, and homogenized briefly in a 7-ml Dounce homogenizer. Whole cells and nuclei were removed from the homogenate by centrifugation for 5 min at 600*g*. The homogenate was then layered on a 25-ml 0–60% discontinuous sucrose gradient and sedimented at 70,000*g* for 10 hr at 4° in a Spinco SW-25.1 rotor. After centrifugation four light scattering bands of cellular material were clearly visible in the gradient and these were collected separately by side puncture of the centrifuge tube. At this stage an aliquot of each fraction was taken to determine the total radioactivity present. The remainder of each fraction was diluted tenfold with RSB and pelleted by centrifugation at 80,000*g* for 1 hr in a Spinco type 30 rotor. Pellets were then solubilized for sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described.

Trypsinization of Cells. Monolayer cultures of L cells were grown as usual in the presence of D-[^3H]glucosamine and washed free of growth medium with PBS. Each plate was then treated with 3.5 ml of 1% (w/v) trypsin (EC 3.4.4.4, Sigma Chemical Co., type III twice recrystallized) in Earle's balanced salt solution (BSS) for 30 min at room temperature. Trypsinized cells detached from the plate and were harvested by centrifugation while control cells which were incubated with BSS only were harvested by scraping.

Cell Surface Labeling with Pyridoxal Phosphate and NaB^3H_4. A modification of the method of Rifkin *et al.* (1972) was used to label free amino groups on the cell surface with pyridoxal phosphate and NaB^3H_4. Monolayer cultures of L cells were grown in 100-mm plastic plates to a density of 3×10^6 cells per plate, washed free of medium, and allowed to react with 5 ml of 0.01 M pyridoxal phosphate in PBS for 30 min at 37°. Excess pyridoxal phosphate was then removed by washing the cells twice with PBS. Coupling of pyridoxal phosphate to the cells was readily apparent by the fact that cells appear slightly yellow at this stage. Reduction was carried out by exposing the pyridoxal phosphate containing cells to a total of 80 μCi per plate of NaB^3H_4 (Amersham, 6.3 Ci/mmol) in 5 ml of PBS for 15 min at 4°. Cells were then washed free of excess NaB^3H_4 and solubilized for gel electrophoresis.

Iodination of Cells. The lactoperoxidase iodination method of Phillips and Morrison (1970) was adapted for use with monolayer cultures. Plastic plates (100 mm) containing $3–4 \times 10^6$ cells were washed free of growth medium and then treated with 0.5 ml of PBS containing 200 μg of lactoperoxidase (Sigma Chemical Co.) and 100 μCi of carrier-free $Na^{125}I$ (Amersham). The enzymatic reaction was initiated by the addition of 10 μl of 0.06% H_2O_2 and identical aliquots of H_2O_2 were added at 2-min intervals of incubation at room temperature. After addition of the last aliquot cells were incubated for a further 10 min with continuous agitation, washed with PBS, and prepared for gel electrophoresis as usual. The total incorporation of ^{125}I into cells by this method amounted to between 1×10^6 and 2×10^6 cpm per 100-mm plate of cells.

Galactose Oxidase Method. The galactose and *N*-acetylgalactosamine portions of cell surface glycoproteins and glycolipids were labeled by first oxidizing these groups with galactose oxidase and then reducing them with potassium

