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Glycoprotein Differences Among Cells of the 14-Day Embryonic Chick Neural Retina

Joel B. Sheffield

Department of Biology, Temple University, Philadelphia, Pennsylvania 19122

In order to test the hypothesis that the progressive layering and differentiation of cell types during the development of the neural retina are associated with cell surface alterations we have separated distinct cell populations from the 14-day embryonic chick retina. Cells of these populations have been shown to differ in associative behavior and intramembrane particle content. We now report that these cells differ in cell surface glycoproteins. Proteins were labeled with two different extrinsic labels and one metabolic label. We used enzymatic transfer of galactose from UDP-gal to cellular acceptors, and borotritide reduction after galactose oxidation as extrinsic labels. Glucosamine incorporation was used as the metabolic label. In all these cases, we were able to identify bands on electrophoretic gels which were unique to individual populations.

Key words: retina, glycoproteins, surface label, cell sorting, embryonic, neural cells

As the neural retina develops, the cells become arranged in layers which are specialized for particular functions. The mechanism of this stratification remains obscure. In order to study the mechanism of this stratification, we have initiated a series of biochemical and morphological studies in the chick retina. It has been shown [1,2] that single cells dissociated from embryonic neural retina and reassociated in cellular aggregates are able to form structures which are analagous to those found in the neural retina, and which continue in development [2-4]. These experiments imply that the ability to form layered structures is, to a significant degree, a property of the individual cells of the tissue. We have proposed that this ability is a consequence of surface differences among the different retinal cells. In support of this hypothesis, we have demonstrated progressive differences in intramembrane particle content among the retinal layers during development [5]. Recently, we have developed a method for the fractionation of the 14-day embryonic chick retina into four cell populations [6,7]. Two of the populations contain greater than 80% of a single cell type, defined on morphological grounds, and the others contain mixtures of cells. Each of the populations develops a characteristic morphology in vitro. Thus the ganglion cell population develops small groups of cells which extend fascicles of axonal processes. We have recently shown [8,9] that cells of the different populations sort out from each other in mixed aggregates.

Since cell sorting behavior may represent a cell surface related activity, we have begun an analysis of the glycoproteins in the surfaces of these isolated cell

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populations. Several radiolabeling procedures have been used. Our results suggest that glycoprotein differences exist among the cells in the different populations.

MATERIALS AND METHODS

1. Tissue Preparation

Retinas were dissected from 14-day-old White Leghorn chicken embryos in Tyrodes solution, transferred to CMF (calcium- magnesium-free Tyrodes [10]) and allowed to incubate at 37°C for 10 min. Dissociation was accomplished by using 1% Trypsin (190 units/mg, Worthington Biochem.) in CMF for 25 min at 37°C. The tissue was rinsed three times with standard culture medium [(MEM) (Flow, Inc.) with glutamine and sodium bicarbonate and supplemented with fetal bovine serum (Flow, Inc.) to 10%, 50 μg/ml deoxyribonuclease (3000 units/mg, Millipore Corp.), 50 μg/ml penicillin-streptomycin, 50 units/ml (Flow), and 2 mM PMSF (phenylmethylsulfonylfluoride, Sigma) as a trypsin inhibitor]. The retinas were then dispersed by aspiration through a fine Pasteur pipet (flame narrowed) 15–20 times.

2. Gradient Preparation

The cells were separated by exploiting buoyant density differences among populations using Percoll (Pharmacia), a high density, low viscosity colloidal silica, and a step gradient as opposed to a linear gradient to facilitate recovery of cells and maintenance of sterility [6,7]. Best separation and maximum survival was attained using standard culture medium with 10% fetal calf serum to dilute the Percoll.

