Biol. 2, 47–110.
Gross, K. J., and Pogo, A. O. (1974), *J. Biol. Chem. 249,* 568–576.
Hamilton, M. G., Faiferman, I., and Pogo, A. O. (1973), *Proc. Int. Congr. Biochem., 9th,* 12.
Henshaw, E. C., and Loebenstein, J. (1970), *Biochim. Biophys. Acta 199,* 405–420.
Kashnig, D. M., and Kasper, C. B. (1969), *J. Biol. Chem. 244,* 3786–3792.
Köhler, K., and Arends, S. (1968), *Eur. J. Biochem. 5,* 500–506.
Lawford, G. R., Sadowski, P., and Schachter, H. (1967), *J. Mol. Biol. 23,* 81–87.
Martin, T. E., and McCarthy, B. J. (1972), *Biochim. Biophys. Acta 277,* 354–367.
Mirsky, A. E., and Pollister, A. W. (1942), *Proc. Natl. Acad. Sci. U.S.A. 28,* 344–352.
Monneron, A., and Bernhard, W. (1969), *J. Ultrastruct. Res. 27,* 266–288.
Moulé, Y., and Chauveau, J. (1968), *J. Mol. Biol. 33,* 465–481.
Niessing, J., and Sekeris, C. E. (1971), *Biochim. Biophys. Acta 247,* 391–403.
Palade, G. E., and Siekevitz, P. (1956), *J. Biophys. Biochem. Cytol. 2,* 171–198.
Parsons, J. T., and McCarty, K. S. (1968), *J. Biol. Chem. 243,* 5377–5384.
Pederson, T. (1974), *J. Mol. Biol. 83,* 163–183.
Penman, S. (1966), *J. Mol. Biol. 17,* 117–130.
Perry, R. P., La Torre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220–226.
Pitot, H. C., and Shires, T. K. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 76–79.
Pogo, A. O., Allfrey, V. G., and Mirsky, A. E. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 550–557.
Pogo, A. O., Littau, V., Allfrey, V. G., and Mirsky, A. E. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 743–750.
Roth, J. S. (1958), *J. Biol. Chem. 231,* 1085–1095.
Samarina, O. P., Krichevskaya, A. A., and Georgiev, G. P. (1966), *Nature (London) 210,* 1319–1322.
Shankar Narayan, K., Steele, W. J., Smetana, K., and Busch, H. (1967), *Exp. Cell Res. 46,* 65–77.
Shiokawa, K., and Pogo, A. O. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2658–2662.
Sibatani, A., De Kloet, S. R., Allfrey, V. G., and Mirsky, A. E. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 471–477.
Sommerville, J. (1973), *J. Mol. Biol. 78,* 487–503.
Spirin, A. S. (1966), *Curr. Top. Dev. Biol. 1,* 1–38.
Spirin, A. S. (1969), *Eur. J. Biochem. 10,* 20–35.
Stévenin, J., and Jacob, M. (1972), *Eur. J. Biochem. 29,* 480–488.
Swift, H. (1963), *Exp. Cell Res., Suppl. 9,* 54–67.
Vesco, C., and Penman, S. (1968), *Biochim. Biophys. Acta 169,* 188–195.
Zimmerman, S. B., and Sandeen, G. (1966), *Anal. Biochem. 14,* 269–277.

Surface Polypeptides of the Cultured Chinese Hamster Ovary Cell†

R. L. Juliano* and Monique Behar-Bannelier

ABSTRACT: The organization of the plasma membrane of logarithmically growing Chinese hamster ovary (CHO) suspension cells has been probed using surface label techniques in conjunction with subcellular fractionation and sodium dodecyl sulfate gel electrophoresis. Five components of apparent molecular weights 137,000, 121,000, 97,000, 67,000, and 57,000 have been shown to be exposed at the outer surface of the cell. These components fully meet the criteria of being (a) reactive with two or more surface label reagents, (b) enriched in a purified plasma membrane fraction, and (c) sensitive to proteolytic digestion of intact cells. Three other components of molecular weights 200,000, 44,000 and 30,000 are also reactive with certain surface label reagents, but fail to meet other criteria for cell surface components. Two polypeptides of molecular weights 180,000 and 37,000 are substantially enriched in the plasma membrane fraction, but are unreactive with surface label reagents. The organization of the CHO cell membrane and the applicability of surface label techniques to cultured cell systems are discussed.

Surface label techniques have yielded much information concerning the molecular architecture of the erythrocyte membrane (Juliano, 1973). In this experimental approach, the intact cells are treated with reagents which can covalently radiolabel polypeptides, but which are excluded by the membrane from the interior of the cell. Thus, only the polypeptides of the cell periphery are labeled and these may be discriminated from other membrane components (Juliano, 1973).

Several workers have applied surface label techniques to the analysis of the membranes of nucleated mammalian cells (Poduslo et al., 1972; Shin and Carraway, 1973; Kinzel and Mueller, 1973; Hunt and Brown, 1974; Huang et al., 1973; Gahmberg and Hakamori, 1973a,b), but few of them have seriously evaluated the pitfalls inherent in the application of these techniques to complex cells. We have previously delineated some of the problems of the surface label approach (Juliano, 1974; Juliano and Behar-Bannel-

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ier, 1975). A serious difficulty is the inevitable presence of a few percent of dead cells in any permanent cell line, or in a cell population freshly isolated from tissue by mechanical or enzymatic treatment. Dead cells lack a permeability barrier to both large and small molecules, allowing reagents to readily penetrate and react with internal sites (Juliano, 1974; Juliano and Mayhew, 1972). Although few in number, the nonviable cells may make a substantial artifactual contribution to further analysis, since a single nonviable cell may contain many more sites for binding and reaction on the cell interior than exist on the outer surface of a viable cell (Juliano, 1974; Juliano and Mayhew, 1972). Fortunately, the contribution of labeled constituents from the interior of nonviable cells can be minimized by careful subcellular fractionation subsequent to the labeling reaction.

Another problem affecting some previous surface label studies is that they have usually relied on a single labeling reagent. This may result in a very misleading picture of the cell surface, since the reagent chosen may react preferentially with a particular surface component, or with an internal component not fully removed by cell fractionation, and have a low degree of reactivity with other important components of the cell surface. In view of these problems, one must conclude that reactivity with a single surface label reagent is a poor criterion for assigning a polypeptide to a location on the outer surface of the cell.

In order to deal with these difficulties, we have utilized a multifaceted approach to the study of mammalian cell surfaces. Our immediate goal is the identification of the polypeptides which are exposed at the cell periphery. To this end we have adopted the following criteria for the identification of putative cell surface polypeptides: (a) the component must be reactive with at least two surface label reagents of differing specificity; (b) the reactive component must be more prominent in a purified plasma membrane fraction, as opposed to the whole cell homogenate; (c) the reactive component should be susceptible to digestion by proteases applied to the intact cell.

Keeping these criteria in mind, we have investigated the membrane organization of the cultured Chinese hamster ovary (CHO) cell. This is a permanent cell line which grows rapidly in suspension or monolayer culture, and which has been extensively characterized from a genetic viewpoint (Thompson and Baker, 1973). It was felt that the CHO cell might be representative of many rapidly proliferating cultured cells, and that it might serve as a proving ground for the development of techniques for the analysis of the plasma membrane of nucleated mammalian cells.