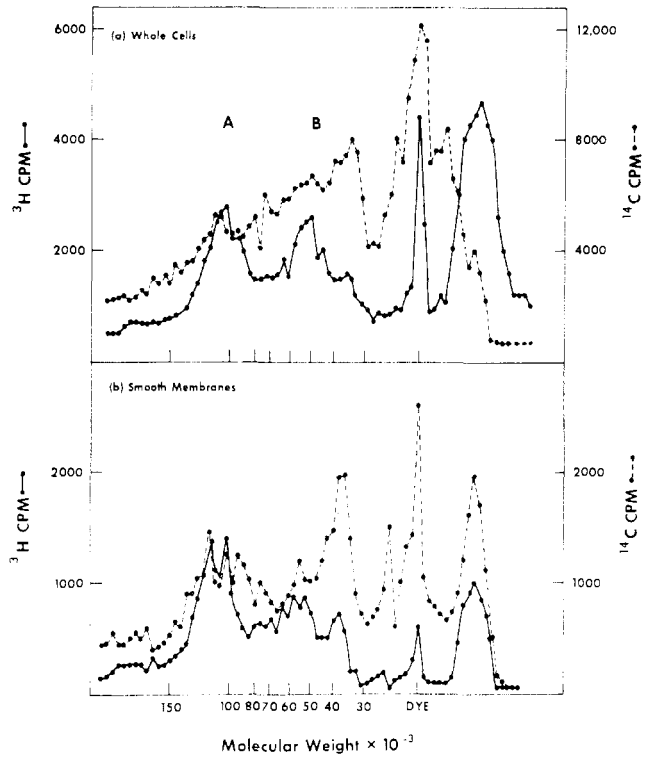

FIGURE 1: Polyacrylamide gel electrophoresis of whole L cells (a) and smooth membranes prepared from L cells (b). Cells were labeled by growing them in the presence of D-[^{3}H]glucosamine (–) and ^{14}C-labeled amino acids (- - -).

borotritide following the method of Gahmberg and Hakomori (1973). As these authors point out, optimal labeling of cells by this method was observed when cells had been previously treated with neuraminidase.

Cells to be labeled by this method were first grown for 48 hr in the presence of ^{14}C-labeled amino acids to provide an internal measure of the total amount of cell protein present. Monolayer cultures grown in this way were harvested by scraping and suspended in PBS at a density of 4×10^6 cells/ml. This suspension was then treated with 0.2 mg/ml of neuraminidase (Worthington Biochemicals) for 10 min at 37°. This treatment was found to solubilize 33% of the total cellular sialic acids when sialic acid was assayed by the method of Warren (1959); neuraminidase was shown to be free of protease activity by the azoalbumin method (Tomarelli *et al.*, 1949). Cells were then washed free of neuraminidase and resuspended in 5 ml of PBS containing 0.3 mg of *Dactylium dendroides* galactose oxidase (EC 1.1.3.9, Sigma Chemical Co.). Incubation was continued for 2 hr at 37° with occasional shaking; cells were then washed twice in PBS to remove excess galactose oxidase. Reduction of oxidized galactose and *N*-acetylgalactosamine groups was carried out by resuspending the cells in 5 ml of PBS containing 1 mCi of potassium borotritide (Amersham, 3 Ci/mmol) and incubating for 30 min at room temperature. The cells were again washed extensively to remove unreacted potassium borotritide and the radioactivity from ^{14}C-labeled amino acids and KB^3H_4 was determined. The cell pellets were then dissolved in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol as described above for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The aliquot of cells for each gel was adjusted so that approximately the same number of ^{14}C counts per minute (from ^{14}C-labeled amino acids) was applied to each gel.

Surface Localization of Labeled Components. The selectivity of the above three biochemical systems for labeling cell surface and not internal structures was estimated by measuring the incorporation of radioactivity into soluble cytoplasmic proteins; since these components are found inside the lipid barrier they should not be well labeled. All three methods were tested similarly. One monolayer culture of 3×10^6 L cells in a 100-mm plate was labeled by the pyridoxal phosphate–NaB^3H_4 method, by the lactoperoxidase iodination technique, or by the galactose oxidase–KB^3H_4 system, respectively, as described above. Labeled cells were then washed in RSB and homogenized in 4 ml of RSB. The homogenate was separated into a supernatant fraction which contained the soluble cytoplasmic proteins and a sedimentable fraction which contained the smooth membranes by centrifugation for 4 hr at 50,000 rpm in a Beckman type SW-50L rotor. Aliquots of each fraction were then precipitated with 5% trichloroacetic acid and filtered on Whatman GF/C glass fiber filters. The dried filters were counted directly in a toluene-based scintillation fluid.

The results are shown in Table I where the total counts per minute (either ^{3}H or ^{125}I) recovered in the supernatant and in the pellet are expressed as a percentage of the total counts per minute present in the original homogenate. In none of the three methods tested was the radioactivity recovered in the supernatant fraction, which contains the soluble cytoplasmic proteins, found to be greater than 3% of the total. This strongly suggests that internal soluble proteins are not well labeled and, therefore, that none of the three probes employed here penetrates appreciably into the interior of the cell.