Before use, the Percoll was adjusted to physiological pH and salinity by adding 10 ml of 10 × Hanks' basic salt solution (HBSS) to 90 ml of Percoll. The gradient consisted of an overlay of 20 ml of standard culture medium, three steps (50 ml each) having respective densities of 1.02, 1.036, and 1.043 gm/ml and a 20 ml step of Percoll. Approximate preparations of the individual steps were prepared from varying proportions of standard culture medium and Percoll and the final density adjustments were made with a refractometer. Dissociated cells were mixed with the 1.036 gm/ml step and the gradient stacked in a 250 ml (61 × 122 mm) polycarbonate centrifuge tube.

Centrifugation was performed in an HS-4 rotor on a Sorvall RC-2B centrifuge equipped with a slow acceleration modification. In order to minimize swirling of the low viscosity gradient, acceleration and deceleration were carried out gradually, allowing the rotor to reach 400 rpm in 7 min, followed by rapid acceleration to 2,000 rpm (770g). Cells were allowed to reach density equilibrium for 1 hr at 4°C, and the rotor was then allowed to decelerate slowly to rest. We allowed 15 min for complete deceleration. Cells were removed from the interfaces between steps and designated populations: I (< 1.02), II (> 1.02, < 1.036), III (> 1.036, < 1.043), and IV (> 1.043). After resuspention in culture medium without serum the cell numbers were determined using a Coulter counter Model B and the quality of the separation was examined with a light microscope. To attain maxiumum purity in Fraction I, a second spin was usually required, consisting of one step of 30 ml and a density of 1.08 gm/ml, on a Sorvall HB-4 rotor at 650g for 1 hr at 4°C.

3. Cell Culture

Freshly isolated cells were allowed to regenerate their surfaces on a gyrotory shaker at 100 rpm in 3 ml of standard culture medium in a 25-ml stoppered Erlenmeyer flask at a density fo 5 × 10^6 cells/ml at 37°C. The higher shaker speed allowed the cells to remain either single or in small aggregates.

4. Cell Labeling Techniques

a. Galactose transfer. Cells were labeled by transfer of tritiated galactose from UDP-galactose to acceptors on the cell surface with exogenous galactosyl transferase [11] galactosyl transferase (lactose synthase, UDP-galactose: D-glucose-4-β-galactosyl transferase; EC 2.4.1.22) was obtained from Sigma Chemical Company (St. Louis, MO). This enzyme transfers galactose to glucose to form lactose, and also transfers galactose from UDP galactose to N-acetyl glucosamine residues [12]. Tritiated UDP galactose was purchased from Amersham (Arlington Heights, IL). Prior to use, the alcohol was removed from the UDP galactose by vacuum. Cells were rinsed three times in phospate-buffered saline (PBS), pH 7.4, once with 0.25 M MOPS buffer, pH 7.4, and resuspended in 50 μl of MOPS. To this was then added 15 μl of a solution containing 2.25 units of enzyme/ml of MOPS 10 μl of 0.15 M $MnCl_2$, and 10 μl of 3-UDP-[^{3}H]-galactose (μCi/ml). The mixture was incubated for 30 min at 37°C, and the cells were recovered and rinsed several times prior to lysis for counting and electrophoresis.

b. Glucosamine incorporation. Glucosamine containing glycoproteins were labeled by metabolic incorporation of 25 μCi of D-[6-^{3}H]glucosamine hydrochloride (38 Ci/μmole, Amersham) added to rotating cultures of isolated cells. Cells were cultured as above for up to 22 hr and recovered by washing three times in PBS at 4°C.

c. Galactose oxidase, borohydride reduction (GOB). We also used the technique of oxidation followed by reduction with tritiated sodium borohydride, using galactose oxidase as an oxidant. The reactions were performed according to the procedures of [13], and [14], as modified by us [15]. Galactose oxidase was purchased from Sigma Chemical Company and Worthington Biochemicals and screened for contaminating protease activity with a nonspecific protease substrate (Azocoll, Calbiochem, La Jolla, CA) and by comparing sodium dodecyl sulfate-gels (SDS-gels) of known substrates after incubation with the enzyme. We found significant proteolytic activity associated with the enzyme obtained from Sigma, and with certain lots from Worthington. We were not able to separate this activity from the enzyme from gel filtration. However, we were able to obtain a preparation of enzyme from Worthington which was free of detectable protease; this enzyme was used for our study.