Materials and Methods

Cells. Chinese hamster ovary (CHO)[1] cells were obtained from the Ontario Cancer Research Institute and were maintained as suspension cultures in a 1:1 mixture of medium α and medium RPMI 1640 plus 10% fetal calf serum. Cells and serum were frequently assayed for mycoplasma contamination and were found to be negative. Cells for experiments were harvested during exponential growth. Viability exceeded 98% in samples obtained directly from culture and was in excess of 90% after surface labeling procedures and at the time of subcellular fractionation. Cell counting was by hemocytometry, and viability was determined by the Trypan Blue exclusion test (Juliano and Mayhew, 1972).

Surface Labeling. Pyridoxal Phosphate-Borohydride. CHO cells were washed in Dulbecco's phosphate-buffered saline (pH 7.2) and labeled in this buffer using a modification (Juliano and Behar-Bannelier, 1975) of the pyridoxal phosphate (pyridoxal-P)-[^{3}H]borohydride technique (Rifkin et al., 1972). Generally, cells were treated with 10 m*M* pyridoxal-P at pH 7.4 for 5 min at 20 or 37°, rewashed, and treated with about 1 mCi of [^{3}H]borohydride for 5 min at 0°. The [^{3}H]borohydride was dissolved in cold 0.01 *N* NaOH just prior to use. Addition of a small aliquot (usually 100 μl) of this material did not change the pH of the reaction mixture. Usually 1-2 $\times$ 10^8 cells were used and the reaction volume was 2 ml. Tritium incorporation with borohydride treatment alone was about one-tenth of that following pyridoxal-P treatment.

Lactoperoxidase. This enzyme catalyzes the iodination of cell surface tyrosine residues (Hubbard and Cohn, 1972; Phillips and Morrison, 1970, 1971; Bauer et al., 1972). Generally a modification (Juliano and Behar-Bannelier, 1975) of the procedure of Bauer et al. was employed and either Na^{131}I or Na^{125}I was used as a radiolabel at the level of 0.1-0.5 mCi per iodination in carrier free form. Incubation times were on the order of 5 min at 37° and usually 10^8 cells (2×10^{-2} g of cell protein) in 2 ml were labeled in each reaction.

Galactose Oxidase. In this system, sialidase is used to cleave sialic acid from cell surface glycoproteins and glycolipids, thus exposing penultimate galactose residues which are then oxidized with the enzyme galactose oxidase. This is followed by a reduction with [^{3}H]borohydride which introduces a radiolabel into the cell surface galactose residues (Gahmberg and Hakamori, 1973a,b; Steck, 1972). In this study, very short reaction times are used so as to minimize cell degradation (Juliano and Behar-Bannelier, 1975). Thus, cells are treated with 50 units of sialidase followed by 15 units of galactose oxidase, each for 5 min at 37°; the enzymes are removed and the cells are treated with 1 mCi of [^{3}H]borohydride for 5 min at 0° in the manner described for pyridoxal-P treatment. Usually 1-2 $\times$ 10^8 cells in a volume of 2 ml of Dulbecco's buffer were utilized. The galactose oxidase induced incorporation of tritium was about 10 times greater than that of samples treated with borotritride alone; these latter were labeled primarily in the lipid fraction. Galactose oxidase labeling in the manner described here does not result in proteolysis of membrane polypeptides as visualized by gel electrophoresis of the membrane fraction. A more detailed account of the galactose oxidase system and of the other labeling methods is given in a previous reference (Juliano and Behar-Bannelier, 1975).

In each case the incorporation of radiolabel subsequent to treatment with pyridoxal-P or with enzymes was at least tenfold greater than that found in the absence of these reagents.

Protease Treatment. Proteolytic dissection (Steck et al., 1971) of cell surface protein was performed by incubating washed intact cells with proteases, usually for 5-30 min at 37°. Reaction was terminated by the addition of 50 vol of cold buffer plus 1% albumin and the cells were washed four times in this solution. The enzymes employed include Pronase, trypsin, and chymotrypsin at levels of 10-1000 μg/ml. The cell concentration was usually 10^8/ml. More detailed information is given in the figure legends.

Subcellular Fractionation. Purified CHO plasma mem-

[1] Abbreviations used are: CHO, Chinese hamster ovary cells; pyridoxal-P, pyridoxal phosphate.

Table I: Cell Fractionation.[a]

	H	M	M/H
$Na^+ + K^+$-ATPase	11.0 ± 8.0	105.0 ± 30.0	9.5
Cytochrome *c* reductase	300.0 ± 45.0	40.0 ± 2.5	0.13
Succinic dehydrogenase	210.0 ± 12.0	0	
DNA	77.0	0	

[a] Enzyme activity in the homogenate (H) or membrane (M) fraction is expressed as nanomoles per milligram of protein per hour. The amount of DNA is expressed as micrograms per milligram of protein. Results represent the mean and arithmetic error from two–three experiments.

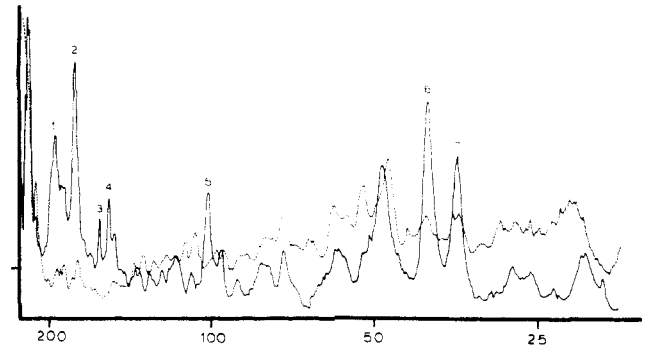

FIGURE 1: Gel electrophoresis of purified plasma membrane and whole cell polypeptides. Equal amounts (30 μg) of protein from a whole cell homogenate (···) or purified membrane (—) were electrophoresed on 5.6% acrylamide gels, stained for protein, and scanned with a densitometer. Molecular weight markers were run in parallel. Components which are distinctly enriched in the plasma membrane are designated by numerals: ordinate, arbitrary optical density units; abscissa, molecular weight scale ($\times 10^{-3}$). In this experiment the membranes were purified by three cycles of the aqueous polymer separation, followed by a sucrose step gradient separation.

branes were isolated by a modification (Juliano and Behar-Bannelier, 1975) of the method of Brunette and Till (1971), employing an aqueous polymer system. Briefly, cells previously treated with surface labels and/or proteolytic enzymes were swollen in cold 1 m*M* $ZnCl_2$ containing 1% albumin for about 5 min. The cells were broken in a Dounce homogenizer and centrifuged at $2 \times 10^4 \times g$/min and the resulting pellet resuspended and washed twice in cold 2 m*M* $MgCl_2$ plus 1% albumin. The pellet was mixed into an aqueous two-phase polymer system also containing 1% albumin and was centrifuged at $2 \times 10^5 \times g$/min. The resulting pellet was discarded and the supernatant was remixed and respun. The interface of the second two-phase centrifugal separation which contained mainly plasma membrane was recovered and washed twice in cold 2 m*M* $MgCl_2$. Albumin was included in the preliminary steps of membrane isolation in order to minimize autodegradation of membrane proteins by cellular proteases (Merrel and Glaser, 1973). In some cases, the product of the two-phase separation was further purified as follows. The material was rehomogenized and subjected to a third two-phase separation. The interface from this separation was washed once in 2 m*M* $MgCl_2$, rehomogenized again, washed in 5% sucrose, and then resuspended in 1.5 cm^3 of 30% sucrose. This was layered over a gradient composed of 1.5-cm^3 steps of 60, 55, 50, 45, and 40% sucrose and centrifuged at $5 \times 10^4 \times g$/min. Membranes were recovered at the 45% interface and were washed twice in 2 m*M* $MgCl_2$ and stored frozen.