TABLE I: Distribution of Radioactivity into Sedimentable and Supernatant Fractions after Cell Surface Labeling.

Labeling Method	% of Total cpm in Supernatant	% of Total cpm in Pellet
Pyridoxal phosphate–NaB^3H_4	3	97
Lactoperoxidase–$Na^{125}I$	1	99
Galactose oxidase–KB^3H_4	<0.1	99

Results

Total Complement of Cellular Glycoproteins. The total complement of L cell glycoproteins was examined by electrophoresis of whole cell homogenates on sodium dodecyl sulfate–polyacrylamide gels. Two monolayer cultures of L cells were grown in 100-mm plastic plates for 48 hr (approximately two generations) in the presence of ^{14}C-labeled amino acids and D-[^{3}H]glucosamine as described under Materials and Methods. Cells were then harvested by scraping, dissolved in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol, and subjected to electrophoresis on 7% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Glycoproteins were localized on the gel by slicing the gel and counting as described above. The result of this experiment is shown in Figure 1a. Nearest the top of the gel (to the left) one finds two separated bands of D-[^{3}H]glucosamine-labeled glycoproteins having apparent molecular weights of 100,000 and 50,000; these we call class A and class B glycoproteins, respectively.

Both of these bands are also labeled with ^{14}C-labeled amino acids. Another peak of glycoprotein or glycopeptide material is found which runs with the Bromophenol Blue tracking dye and a fourth peak of radioactivity most probably consisting of glycolipid is found just ahead (to the right) of the dye. We will not discuss these latter two components further, but rather restrict our attention to class A and class B glycoproteins only.

Criteria for "Surface" Glycoprotein. Our problem, therefore, was to establish whether class A or class B glycoproteins are or contain cell surface components. For this purpose, we have adopted three criteria commonly employed to differentiate between cell surface and internal structures. First, cell surface glycoproteins ought to be isolated with the smooth membrane fraction of cell homogenates and not, for example, with the soluble, nuclear, or rough membrane fractions. Second, surface glycoprotein components should be digested when intact cells are treated with proteolytic enzymes. Since these enzymes do not enter or lyse the cells, only external and not internal glycoproteins are degraded. Third, surface glycoproteins ought to be labeled preferentially over internal glycoproteins by reactive chemical groups which do not penetrate the plasma membrane. A number of these methods are now available including the pyridoxal phosphate–sodium borotritide system, the lactoperoxidase method, and the galactose oxidase technique which we have used with L cells as described below. Cellular glycoproteins meeting all three of these criteria are very likely to be situated on the outer surface of the cell. Our experimental studies, therefore, were designed to apply these criteria to the glycoprotein components of L cells.

Glycoproteins Isolated with Smooth Membranes. The glycoproteins isolated with L cell smooth membranes prepared by the method of Wagner *et al.* (1972) were examined by electrophoresis of solubilized membranes on sodium dodecyl sulfate–polyacrylamide gels. Two 100-mm plates of L cells were first grown in the presence of D-[^3H]glucosamine and ^{14}C-labeled amino acids, harvested, and homogenized as previously described; subcellular fractions were prepared from the homogenate by sucrose density gradient centrifugation. After centrifugation four light scattering bands of cellular material were clearly visible in the gradient and these were collected separately. As shown in Table II, approximately 60% of the D-[^3H]glucosamine-labeled material applied to the gradient was found in the uppermost (band 1) of these bands which contains the smooth membranes. A significant amount of the total label is also found in band 2 which consists mostly of rough endoplasmic reticulum while the lower two bands, 3 and 4, contained only a small fraction of the total D-[^3H]-glucosamine label. These latter three bands were not examined further.

Smooth membranes were then concentrated from band 1 by centrifugation, solubilized, and subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. The results of this experiment are shown in Figure 1b. It is clear from the pattern of D-[^3H]glucosamine label that class A and class B glycoproteins are prominent features of the smooth membrane fraction and that they occur in roughly the same proportion to each other in the smooth membrane fraction as in the whole cell homogenate. As in the case of gels run on whole cells, both the class A and class B glycoprotein regions of the gel also contain label derived from ^{14}C-labeled amino acids.