In order to retain cell integrity, the reactions were carried out in HBSS to which we added 2 mM PMSF. Fifteen million cells which had been allowed to recover from trypsinization for 24 hr were incubated in serum-free medium for 3 hr, rinsed in HBSS, and resuspended in a volume of 200 μl. Galactose oxidase, 4.5 units, was added and the cells were incubated for 15 min at 37°C. After three rinses in HBSS, the cells were resuspended in 100 μl of 0.01 N NaOH containing 1 μCi of [^{3}H]sodium borohydride and incubated for 15 min at room temperature. The cells were then rinsed three times in HBSS and resuspended in lysis buffer.

5. Electrophoresis

Samples of electrophoresis were initially suspended in a detergent solution [½% DOC (7-deoxycholic acid, Sigma), ½% Nonidet P40 (Bethesda Research Laboratories), 100 mM Tris-HCl, pH 8, 5 mM NaCl, 50 μg/ml RNase (Sigma), 1 mg/ml DNase I (Worthington), 2 mM PMSF] which was stored as frozen aliquots. Lysis was accomplished through two cycles of freezing and thawing and enzymatic digestion was allowed to proceed for a minimum of 4 min at 4°C. Before electrophoresis, the extract was added to an equal volume of buffer containing 2% SDS, 40% glycerol, 1% β-mercaptoethanol, and 0.08 M Tris-HCl, pH 6.8.

Acrylamide gels were prepared as described by Laemmli [16]. Acrylamide gels (7.5%) were used. Equal numbers of dpm were applied to each well and electrophoresis was carried out at 22 mA/gel until the dye marker had reached the bottom. Autofluorographs were prepared by the methods of Laskey and Mills [17]. Exposed films were developed with Kodak DX-80.

Each labeling procedure was performed on at least three independent sets of the cell populations, and the fluorograms were compared for reproducibility. We have discussed only those bands which were consistently visible.

6. Nomenclature

Nomenclature of materials on gels is modified from that used extensively in virus study [18]. Each band is assigned an apparent molecular weight, based on its mobility in SDS-acrylamide gels, and a prefix is attached identifying the band as a protein (p) or glycoprotein (gp). Thus, all of the material that we identify with our labeling procedures will be prefixed with "gp."

RESULTS

The results have been summarized in Table I, although it should be realized that indications of presence (+) or absence (−) are probably an oversimplification of the quantitative differences seen on the gels.

1. Galactose Transfer

Initial studies to determine optimum conditions were performed with freshly dissociated cells from 14-day retinas. Cell populations were not separated out. Cells were placed in culture for varying time periods, and then subjected to the labeling procedure. As can be seen in Table I, it requires 24 hr after trypsinization for cells to incorporate maximal levels of label. We have found, in agreement with [20,21], that a significant fraction of the label that is incorporated is due to cell associated endogenous galactosyl transferase activity. This implies that the results we see are due to a combination of the effects of the endogenous with the exogenous enzymes.

An example of the distribution of glycoproteins labeled with this procedure is presented in Figure 1. The cells were separated into populations, and each population was grown in medium for 24 hr prior to labeling. After labeling, the cells were lysed and electrophoresed. In the fluorogram of Figure 1, the first track contains the molecular weight markers, the next four contain the different fractions, and the final track is an unfractionated retina, trypsinized and cultured as