Subcellular fractionation was monitored by measuring levels of alkali cation ATPase (Kimelberg and Papahadjopoulos, 1972) as a plasma membrane marker, cytochrome *c* reductase (Mackler, 1967) as an endoplasmic reticulum marker, succinic dehydrogenase as a mitochondrial marker (Reid, 1972), and DNA as a marker for the nucleus (Schneider, 1957). More detailed characterizations of CHO membranes prepared by the two-phase polymer technique have been reported previously (Juliano and Behar-Bannelier, 1975; Juliano and Gagalang, 1975).

Polyacrylamide Gel Electrophoresis and Radiation Counting. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and staining for protein were performed according to the methods of Fairbanks et al. (1971). Molecular weight standards employed included bovine serum albumin, ovalalbumin and chymotrypsinogen, all 3× crystallized, and whole erythrocyte membrane proteins. The distribution of radioactivity in gels was determined by slicing 12-cm gels into approximately 80 sections using a jig and scalpel blade. For analysis of tritium-labeled samples, the slices were extracted overnight at 60° with 0.5 cm^3 of a 10:9:1 mixture of toluene, Protosol, and water, after which 10 cm^3 of scintillator (toluene–Liquiflor–Protosol, 20:1:2) was added and samples were incubated an additional 24 hr at 25°. Tritium samples were counted on a Packard Tri-Carb with automatic external standardization. Iodine was determined with a Nuclear-Chicago γ counter.

Autoradiography. Autoradiograms of ^{131}I-containing gels were obtained on longitudinal sliced sections or on gel slabs according to the methods described by Knauf et al. (1974). Molecular weight standards for autoradiography were prepared by reductive alkylation of 3× crystallized albumin, ovalbumin, and chymotrypsinogen with [^{14}C]formaldehyde (Rice and Means, 1971).

Materials. Tissue culture supplies including medium RPMI 1640, fetal calf serum, and Dulbecco's buffered salt solutions were purchased from Grand Island Biological Company. Medium α was obtained from the Ontario Cancer Research Institute. Tritiated borohydride (100 mCi/mmol), radioiodine ($^{125}I \sim 17$ Ci/mg, $^{131}I \sim 25$ Ci/mg), and scintillation solvents were obtained from New England Nuclear. Lactoperoxidase, sialidase, and Pronase (45 U/mg) were purchased from Calbiochem, while galactose oxidase and 3× crystallized trypsin (10,000 U/mg) and chymotrypsin (50 U/mg) were purchased from Sigma. All other chemicals were of the highest grade commercially available and were used without further purification.

Results

(1) *Membrane Characterization.* A key step in the analysis of cell surface polypeptides is the preparation of a subcellular fraction substantially enriched in plasma membrane material. Evidence that this has been attained in the present instance is presented in Table I. The levels of the plasma membrane marker enzyme $Na^+ + K^+$-ATPase have been enriched by about tenfold, while the levels of intracellular markers have been substantially reduced. A purification of tenfold is less than that achieved by some workers (Charlampos et al., 1973), but it does allow the discrimination of components which are characteristic of the membrane.

Electrophoretograms of equal amounts of protein from the plasma membrane and whole cell homogenate show that certain bands are distinctly enriched in the membrane fraction and thus may be polypeptides characteristic of the membrane (Figure 1). Other bands are relatively depleted

Table II: Apparent Molecular Weights of Proteins Enriched in the Plasma Membrane Fraction (See Figure 1).

Designation	Mol wt	Designation	Mol wt
1	200,000	5	105,000
2	180,000	6	42,000
3	165,000	7	37,000
4	155,000		

Table III: Distribution of Surface Label Radioactivity in Subcellular Fractions.[a]

Surface Label Technique	Cell Fraction	% Total Protein	% Total Radio-act.	cpm/mg of Protein
[^{125}I] Lactoperoxidase	Homogenate	100.0	100.0	27,466
	Supernatant	32.2	1.5	1,299
	Pellet	46.5	117.0	
	Membrane			64,522
Galactose oxidase	Homogenate	100.0	100.0	5,500
[^{3}H] borohydride	Supernatant	36.5	7.0	1,106
	Pellet	48.5	82.0	
	Membrane			37,500
Pyridoxal-P–	Homogenate	100.0	100.0	16,158
[^{3}H] borohydride	Supernatant	45.5	14.0	5,110
	Pellet	48.4	110.0	
	Membrane			34,100

[a] Cells were treated with surface label reagents as described under Materials and Methods, and washed free of excess radioactivity. A portion of each sample was used to prepare a plasma membrane fraction via two cycles of the aqueous polymer separation method. Another portion was homogenized to 90% breakage in 5 m*M* $MgCl_2$ and centrifuged at 39,000 rpm for 1 hr in an IEC SB283 rotor, and the pellet and supernatant were recovered. The amount of protein and the Cl_3CCOOH precipitable radioactivity were determined for the fractions. The pellet of the 39,000 rpm centrifugation contains plasma membranes, nuclear material, and other particulate material.

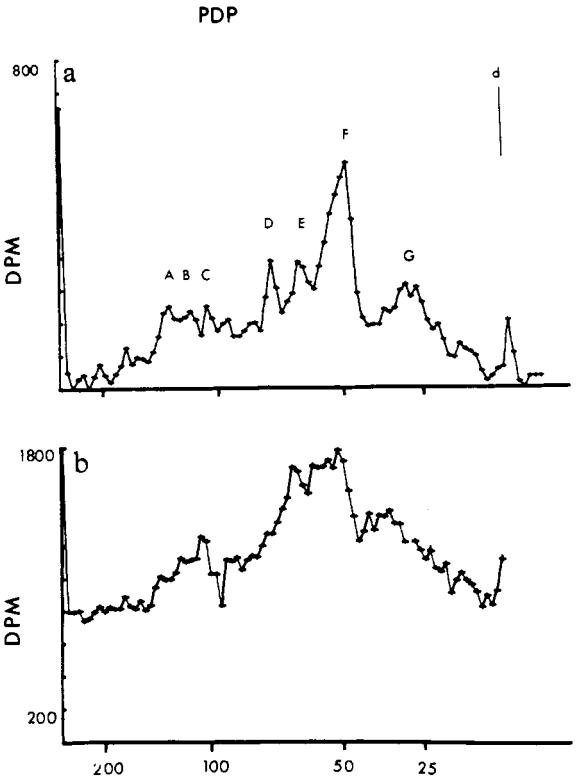

FIGURE 2: Gell electrophoresis of fractions derived from cells labeled by the pyridoxal-P–borotritide method. The ordinate is normalized on the basis of the total radioactivity per gel. A molecular weight scale is given on the abscissa and the position of the dye (d) marker is indicated: (a) purified plasma membrane fraction; (b) whole cell homogenate. The acrylamide concentration was 5.6% and the labeling was done at 37°.

in the membrane and may represent polypeptides from other cellular loci, while yet another set of bands shows equal intensity in the two preparations and may represent components which are shared by the plasma membrane and by other cellular organelles. The possibility of nonmembrane protein segregating with the plasma membrane fraction as an artifact of subcellular fractionation must also be considered. The molecular weights of selected cell polypeptides are shown in Table II.