Trypsin-Sensitive Glycoproteins. In order to determine whether class A and B glycoproteins would be sensitive to proteolytic digestion from the exterior surface of the cell, whole L cells were treated with the proteolytic enzyme trypsin. Four replicate cultures of cells were grown on 100-mm plates in the presence of D-[^3H]glucosamine to a density of 3.5×10^6 cells per plate as previously described. Two of these were then treated with 1% trypsin in BSS for 30 min at room temperature while the other two were incubated with BSS only. Trypsinized cells and control cells were then harvested separately, washed, dissolved in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol, and examined for their content of class A and class B glycoproteins by polyacrylamide gel electrophoresis.

TABLE II: Recovery of Radioactivity in Subcellular Fractions Separated by Sucrose Density Gradient Centrifugation.[a]

Light Scattering Band	^3H Label Derived from D-[^3H]-Glucosamine		^{14}C Label Derived from ^{14}C-Labeled Amino Acids		
	cpm Recovered	%	cpm Recovered	%	$^3H/^{14}C$
1	162,675	62	555,125	51	0.293
2	64,550	24	248,675	23	0.258
3	28,625	11	210,475	19	0.136
4	11,475	4	69,050	6	0.161

[a] A Dounce homogenate was prepared from 7×10^6 cells grown in the presence of D-[^3H]glucosamine and ^{14}C-labeled amino acids. Homogenate (4 ml) containing a total of 2.63×10^5 cpm of ^3H label and 1.25×10^6 cpm of ^{14}C was separated into subcellular fractions by sucrose density gradient centrifugation as described under Materials and Methods. The total radioactivity present in each fraction was determined by counting an aliquot directly in a liquid medium containing one part Triton X-100 and two parts toluene plus fluors.

The results of this experiment are presented in Figure 2a,b. Figure 2a shows the familiar pattern of D-[^3H]glucosamine label from untrypsinized cells in which class A and class B glycoproteins are clearly resolved. A significant decrease in the yield of both classes of glycoproteins was observed after trypsin treatment as shown in Figure 2b, although a small residue of class A and class B glycoproteins always remained. We found no clear evidence of new species of glycoprotein material, perhaps corresponding to degradation products of intact glycoproteins, associated with trypsinized as compared to control cells, Thus, proteolysis of surface glycoproteins must yield primarily soluble, not cell-associated, glycopeptide fragments. Trypsinization of cells did not cause a significant change in either the amount of glycolipid or the amount of glycopeptide migrating in the marker dye region of the gel.

Selective Labeling of Cell Surface Components. The ability of class A and class B glycoproteins to react with nonpenetrating biochemical reagents was tested by applying three of these methods to cultures of L cells. The three methods employed were the pyridoxal phosphate–sodium borotritide method recently described by Rifkin *et al.* (1972), the lactoperoxidase method of Phillips and Morrison (1970), and the galactose oxidase–potassium borotritide technique (Gahmberg and Hakomori, 1973); all three of these methods are designed to introduce radioactive label specifically into cell surface structures. The pyridoxal phosphate–sodium boro-