TABLE I. Summary of Bands Observed in the Different Labeled Preparations

	Gal transfer				Glucosamine				Gal oxidase-BT_4			
Band	I	II	III	IV	I	II	III	IV	I	II	III	IV
gp230	±	−	−	−	+	−	−	−				
gp173	+	+	+	+	+	+	+	+	+	+	+	+
gp115	+	−	−	−								
gp100	+	+	+	+	+	±	±	±	+	+	+	+
gp 93	−	+	+	+	±	+	+	+				
gp 66	+	+	+	+					+	+	+	+
gp 50	−	±	±	+	−	−	−	+				
gp 46	+	+	+	+	+	+	+	+				
gp 44	+	+	−	−								
gp 41	−	−	−	+								

were the others. There are about 20 individual bands visible in each track. Of these, all but 6 are shared by all of the cell populations. Of particular interest are the bands which differ among the populations. These consist of bands gp230, gp115, gp93, gp50, gp44, and gp41. Gp230 is barely detectable in the photographs, but a small amount is visible in population I in the original fluorograms. It is not detectable in the other populations, although it is possible that subthreshold amounts exist. Gp115 is present in population I, but absent from the other three populations. Gp93, on the other hand, is absent from population I and present in the others. The region from 46 to 50 kd molecular weight appears to contain several components. There is an increase in the amount of gp50 and a loss of gp46 as one looks at populations I, II, III, and IV in order. Gp 44 appears to be unique to population I and gp41 is found only in population IV.

In all of the samples, there is a significant amount of material that moves with the dye front. This may be low-molecular-weight components or glycolipid. Wallenfels [11] reports that in the mouse retina, 5% of the incorporated label can be extracted by organic solvents and is lipid. Five percent of the total mixture would be as many counts as in many of the bands we visualize, and therefore would appear as a significant region in the gels.

2. Glucosamine

The time course of incorporation of glucosamine by dissociated unfractionated 14-day retina cells is given in Figure 2. We used a 24-hr labeling period for the isolated cell populations. A typical fluorogram of an SDS-polyacrylamide gel is presented in Figure 3. The arrangement of the tracks is the same as that with galactose transfer. Most of the bands are shared by all of the populations. The overall labeling pattern is similar but not identical to that obtained with galactose transfer. Several of the unique bands detected by galactose transfer, gp230, gp93, and gp50, are also visible in the glucosamine labeled populations. However, gp75, which is intensely labeled by galactose transfer, is not strongly labeled by glucosamine. Other differences can be found. The material of gp100, which appeared to be common to all fractions after galactose transfer, is unevenly distributed among the populations after glucosamine labeling. The labeling of gp100 is significantly less, as a function of the total label, in populations II, III, and IV, than in I.