(2) *Surface Label Studies.* (a) Localization of Labeled Components at the Cell Periphery. The three surface label techniques employed here primarily label components exposed at the cell periphery. This is shown by the experiments presented in Table III where intact cells were treated with surface label reagents and then subjected to subcellular fractionation. The high-speed supernatant fraction, which presumably derives largely from soluble cytoplasmic components, contained 32–46% of the total cell protein, but only a small percentage of the incorporated radioactivity. In addition, the cytoplasmic fraction had a specific activity (counts per minute per milligram) many fold lower than that of the plasma membrane fraction. Both of these observations indicate that the surface label techniques used here primarily radiolabel components which are exposed at the outer surface of the CHO cell and do not readily label internal components. In the experiments described here, the lactoperoxidase system resulted in the lowest degree, and the pyridoxal-P technique in the highest degree, of undesirable internal labeling (see Juliano and Behar-Bannelier (1975) for a further discussion of this issue).

(b) Pyridoxal Phosphate–Sodium Borotritide. When intact cells are labeled with this system, then subjected to cell fractionation and the fractions analyzed by gel electrophoresis, the following patterns are observed. Whole cell homogenates display a diffuse distribution of radioactivity indicating a heterogeneous array of labeled components, with broad peaks centering on molecular weights of about 120,000, 60,000, and 35,000. This result is similar to those observed by previous investigators in the L cell line (Hunt and Brown, 1974). By contrast, the plasma membrane fraction displays a complex, but distinct series of seven peaks, which we have chosen to label A–G (see Figure 2a,b). These components were consistently observed in a series of experiments; in addition, a few minor peaks were occasionally observed in individual instances. The apparent molecular weights of peaks A–G were maintained when samples were electrophoresed on gels of different acrylamide concentrations. The most distinctive membrane components of cells labeled with pyridoxal-P and borotritide are peaks D, E, F, and G of apparent molecular weights 69,000, 59,000, 44,000, and 29,000, respectively. These components were intensely labeled during short (5 min) incubations with pyridoxal-P at 37 or at 20°. Labeling with [^{3}H]borohydride without prior pyridoxal-P treatment results in a low degree of incorporation; the labeling is confined to a substantial peak which co-migrates with the dye marker and a minor peak in the 65,000–70,000 mol wt range. The observation of a diffuse labeling pattern in homogenates, as opposed to a more sharply defined pattern, and the enrichment of certain

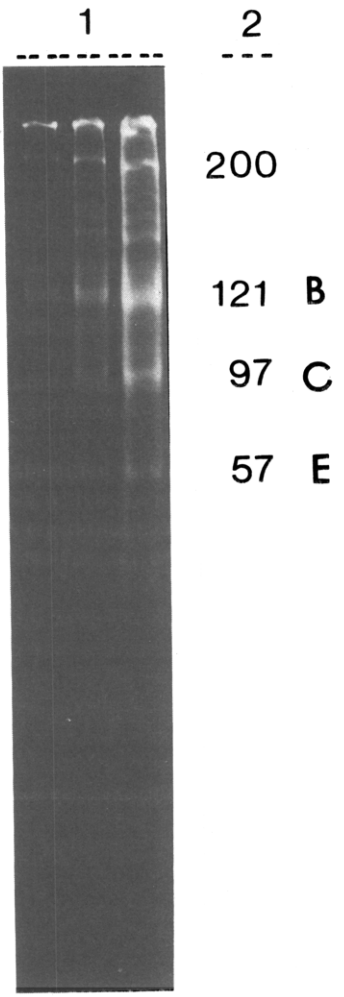

FIGURE 3: Autoradiograms of gels of plasma membranes derived from cells labeled with ^{131}I by the lactoperoxidase method. Slab gel electrophoresis showing (1) lactoperoxidase labeled bands and (2) a molecular weight scale ($\times 10^{-3}$). The photograph illustrates some of the fine structure which is not apparent in gels sectioned and counted for radioactivity.

labeled peaks (D, E, F, G) in purified membranes, are quite characteristic of surface label techniques as applied to CHO cells (Juliano and Behar-Bannelier, 1975). Because of the complexity of the pyridoxal-P induced labeling pattern, further analysis utilizing proteolytic dissection was not successful.

(c) Lactoperoxidase. Plasma membranes derived from intact cells radioiodinated with lactoperoxidase display an electrophoretic pattern consisting of three intensely labeled components and a number of lesser components. The major components have apparent molecular weights of 121,000, 97,000, and 57,000, while other components are approximately 200,000, 158,000, 44,000, and 28,000 in size and may be most readily visualized in autoradiograms (Figure 3). The 200,000 mol wt component which is quite prominent in autoradiography seems smaller in some fractionated gels (Figure 4a), but is fairly prominent in other experiments. This variability may be due to the fact that this peak is very sensitive to proteolysis (our unpublished observations) and may have been degraded during cell fractionation in some experiments. Lactoperoxidase labeled membrane components which coincide in molecular weight with pyridoxal-P–borotritide labeled components are designated by the same letter. The pattern of lactoperoxidase induced labeling was the same in membranes prepared by two cycles of the aqueous polymer separation, as in membranes further purified by an additional polymer separation and by a sucrose step gradient.

Proteolytic dissection of intact cells labeled by the lactoperoxidase technique reveals that component B is particularly sensitive to tryptic digestion. There is also an indication of a reciprocal relation between the peak heights of components B and F, suggesting that F may be a proteolysis product of B (Figure 4a,b). Digestion of intact labeled cells with chymotrypsin and Pronase results in the progressive loss of peaks B, C, and E and the accumulation of labeled low molecular weight proteolysis products (Figure 4c,d). However, no selectivity of proteolysis is demonstrable and there is little indication of the accumulation of distinct products of moderate molecular weight as is the case during proteolytic dissection of the erythrocyte membrane (Juliano, 1973). Digestion with trypsin for longer periods of time also results in the gradual degradation of peaks C and E, as well as the loss of peak B.

The specificity of proteolytic dissection for cell surface components is shown by the fact that, under the conditions used in this study, the enzymes do not degrade proteins which are presumably located at the cell interior. For example, component 2 of Table II may be a membrane polypeptide which is not exposed at the cell surface since it is enriched in the membrane fraction, but not reactive with surface label reagents. This component remains undegraded in membranes isolated from cells exposed to proteases under the conditions specified under Materials and Methods and in the figure legends. Likewise, the protein patterns of homogenates from intact cells treated with proteases are essentially identical with the control patterns, as one would expect, since the majority of the cell polypeptides stem from interior locations and thus are not exposed to proteolytic attack.

Homogenates of lactoperoxidase labeled normal cells show the same peak pattern as the membrane with the addition of a few peaks at low molecular weight, but the major membrane components (B, C, E) are less prominent and there is a much higher background of diffuse labeling. The low molecular weight peaks may represent labeled internal components from nonviable cells.

(d) Galactose Oxidase. Membranes from intact cells labeled with the galactose oxidase borotritide system possess an electrophoretic pattern consisting of four major peaks of molecular weights 139,000, 101,000, 64,000, and 54,000. These components are designated A, C, D, and E and they correspond to components of the same designation visualized by treatment with lactoperoxidase and pyridoxal phosphate–borotritide (see Figure 5a).