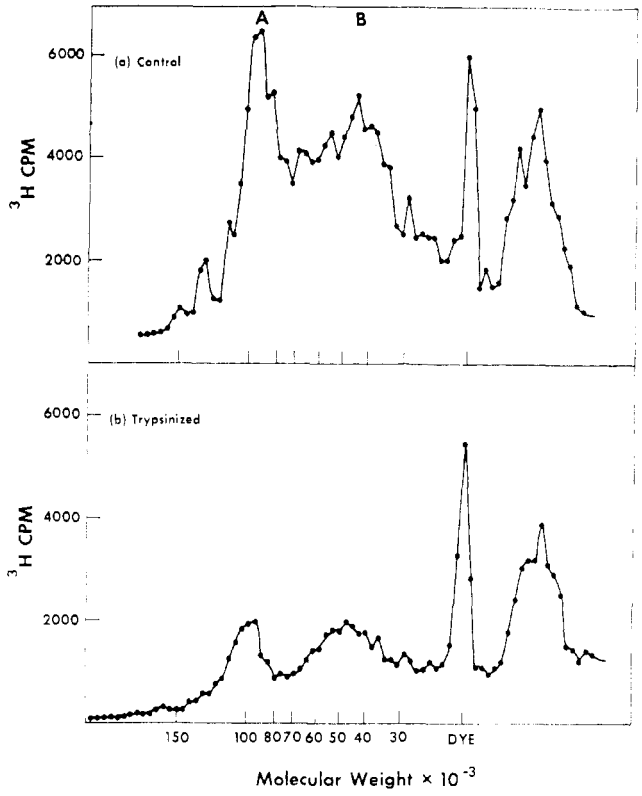

FIGURE 2: Polyacrylamide gel electrophoresis of control (a) and trypsinized (b) L cells grown in the presence of D-[^{14}C]glucosamine. Control cells and cells that had been treated for 30 min with 1% (w/v) trypsin at room temperature were solubilized and subjected to electrophoresis on 7% polyacrylamide gels.

tritide system achieves this result by reducing with NaB^3H_4 the Schiff's base formed by reaction of pyridoxal phosphate with free amino groups on the cell surface. Since pyridoxal phosphate does not enter the cell this results in the introduction of ^{3}H label specifically into cell surface structures.

Cells to be reacted with pyridoxal phosphate and sodium borotritide were first grown for 48 hr in the presence of D-[^{14}C]glucosamine in order to provide internal ^{14}C-labeled markers for all cellular glycoprotein classes. One plate of cells grown in this way was treated with pyridoxal phosphate and NaB^3H_4 as described under Materials and Methods; labeled components were then separated on sodium dodecyl sulfate–polyacrylamide gels.

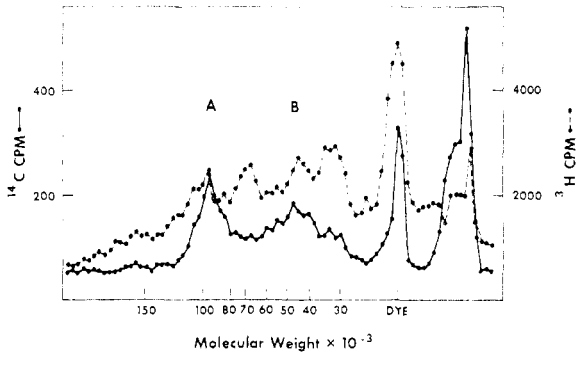

FIGURE 3: Polyacrylamide gel electrophoresis of L cells treated with pyridoxal phosphate and reduced with NaB^3H_4. Cells were grown in the presence of D-[^{14}C]glucosamine (–), treated with pyridoxal phosphate reduced with NaB^3H_4 (- - -), and subjected to polyacrylamide gel electrophoresis.

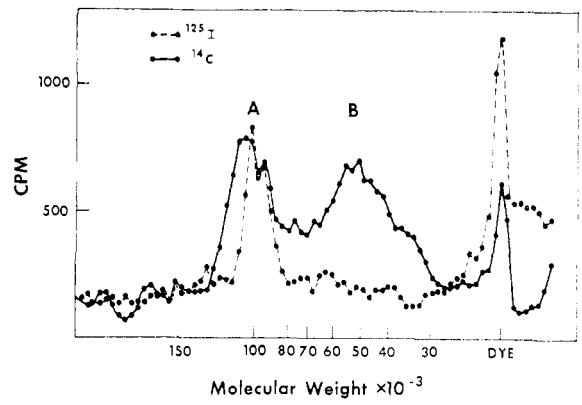

FIGURE 4: Lactoperoxidase-catalyzed iodination of L cells. Cells grown on D-[^{14}C]glucosamine (–) and cells iodinated with Na^{125}I (- - -) were subjected to electrophoresis on separate gels.