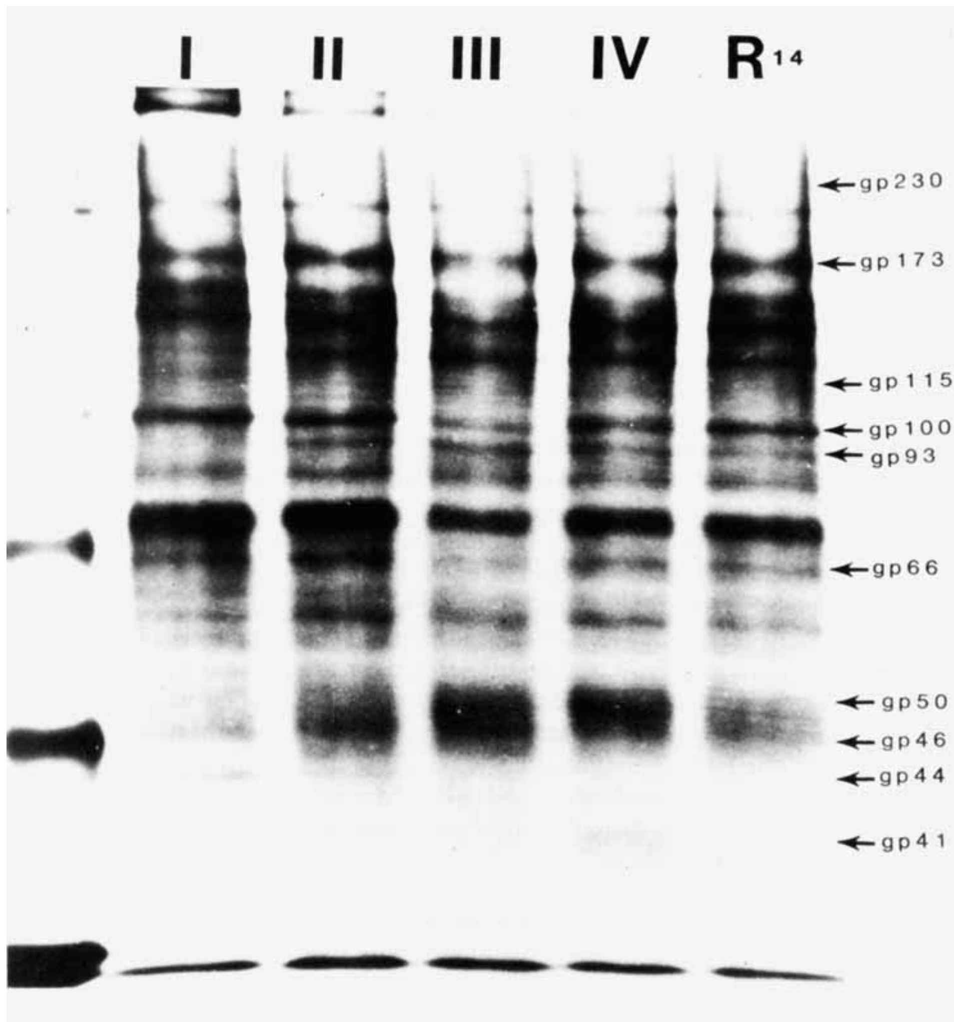

Fig. 1. Radiofluorogram of a 7.5% polyacrylamide gel of glycoproteins labeled by galactose transfer. The different tracks are from left to right; molecular weight markers (myosin, phosphorylase b, bovine serum albumin, ovalbumin), population I, population II, population III, population IV, unfractionated retina.

3. Galactose Oxidase-Borohydride Reduction

The labeling pattern of the four populations after enzymatic galactose oxidation and borotritide reduction is presented in Figure 4. Relatively few bands are clearly visible as compared with the other labeling procedures. Each of these bands has a corresponding region in the other gels. Gp173 appears to be present in lower amounts in population I than in the other populations, whereas gp69 is enriched in fractions I, II, and III. Gp100 is the band that is shared by all populations after galactose transfer but is labeled to a greater extent in population I after glucosamine label.

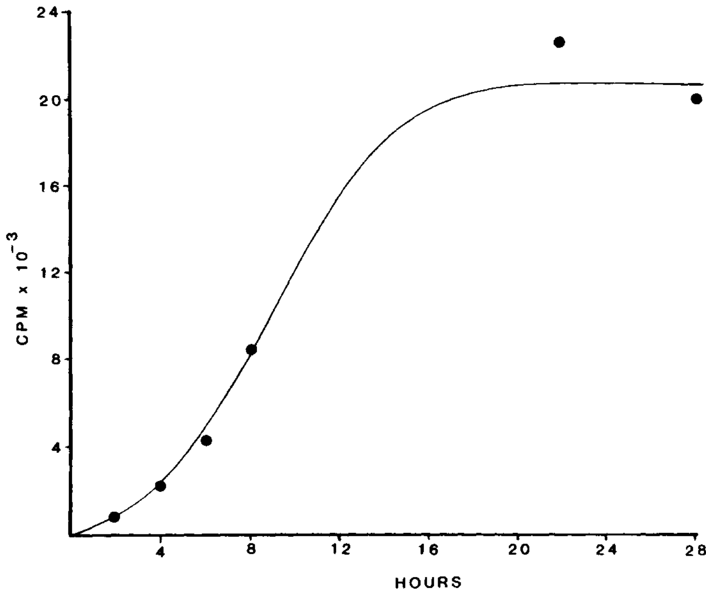

Fig. 2. Incorporation of glucosamine into trichloroacetic acid-precipitable material after trypsinization of 14-day neural retina and culture at various times.

A large amount of label moves in front of the dye marker. This may represent reduction by borohydride of unsaturated bonds in lipid.