Tryptic digestion reveals that none of the labeled components is selectively sensitive to the enzyme (Figure 5a,b). This test readily discriminates components A and B, although they are close in molecular weight, since the latter component, visualized by lactoperoxidase treatment, is selectively digested by trypsin, while the former is not. Digestion with less specific proteases such as chymotrypsin reduces peaks A, C, D, and E with no evidence of selectivity (Figure 5c,d). It should be noted that there is no evidence of the accumulation of labeled low molecular weight proteolysis products, as in the case of digestion of lactoperoxidase labeled cells. This suggests that proteolytic enzymes cleave surface glycopeptides at a locus between the site of galactose oxidase induced labeling and the surface of the mem-

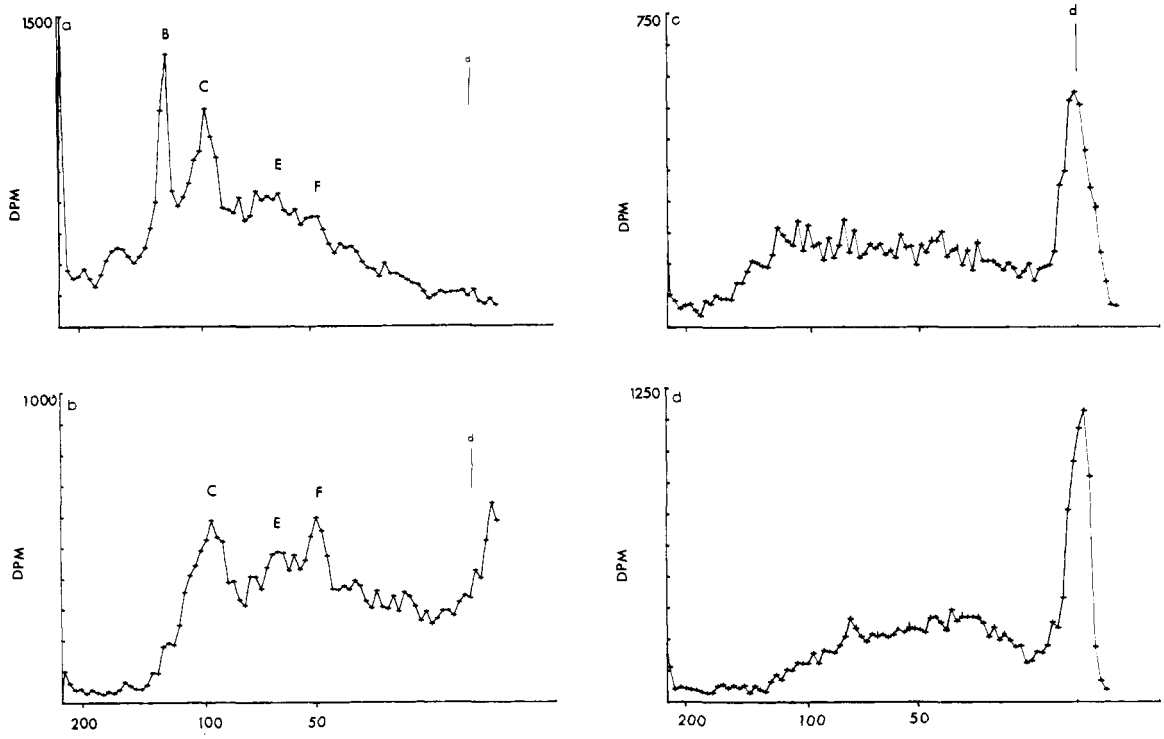

FIGURE 4: Gel electrophoresis of plasma membranes from cells labeled by the lactoperoxidase method and further treated with proteolytic enzymes. The abscissa shows a molecular weight scale, and the position of the Bromophenol Blue (d) marker is indicated. The gel concentration was 5.6% throughout. ^{131}I was used in a and b, while ^{125}I was used in c and d: (a) control (82,000 dpm/gel); (b) treated with 100 μg/ml of trypsin for 5 min at 37° (55,000 dpm/gel); (c) treated with 1 mg/ml of chymotrypsin for 30 min at 37° (18,000 dpm/gel); (d) treated with 100 μg/ml of Pronase for 30 min at 37° (25,000 dpm/gel).

brane and that the labeled proteolysis products are released from the cells.

As we have reported previously (Juliano and Behar-Bannelier, 1975), the labeling pattern of whole cell homogenates from galactose oxidase treated cells has a broad major peak at 35,000 mol wt and is quite different from the pattern of the membrane (Figure 5e). The major peak of the whole cell homogenate may represent surface components which are lost upon plasma membrane isolation. However, we feel that they more likely represent labeled cytoplasmic components from the interior of the subpopulation of nonviable cells (Juliano and Behar-Bannelier, 1975). This observation reinforces the need for cell fractionation studies in conjunction with surface label experiments. It can be seen (Figure 5c,e) that components A, C, and D are more prominent in the purified membrane fraction than in the homogenate. Treatment with borotritide alone results in a pattern showing a rapidly migrating peak ($R_f > 1.0$) which presumably is lipid, as well as a small peak at mol wt 64,000. This latter peak is substantially enhanced in samples pretreated with galactose oxidase.

(e) Correspondence of Labeled and Protein Stained Components. In order to compare the pattern of radioactivity introduced by a surface label with the polypeptide pattern of the membrane, a procedure involving autoradiography of stained, dried gel sections was employed. CHO cells were labeled with ^{131}I using the lactoperoxidase method. Membranes were prepared and run on dodecyl sulfate gels and the gels fixed and stained for protein (Fairbanks et al., 1971). The stained gels were then dried and used for autoradiography (Knauf et al., 1974). The results, presented in Figure 6, revealed that two of the major iodinated bands (B and C) do not correspond to major protein staining bands. Thus, either the labeled bands are quantitatively minor components in terms of membrane protein, or they fail to stain well with Coomassie Blue dye. This latter alternative is frequently true in the case of glycoproteins (Juliano, 1973).[2] The third major iodinated band (E) corresponds with a protein band which is prominent in the plasma membrane (see Figure 1). Some of the other iodinated bands also seem to corrrespond to protein components; thus, the sharp band at mol wt 200,000 corresponds to band 1 which is a component definitely enriched in the plasma membrane fraction (see Table II).

(f) Summary of Surface Label Experiments. The results of our studies employing surface labels and proteolytic dissection are summarized in Table IV. As before, components which are reactive with more than one of the surface label reagents and which coincide in molecular weight are given the same designation. The five components A–E are reactive with at least two surface label systems and are cleaved by proteolytic digestion. Two other components F and G are labeled strongly in the pyridoxal-P borotritide system, but are not intensely labeled by the other reagents. The proteolytic sensitivity of these two components is unknown. Autoradiography of lactoperoxidase labeled samples reveals a sharp band of mol wt 200,000 which corresponds with a protein component which is enriched in the plasma membrane fraction (Figure 6). We have observed that this band is very sensitive to tryptic digestion of intact cells (our un-

[2] The region between 90,000 and 130,000 mol wt is heavily labeled in gels of membranes prepared from CHO cells which have been allowed to incorporate [^{14}C]glucosamine (Juliano and Gagalang, 1975). Thus, peaks B and C (mol wt 121 and 97,000) may well be glycosylated proteins.