The results of this experiment are shown in Figure 3. Radioactive label derived from D-[^{14}C]glucosamine (solid curve) indicates the positions of class A and class B glycoproteins on the gel. The dashed curve represents ^{3}H label from NaB^3H_4. It shows that regions of the gel corresponding to both class A and class B glycoproteins are well labeled by the pyridoxal phosphate–sodium borotritide system. In addition, two regions of the gel are labeled with NaB^3H_4 which do not correspond to known glycoprotein classes; these occur between class A and class B glycoproteins and just ahead of class B glycoproteins, respectively. ^{3}H radioactivity in these regions is most probably due to labeling of surface proteins which do not contain carbohydrate side chains and therefore are not labeled with D-[^{14}C]glucosamine. Material which runs with the Bromophenol Blue dye and the glycolipid region of the gel is also labeled. Pre-reduction of cells with excess nonradioactive $NaBH_4$ prior to treatment with pyridoxal phosphate and NaB^3H_4 did not significantly alter either the pattern of radioactivity observed on the gel or the total amount of ^{3}H label incorporated into cells. Less than 5% of the total NaB^3H_4 labeling was observed if the pyridoxal phosphate step was omitted.

Like the pyridoxal phosphate–sodium borotritide system, the lactoperoxidase iodination method is designed to introduce radioactive label specifically into cell surface structures. In this case, whole cells are allowed to react with the enzyme lactoperoxidase in the presence of H_2O_2 and Na^{125}I; this results in the iodination of exposed proteins primarily at available tyrosine residues. Since lactoperoxidase cannot penetrate the lipid bilayer only cell surface structures are iodinated.

L cells (3×10^6) in one 100-mm plate were iodinated by this method, solubilized, and analyzed by electrophoresis on polyacrylamide gels. For comparison, a separate gel was run simultaneously on cells labeled by growing them in the presence of D-[^{14}C]glucosamine. The results of this experiment are shown in Figure 4 where the profiles obtained from both gels are plotted together. The solid curve shows the position of D-[^{14}C]glucosamine-labeled bands corresponding to class A and class B glycoproteins. The dashed curve shows ^{125}I label in two regions of the gel, one roughly coincident with class A glycoproteins and the other at the position of the dye marker. No labeling is observed in the region of class B glycoproteins. Labeling of class B glycoproteins could not be induced either by mild trypsinization of the cells before iodination (Phillips and Morrison, 1973) or by extending the time of incubation with lactoperoxidase.

In contrast to the pyridoxal phosphate–NaB^3H_4 and the lactoperoxidase methods which label the polypeptide backbone of cell surface glycoproteins, the galactose oxidase method preferentially labels the carbohydrate portions of these structures; galactose and *N*-acetylgalactosamine groups are oxidized enzymatically by galactose oxidase and reduced with KB^3H_4. Since the enzyme galactose oxidase cannot penetrate the lipid bilayer only cell surface structures are labeled by this method. However, in agreement with the results of Gahmberg and Hakomori (1973) we found that optimal labeling by the galactose oxidase technique was observed when cells had been pre-treated with neuraminidase. This treatment probably makes oxidizable groups on the cell surface more accessible to the enzyme galactose oxidase.

Twenty-four plates of cells were grown to a density of 3×10^6 cells per plate in the presence of ^{14}C-labeled amino acids to provide an internal marker for proteins after sodium dodecyl sulfate–polyacrylamide gel electrophoresis and to obtain an estimate of the total amount of protein applied to each gel. Cells were harvested as usual and divided into three equal aliquots.

One aliquot of cells was treated with neuraminidase before oxidation with galactose oxidase, another was treated with galactose oxidase only, and a third received no enzyme treatment at all. All cells were then reduced with KB^3H_4 as described under Materials and Methods, solubilized in 1% sodium dodecyl sulfate–1% 2-mercaptoethanol, and analyzed on sodium dodecyl sulfate–polyacrylamide gels.

Figure 5a (solid curve) shows the gel profile obtained when cells were treated with both neuraminidase and galactose oxidase prior to reduction. Of the four regions of the gel labeled in this way two correspond to class A and B glycoproteins, respectively; class A glycoproteins are somewhat more heavily labeled than class B. The tracking dye and glycolipid regions of the gel are also labeled. However, when neuraminidase treatment is omitted (dashed curve) only the latter two bands are labeled by KB^3H_4. No radioactivity is found in the regions of class A or B glycoproteins. A similar result was obtained in the case of cells treated only with KB^3H_4 as shown in Figure 5b (solid curve). The tracking dye and glycolipid regions of the gel are well labeled while class A and B glycoproteins are not.