DISCUSSION

We have demonstrated that the glycoproteins of different populations of cells from the 14-day embryonic chick neural retina can be labeled by several techniques. When these labeled glycoproteins are separated on SDS-polyacrylamide gels, about 20 different bands can be resolved, depending on the labeling procedures. Of these bands, 6 are unique to certain populations of cells, and the rest are shared by all.

The techniques we have used for labeling depend on different characteristics of the cells for their specificity. Glucosamine is incorporated into newly synthesized glycoproteins during the recovery of cells from trypsinization, and its incorporation is dependent on the metabolism of the cell. Galactose transfer and GOB, on the other hand, are reactions which are mediated by an exogeneous enzyme and do not require cellular metabolism. Each technique introduces radioactive label at different positions in the glycoproteins. Glucosamine is incorporated essentially unchanged into growing polysaccharide chains, while the galactose transfer procedure adds label to terminal N-acetyl glucosamine residues. The galactose oxidase-borotritide reduction procedure introduces tritium into accessible galactose residues. It appears from the fluorograms that some of the glycoproteins we have identified share sites which are accessible to each of the procedures. Gp173 and gp100 can be seen in all three preparations, and gp230 and gp50 are detectable both with glucosamine incorporation and galactose transfer.

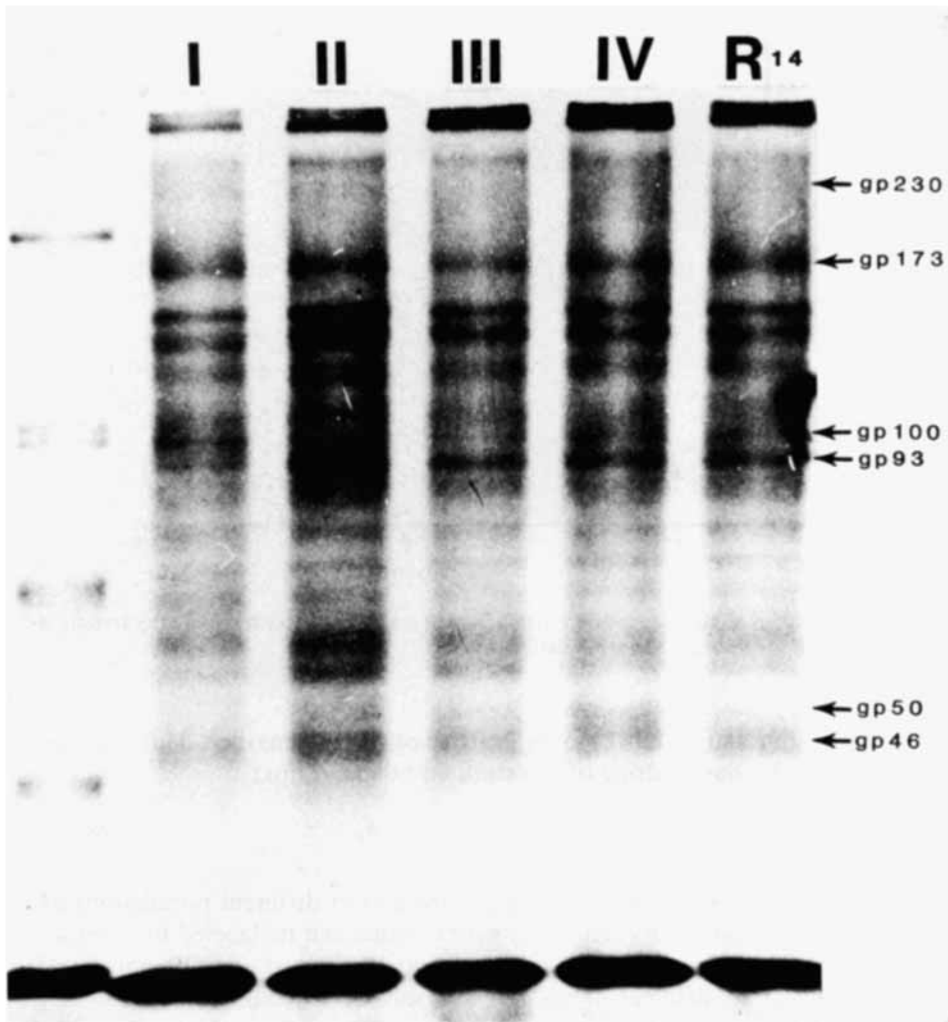

Fig 3 Radiofluorogram of a 7 5% polyacrylamide gel of glycoproteins labeled by glucosamine incorporation The tracks are the same as those for Figure 1

TABLE II. Labeling of Proteins by Galactose Transfer at Times After Trypsinization

Time (hr)	Enzyme	cpm bound/5 × 10^6 cells
0 5	+	2,200
1	+	3,910
3	+	3,680
24	+	14,020
24	−	6,025

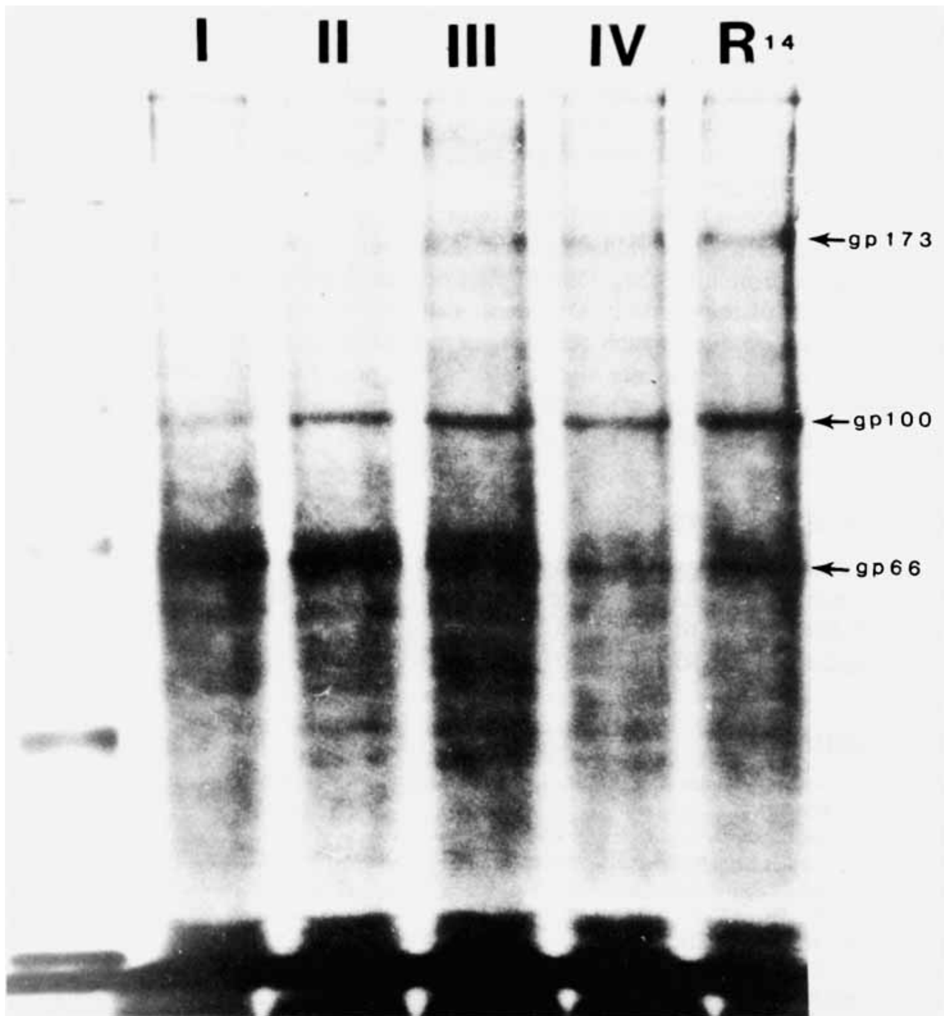

Fig 4 Radiofluorogram of a 7 5% polyacrylamide gel of glycoproteins labeled by galactose oxidase-borotritide reduction The tracks are the same as those for Figure 1