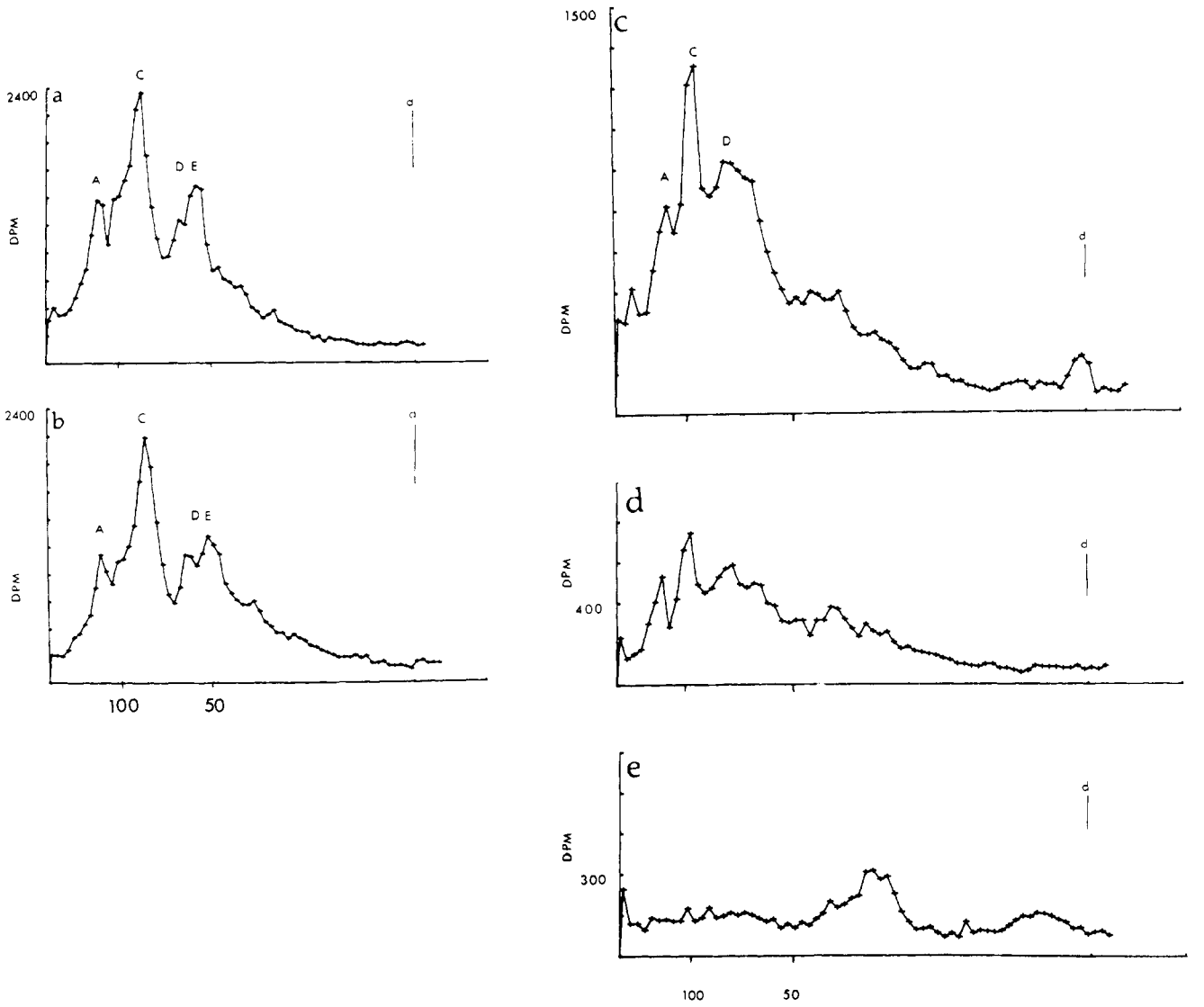

FIGURE 5: Gel electrophoresis patterns of galactose oxidase treated cells and membranes: (a) plasma membrane control; (b) plasma membranes from cells post-treated for 5 min at 37° with 100 μg/ml of trypsin; (c) control plasma membranes; (d) plasma membranes from cells post-treated for 30 min at 37° with 1 mg/ml of chymotrypsin; (e) control whole cell homogenate. In c, d, and e, the ordinates are normalized in terms of micrograms of protein per gel, and in a and b in terms of counts per minute per gel. The gel concentration is 7.5% throughout.

published observations). Several minor components are also visualized by autoradiography of radioiodine labeled samples (Figures 3 and 6). In most experiments, some material appears at the top of the gel. We believe this is an aggregate rather than a high molecular weight species, as it can be reduced by centrifugation of the solubilized membranes. The amount of aggregated material is less in preparations from intact cells (Figure 4a,b) which have been exposed to proteases, but the significance of this observation is unclear.

Conclusions

We have adopted a multifaceted approach to the study of membrane organization in nucleated mammalian cells, employing conventional cell fractionation techniques, as well as the newer tool of surface labeling. Our results have certain implications concerning the surface label approach to membrane structure in general as well as implications for the specific problem of the organization of the CHO cell membrane.

Let us first deal with the general problem of surface labeling. As we see in Table IV, the set of membrane components most reactive with one surface label reagent, such as lactoperoxidase, may be quite different from the set of components most reactive with another surface reagent, such as pyridoxal phosphate or galactose oxidase. This is not surprising as the reagents differ in chemical specificity as well as in size, and thus may be reactive with, or have access to, different groups at the cell periphery. There are some components, C and E for example, which are labeled by all reagents, but other components such as the 200,000 mol wt polypeptide and component A are well labeled by one reagent, but not by others. These results illustrate the concept that studies employing a single surface label technique may lead to spurious results. On the one hand, a particular reagent may fail to label an important component (as apparently is the case for component A with lactoperoxidase), but on the other hand, a reagent may have a particularly strong reaction with a minor surface component, or with a contaminant, thus giving a misleading picture of its importance (this may be true in the case of component G). Such spurious findings may be minimized by the application of several surface label systems to the study of a particular cell type.

Table IV: Apparent Molecular Weights and Protease Sensitivity of Polypeptides Reactive with Surface Labels.[a]

Designation	Mol Wt × 10^{-3}				
	Pyridoxal-P	Lactoperoxidase	Galactose Oxidase	Trypsin	Chymotrypsin or Pronase
		200+		+++	+
A	137 ± 8 (*n* = 5)		*139 ± 8* (*n* = 4)	+	+
		158+			
B	121 ± 6 (*n* = 4)	*121 ± 3* (*n* = 3)		+++	+
C	106 ± 3 (*n* = 6)	*97 ± 4* (*n* = 3)	*1.01 ± 3* (*n* = 4)	+	+
D	*69 ± 3* (*n* = 5)		*64 ± 2* (*n* = 4)	+	+
E	*59 ± 2* (*n* = 6)	*57 ± 3* (*n* = 3)	*55 ± 3* (*n* = 4)	+	+
F	*44 ± 3* (*n* = 6)	42+			?
G	*30 ± 2* (*n* = 5)	28			?

[a] The peaks which are most intensely labeled and which are most prominent in the plasma membrane fraction relative to the homogenate are in italics. The molecular weight values presented here were determined using 5.6% gels.

It seems more likely that a polypeptide may truly be located at the cell periphery if it is reactive with several surface label reagents of differing chemical specificity and physical properties.