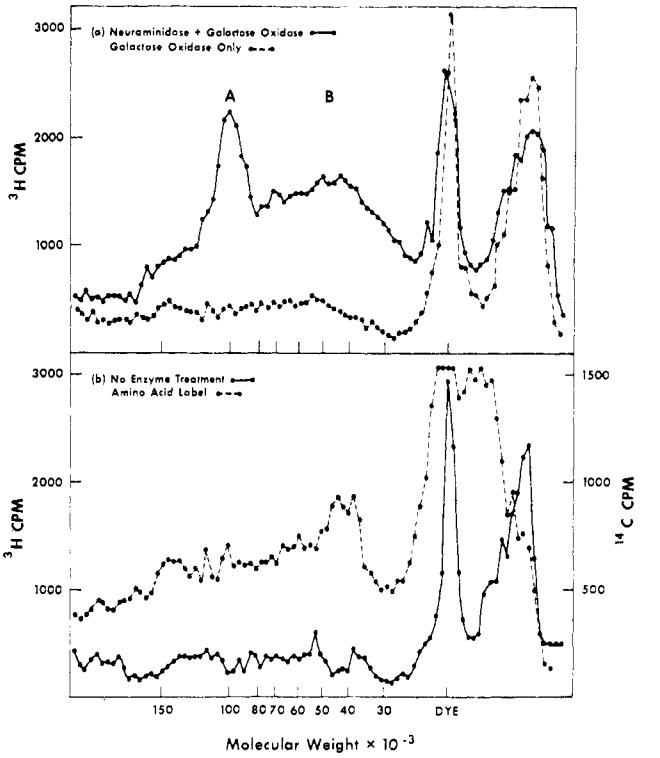

FIGURE 5: Polyacrylamide gel electrophoresis of L cells treated with galactose oxidase and subsequently reduced with KB^3H_4: (a) cells treated with neuraminidase followed by galactose oxidase (–) or galactose oxidase only (- - -); (b) cells reduced with KB^3H_4 without prior enzyme treatment (–) and the electrophoretic pattern of ^{14}C-labeled amino acids on which the cells had been grown prior to enzyme treatment and/or reduction (- - -).

Discussion

The work described here provides two main contributions to studies of cell surface glycoproteins. First, attention has been drawn to the fact that the glycoprotein components of mouse L cells can be separated into two major classes by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. The two classes, called class A and B glycoproteins, have apparent molecular weights of approximately 100,000 and 50,000, respectively. The true molecular weights, however, may be somewhat different (probably smaller) due to the fact that glycoproteins migrate in an anomalous fashion compared to globular proteins on sodium dodecyl sulfate–polyacrylamide gels (Bretscher, 1971). These glycoproteins become radioactively labeled when cells are grown in the presence of ^3H- or ^{14}C-labeled D-glucosamine and are detected on polyacrylamide gels as bands of radioactivity. It is possible, therefore, that other classes of glycoproteins exist which are not labeled in this way. However, no new classes of glycoproteins were revealed by analyzing cells labeled by growth in the presence of [^3H]fucose, [^3H]mannose, or [^{14}C]mannosamine. Bands of radioactivity on the gel corresponding to class A and B glycoproteins are somewhat broader and more irregular in shape than bands produced, for instance, by single globular protein species. It is, therefore, possible that each glycoprotein class consists of more than a single species of glycopolypeptide.

Second, criteria have been adopted for distinguishing cell surface glycoproteins from internal cytoplasmic components and these have been applied experimentally to class A and B glycoproteins of L cells. In particular, a glycoprotein class was regarded as a "surface" component if it was found to be isolated with the smooth membrane fraction of Dounce-homogenized cells, degraded when monolayer cultures of intact cells were treated with trypsin, and labeled from the outer cell surface by biochemical methods designed to introduce radioactive label selectively into the exposed surface structures of intact cells. The methods examined here were the pyridoxal phosphate–NaB^3H_4 system, the lactoperoxidase iodination method, and oxidation by galactose oxidase followed by reduction with KB^3H_4. The results of these experiments are summarized in Table III. Here we have listed the five types of experiments carried out to identify cell surface components and noted whether or not class A and B glycoproteins qualify as surface structures by that criterion. The results are clearest in the case of class A glycoproteins; these fulfill all our criteria for surface structures so we conclude that at least some class A glycoproteins are found on the outer surface of L cells. Class B glycoproteins are also most likely to be surface components since they fulfill all our criteria except for iodination by lactoperoxidase. It is not yet clear why class B glycoproteins are resistant to iodination *in situ* although the reason may be unimportant in the present

TABLE III: Summary of Results.