One of our concerns was that the serum in the medium in which the cells were grown after isolation would adhere to the cells and be labeled by the galactose transfer and borohydride reduction techniques. Our finding of similar bands after metabolic labeling with glucosamine implies that the bands that we have seen are indeed cellular constituents.

An interesting finding is that some bands differ among populations after one labeling procedure, but not after another. Gp100 is uniformly labeled by galactose transfer, but is labeled with glucosamine primarily on the cells in population I. This may indicate that there are subtle cell type specific differences in the glycosylation of particular proteins.

Several workers [11,19] have reported changes in glycoproteins during retinal development. The procedures used by these authors did not allow discrimination among cell types. Our observations indicate that glycoprotein differences exist among the cells within the retina of 14-day chick embryo. Recent immunological studies [22] indicate that antigenic differences exist among mature retinal cells. Presumably, some of these might be cell surface components, and would be expected to be glycoproteins.

We have shown that the different retinal cell populations possess distinct associative properties. Several cell association systems have been shown to be mediated by glycoprotein [23,24]. Our finding of significant glycoprotein differences among the cells of the retina is consistent with the hypothesis that these associative differences are due to such cell surface molecules. In order to establish that these glycoproteins play a role in cell association, and ultimately in the normal development of the retina, it will be necessary to isolate these molecules and demonstrate a direct role for them in cell association.

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REFERENCES

1. Moscona AA: In Willmer EN (ed): "Cells and Tissues in Culture." New York: Academic Press, 1965, pp 489–529.
2. Sheffield JB, Moscona AA: Dev Biol 23(1):36, 1970.
3. Vogel Z, Daniels MP, Nirenberg M: Proc Natl Acad Sci USA 73:2370, 1976.
4. Stefanelli AA, Zacchei AM, Caravita S, Cataldi A, Teradi LA: Experientia 23:199, 1967.
5. Sheffield JB: Tissue Cell 12:355, 1980.
6. Sheffield JB, Pressman D, Lynch M: Science 209:1043, 1980.
7. Sheffield JB, Lynch M: In Hilfer RS, Sheffield JB (eds): "Ocular Size and Shape Regulation in Development." New York: Springer Verlag, 1981, pp 99–122.
8. Sheffield JB: J Supramol Struct Cell Biochem Suppl 5:293, 1981.
9. Sheffield JB: Devel Biol (in press).
10. Moscona AA: Exp Cell Res 3:535, 1952.
11. Wallenfels B: Proc Natl Acad Sci USA 76:3223, 1979.
12. Schindler M, Mirelman, Schwarz U: Eur J Biochem 71:131, 1976.
13. Gahmberg CG, Hakamori SI: J Biol Chem 248:4311, 1973.
14. Steck TL: J Cell Biol 62:1, 1974.
15. Sheffield, JB, Daly T: Virology 70:247, 1976.
16. Laemmli UK: Nature (London) 227:680, 1970.
17. Laskey RA, Mills AD: Eur J Biochem 56:335, 1975.
18. August JT, Bolognesi DP, Fleissner E, Gilden RV, Nowinski RC: Virology 60:595, 1974.
19. Mintz G, Glaser L: J Cell Biol 79:132, 1978.
20. Roth S, McGuire EF, Roseman S: J Cell Biol 51:536, 1971.
21. Porzig EF: Dev Biol 67:114, 1978.
22. Barnstable CJ: Nature (London) 286:231, 1980.
23. Thiery JP Brackenbury R, Rutishauser U, Edelman G: J Biol Chem 252:6841, 1977.
24. Hausman RE, Knapp LW, Moscona AA, J Exp Zool 198(3):417, 1976.