The need for cell fractionation studies in association with surface label studies is brought out by inspection of Figure 2 and Figure 5. The surface label pattern of the whole cell homogenate is invariably more diffuse than that of the isolated membrane, suggesting greater heterogeneity of labeled components in the homogenate. Moreover, at least in the case of the galactose oxidase system, the major labeled component in the homogenate is completely absent from the isolated membrane and may not represent a membrane component at all. Thus, subcellular fraction must go hand in hand with surface label studies (Juliano and Behar-Bannelier, 1975).

Now let us turn to the question of the organization of the CHO cell membrane. In the discussion which follows, we make what seems to us to be a reasonable assumption concerning the identity of labeled components. We assume that if two surface label systems result in major peaks of activity which are coincident in molecular weight, then the coincident peaks most likely represent the same polypeptide or glycopeptide component. We realize the possible existence of two or more components which are close in molecular weight, but are reactive with different surface labels. We cannot rule out this possibility, but we can argue against it with the observation that peaks which are coincident remain so on gels of different acrylamide concentration.[3] The question of molecular heterogeneity among labeled components very close in molecular weight remains open in this study, as it does in almost all surface label studies, including those concerning the erythrocyte membrane (Cabantchik and Rothstein, 1974).

In the introductory statement we formulated three criteria for the identification of cell surface polypeptides. These criteria are as follows: (a) reactivity with two or more surface labels; (b) enrichment of the reactive component in the plasma membrane fraction relative to the whole cell homogenate; (c) susceptibility to digestion by proteases applied to intact cells. From the data in Table IV, we see that five of the labeled components satisfy all three criteria; these are components A, B, C, D, and E. In all probability then, these entities represent polypeptides which are exposed at the outer surface of the cell. Components A, C, D, and E are reactive with the galactose oxidase system and thus are presumably glycopeptide in nature. Two other components, F and G, are reactive primarily with one reagent, namely pyridoxal phosphate. These labeled peaks are enriched in the plasma membrane and they coincide in molecular weight with bands which stain for protein and which are present in the membrane. Thus, it seems likely that F and G are mem-

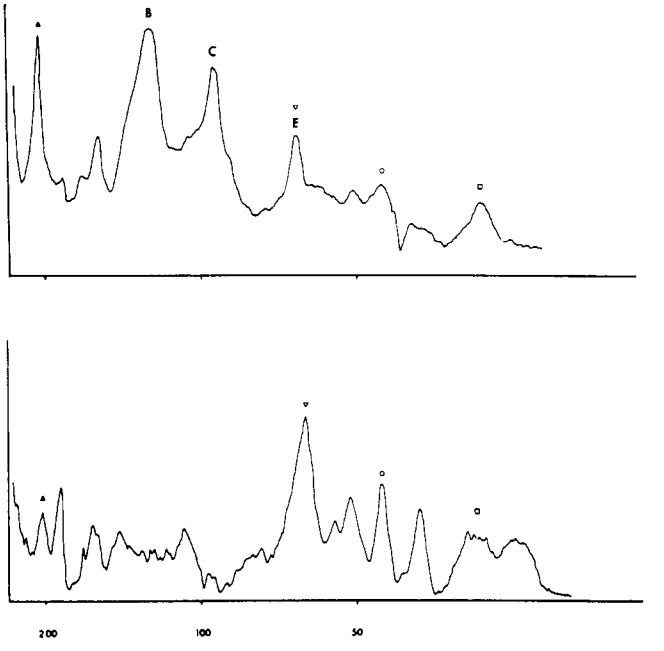

FIGURE 6: Protein staining patterns and autoradiograms of sodium dodecyl sulfate gels of CHO cell plasma membranes. Membranes from CHO cells labeled with ^{131}I by the lactoperoxidase method were run on 5.6% slab gels; the gels were stained for protein and prepared for autoradiography. This approach allows a direct comparison of the staining pattern and labeling pattern. Photographic negatives of the dried and stained gels and of the corresponding autoradiograms were scanned on a densitometer. Band designation follows Tables II and IV; corresponding bands are indicated by symbols: (upper pattern) autoradiogram; (lower pattern) Coomassie Blue stain. In this experiment, the membranes were prepared by two cycles of aqueous polymer separation.

[3] Component C has a somewhat lower molecular weight (95,000) when electrophoresed on 7.5% acrylamide gels than when electrophoresed on 5.6% gels (101,000). This component seems to contain a good deal of carbohydrate, as evidenced by the intense labeling with galactose oxidase and, thus, may show anomalous migration behavior in sodium dodecyl sulfate gels (Juliano, 1973).

brane polypeptides, but we cannot definitely assign them to a position at the cell periphery. The 200,000 molecular weight component, which is prominently labeled in lactoperoxidase treated samples, is not readily visualized by the other two surface label techniques. Nonetheless, this component is probably a cell surface polypeptide since it is very sensitive to tryptic digestion and it corresponds to a polypeptide which is markedly enriched in the plasma membrane fraction. In Table II, one finds several polypeptides such as 2 and 7 which are quite enriched in the plasma membrane, but which do not correspond in molecular weight with one of the components strongly reactive with surface labels. It seems possible, then, that these unlabeled components represent membrane polypeptides which are not exposed at the cell surface.

Our findings suggest that at least five, and possibly as many as eight, distinct components can be detected at the CHO cell surface with the three labeling techniques employed in this study. One must bear in mind that some of these components may, upon further investigation, prove to be heterogeneous. These results may be compared with the findings of previous workers who have discriminated five surface components on HeLa cells (Huang et al., 1973), and about the same number on hamster fibroblasts (Hynes, 1973) by lactoperoxidase labeling, while five–six components have been visualized on mouse and hamster fibroblasts by galactose oxidase labeling (Gahmberg and Hakamori, 1973a,b). In common with a previous study (Hynes, 1973), we have found certain labeled surface components (mol wt 200,000 and 121,000) which are selectively sensitive to tryptic cleavage. One should note that the previous studies cited usually relied on a single labeling reagent, and in some cases no cell fractionation was performed. Thus, in the light of the discussion above, some of the components reported by previous workers may not be truly associated with the cell surface. In any case, it seems likely that the surfaces of cultured cells may be more complex than those of red cells (Juliano, 1973) or fat cells (Czech and Lynn, 1973) where two–five components seem to be present at the cell periphery.

The conclusions presented above are subject to several limitations. Firstly, there may be other surface polypeptides which are simply not reactive with the reagents used and thus have not been identified. Secondly, we can say nothing concerning the association of the membrane polypeptides into oligomeric units, nor can we assess the degree to which they penetrate or traverse the lipid bilayer. Thirdly, in the present study we have examined the membranes of asynchronous exponentially growing cultures, and thus the surface configuration described here represents a sample averaged over the cell cycle. Other studies (Hynes and Bye, 1974; Mastro et al., 1974) have suggested that the surface protein pattern of fibroblasts may vary as a function of the growth state and state of attachment of the cells. Electron microscopic studies of CHO cells have revealed that substantial surface changes occur during the cell cycle (Porter et al., 1973). Thus, subtle differences in labeling patterns between experiments may be a reflection of cellular growth parameters. In addition, one might expect that the surface configuration at one particular stage of the cell cycle might be quite distinct from that at another stage. Finally, we must consider the possibility that some serum proteins from the culture media may be so firmly attached to the cell surface as to resist extensive washing, and thus may be visualized by surface labeling techniques, although this was not found to be a problem in other surface label studies (Hynes, 1973). Given these limitations, our observations provide a first, rough view of the molecular organization of the CHO cell surface. Hopefully, further studies will lead to a more refined analysis.