Criterion for "Surface" Structure	Glycoprotein Class A	Glycoprotein Class B
Isolation with smooth membranes	Yes	Yes
Trypsin sensitive *in vivo*	Yes	Yes
Labeled with pyridoxal phosphate–NaB^3H_4	Yes	Yes
Iodinated by lactoperoxidase method	Yes	No
Labeled with galactose oxidase–KB^3H_4	Yes	Yes

context. For example, class B glycoproteins may have no exposed tyrosine residues available for iodination at the cell surface. Furthermore, recent biochemical studies of the mouse H-2 histocompatibility alloantigens support the view that at least some class B glycoproteins are cell surface components (Schwartz *et al.*, 1973). These glycoprotein antigens, which must be located on the outer surface of the cell since they are strong transplantation antigens, have been shown to have molecular weights (43,000 for H-2D^d and 47,000 for H-2K^d) consistent with their being class B glycoproteins.

It is clear, however, that both class A and B glycoproteins may be heterogeneous with respect to cell surface and internal glycoproteins; both classes may contain some cell surface and some internal components. The most likely candidates for internal class A and B glycoproteins are the trypsin-resistant fractions of class A and B glycoproteins, respectively, as shown in Figure 2. Furthermore, heterogeneity may exist even among the cell surface components of class A and B glycoproteins. For example, class A glycoproteins may consist of several cell surface components. Direct isolation and purification of these structures are probably the most straightforward ways to examine the heterogeneity of these glycoprotein classes and studies of techniques for glycoprotein isolation are currently in progress in our laboratory.

Finally, since we have drawn attention to two classes of L cell surface glycoproteins, it is natural to inquire about how other cell types will compare. One wants to know, for instance, whether all mouse cells will have only class A and B surface glycoproteins or whether cells with specialized functions will also have distinctive surface glycoprotein components. Information of this type ought to be most useful in attempting to define the biological function of the cell surface glycoproteins.

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References

Atkinson, P. H., and Summers, D. F. (1971), *J. Biol. Chem. 246*, 5162.
Bretscher, M. S. (1971), *Nature (London), New Biol. 231*, 229.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Evans, W. H. (1970), *Biochim. Biophys. Acta 211*, 578.
Gahmberg, C. G., and Hakomori, S. (1973), *J. Biol. Chem. 248*, 4311.
Glossmann, H., and Neville, D. M., Jr. (1971), *J. Biol. Chem. 246*, 6339.
Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., and Scott, R. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1445.
Nachman, R. L., Hubbard, A., and Ferris, B. (1973), *J. Biol. Chem. 248*, 2928.
Phillips, D. R. (1972), *Biochemistry 11*, 4582.
Phillips, D. R., and Morrison, M. (1970), *Biochem. Biophys. Res. Commun. 40*, 284.
Phillips, D. R., and Morrison, M. (1973), *Nature (London), New Biol. 242*, 213.
Rifkin, D. B., Compans, R. W., and Reich, E. (1972), *J. Biol. Chem. 247*, 6432.
Schwartz, B. D., Kato, K., Cullen, S. E., and Nathenson, S. G. (1973), *Biochemistry 12*, 2157.
Segrest, J. P., Kahane, I., Jackson, R. L., and Marchesi, V. T. (1973), *Arch. Biochem. Biophys. 155*, 167.
Tomarelli, R. M., Charney, J., and Harding, M. L. (1949), *J. Lab. Clin. Med. 34*, 428.
Wagner, R. R., Kiley, M. P., Snyder, R. M., and Schnaitman, C. A. (1972), *J. Virol. 9*, 672.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Winzler, R. J. (1969), *in* Red Cell Membranes: Structure and Function, Jamieson, G. A., and Greenwalt, T. J., Ed., Philadelphia, Pa., Lippincott, p 157.