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References

Bauer, S., Schenkein, I., and Uhr, J. W. (1972), *J. Immunol. 180,* 748.

Brunette, D. M., and Till, J. E. (1971), *J. Membr. Biol. 5,* 215.

Cabantchik, Z. I., and Rothstein, A. (1974), *J. Membr. Biol. 15,* 227.

Charlampos, F. C., Gonatas, N. C., and Melbourne, A. D. (1973), *J. Cell Biol. 59,* 421.

Czech, M. P., and Lynn, W. S. (1973), *Biochemistry 12,* 2597.

Fairbanks, C., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Gahmberg, C. G., and Hakamori, S. I. (1973a), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3329.

Gahmberg, C. G., and Hakamori, S. I. (1973b), *J. Biol. Chem. 248,* 4311.

Gates, L., and Morrison, M. (1974), *Exp. Cell Res. 83,* 344.

Huang, C. C., Tsai, C. M., and Cannelakis, E. S. (1973), *Biochim. Biophys. Acta 332,* 59.

Hubbard, A. L., and Cohn, Z. A. (1972), *J. Cell Biol. 55,* 390.

Hunt, R. C., and Brown, J. C. (1974), *Biochemistry 13,* 22.

Hynes, R. O. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3170.

Hynes, R. O., and Bye, J. (1974), *Cell 3,* 113.

Juliano, R. L. (1973), *Biochim. Biophys. Acta 300,* 341.

Juliano, R. L. (1974), *Exp. Cell Res. 86,* 181.

Juliano, R. L., and Behar-Bannelier, M. (1975), *Biochim. Biophys. Acta 375,* 249.

Juliano, R. L., and Gagalang, E. (1975), manuscript in preparation.

Juliano, R. L., and Mayhew, E. (1972), *Exp. Cell Res. 73,* 3.

Kimelberg, H. K. and Papahadjopoulos, D. (1972), *Biochim. Biophys. Acta 282,* 277.

Kinzel, V., and Mueller, G. C. (1973), *Biochim. Biophys. Acta 322,* 337.

Knauf, P. A., Proverbio, F., and Hoffman, J. F. (1974), *J. Gen. Physiol. 63,* 305.

Mackler, B. (1967), *Methods Enzymol. 10.*

Mastro, A., Beer, C. T., and Mueller, G. (1974), *Biochim. Biophys. Acta 352,* 38.

Merrel, R., and Glaser, L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2794.

Phillips, D., and Morrison, M. (1970), *Biochem. Biophys. Res. Commun. 40,* 284.

Phillips, D., and Morrison, M. (1971), *Biochemistry 10,* 1766.

Poduslo, J., Greenberg, C. S., and Glick, M. S. (1972), *Biochemistry 11,* 2616.

Porter, K., Prescott, D., and Frye, J. (1973), *J. Cell Biol.*

57, 815.
Reid, E. (1972), in "Subcellular Components", Birnie, G. D., Ed., London, Butterworths.
Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246,* 831.
Rifkin, D. B., Compans, R. W., and Reich, E. (1972), *J. Biol. Chem. 247,* 6432.
Schneider, W. C. (1957), *Methods Enzymol. 3.*
Shin, B. C., and Carraway, K. L. (1973), *Biochim. Biophys. Acta 330,* 245.
Steck, T. L. (1972), in "Membrane Research", Fox, F., Ed., New York, N.Y., Academic Press.
Steck, T. L., Fairbanks, C., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2617.
Thompson, L. H., and Baker, R. M. (1973), *Methods Cell Biol. 6,* 209.

Chemical and Physical Properties of the Disulfides of Bovine Neurophysin-II†

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ABSTRACT: Bovine neurophysin-II is shown to be very susceptible to partial reduction in the absence of urea. Reduction of an average of one disulfide leads to major changes in conformation and disulfide optical activity, manifest in part by pronounced far-uv ellipticity changes, complete loss of the 248-nm ellipticity band, and a shift of the 278-nm ellipticity band to shorter wavelengths with loss of half its intensity; the reduction process generates a mixture of products and appears to be accompanied by disulfide interchange. The circular dichroism data indicate that the disulfide(s) most susceptible to reduction or interchange are either the principal contributors to the 248- and 278-nm ellipticity bands or that the optical activity of other disulfides is dependent on their integrity. Peptides that bind to the hormone-binding site of neurophysin-II protect against reduction. On reoxidation of partially reduced neurophysin-II there is only a partial return of the native circular dichroism spectrum and electrophoretic behavior. The percentage of native protein in samples reoxidized following different degrees of reduction was estimated by comparison of the circular dichroism spectra of these samples with those of the fractionated native and denatured components of monoreduced-reoxidized neurophysin. Under our reoxidation conditions, less than 50% native protein was found in monoreduced-reoxidized neurophysin and less than 10% native protein was found in completely reduced-reoxidized neurophysin. The results are interpreted with qualified reference to a model in which one or more disulfides are "strained" in the native state and in which the native protein is unstable relative to species in which the disulfides are differently paired.

The neurophysins, carrier proteins for oxytocin and vasopressin within the neurohypophyseal system, are rich in disulfides, bovine neurophysins each having seven disulfide bonds per monomer of 10,000 molecular weight; additionally, the neurophysins have a low content of aromatic amino acids, the bovine proteins containing no tryptophan, one tyrosine, and three phenylalanine residues per monomer (Rauch et al., 1969; Breslow et al., 1971; Schlesinger et al., 1972). The optical activity of neurophysin disulfides can therefore be defined with less ambiguity than with most other proteins, and two neurophysin near-uv ellipticity bands, at 278 and 248 nm, respectively, have been assigned almost exclusively to disulfide transitions (Breslow, 1970). Relationships between disulfide geometry, environment, and optical activity are inadequately understood. In particular, on the basis of existing theory, it is not possible a priori to evaluate the contribution of any single neurophysin disulfide bond to the 248- and 278-nm ellipticity bands. For example, the wavelength of an individual disulfide transition in the near-uv depends on the disulfide dihedral angle (Barltrop et al., 1954; Bergson et al., 1962; Beychok, 1965) and the sign of the longest wavelength ellipticity band depends at least in part on the disulfide screw sense (Beychok, 1965; Carmack and Neubert, 1967; Claeson, 1968; Imawishi and Isemura (1969). However, due to the compound nature of the near-uv disulfide transition, a single disulfide can exhibit one or two near-uv ellipticity bands of opposite sign, the number, position, and relative intensity of the two bands being dependent on geometry and environment (Linderberg and Michl, 1970; Casey and Martin, 1972; Sears and Beychok, 1973).

In an attempt to resolve the contributions of different neurophysin disulfides to neurophysin near-uv optical activity, we have studied the effect of varying degrees of reduction on neurophysin circular dichroism (CD) spectra. In the course of these studies, we observed that neurophysin is highly susceptible to partial reduction and we therefore additionally probed aspects of the reversibility of the reduction–reoxidation process. Studies reported here were carried out with bovine NP-II,[1] but preliminary parallel studies

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[1] Abbreviations used are: NP, neurophysin; DTT, dithiothreitol; DTE, dithioerythritol.