*Biochimica et Biophysica Acta*, 298 (1973) 195–208


BBA 76243

# STUDIES ON CELL-COAT MACROMOLECULES IN NORMAL AND VIRUS-TRANSFORMED $BHK_{21/C13}$CELLS

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(Received October 2nd, 1972)

## SUMMARY

Cells have been grown *in vitro* in the presence of radioactive precursors; much of the glucosamine label is incorporated into the surface material, part of it is then spontaneously released into the medium, part is readily removed by an EDTA wash, and further amounts may be released by a mild trypsin treatment or by a period of chase.

The material in the EDTA wash from variously labelled cultures has been fractionated by Sephadex G-200 chromatography; the excluded peak has been analysed by Sepharose 6B and DEAE-cellulose chromatography and by electrophoresis on large pore polyacrylamide gels. Its sensitivity to pronase and hyaluronidase has been determined, as well as its sugar, lipids and amino acid composition.

Cell-coat material from Polyoma and Rous sarcoma virus-transformed cell lines failed to show significant differences in chromatographic properties and in sugar and amino acid composition. Minor but reproducible differences were found in the polyacrylamide electrophoresis migration patterns, when comparing glucosamine-labelled materials.

## INTRODUCTION

Cellular surfaces are attracting considerable interest because of their involvement in contact-mediated phenomena, including control mechanisms of cellular multiplication. Alterations at their level have been postulated to represent key steps in the expression of malignancy, and their structural and behavioural changes have, in fact, been shown to be correlated with cell transformation. Particularly relevant seem to be the altered reactivity of tumour cells vis à vis plant agglutinins[1,2], the changes of plasma membrane composition[3–5], and the morphological variations of cell-coat thickness[6,7]. Further progress in this area is hampered by the technical difficulties connected with the extreme lability of the outermost cellular layers, and by the lack of detailed knowledge about the nature and function of the cell coat. For tissue culture cells the latter appears as a fuzzy coat up to 5 times as thick as the plasma membrane proper[7], and it obviously plays an important role in cell adhesion and contact inhibition. Most of the cell-coat material is certainly lost from the cell membranes in the process of their isolation; in fact, electron microscopic

evidence shows that the cell coat is lacking in protease-treated cells, and that even gentle washes may remove or reduce it.

Having engaged in a biochemical comparison of normal and virus-transformed $BHK_{21}$ cell surfaces, we have focused on the macromolecular material which is released into the medium or which is only loosely bound to cell surfaces. We report here our studies on such complex material, whose biological activity has still to be defined and correlated with several well-known phenomena.

## MATERIALS AND METHODS

### *Cells and viruses*

The BHK-Py cell line has been derived from $BHK_{21/C13}$ after transformation with the LP strain of Polyoma virus; the BHK-B4 cell line has been kindly supplied by Dr J. Macpherson after transformation of the same $BHK_{21/C13}$ cell line with the Carl Brian strain of Rous sarcoma virus.

Cell lines are passaged in minimal essential medium (GIBCO-F15) with 10% tryptose phosphate broth and 10% calf serum. $BHK_{21/C13}$ cells show an ordered growth pattern and do not produce tumours; BHK-Py cells show random growth, are oncogenic for hamster, have complement-fixing T-antigen, are agglutinated by concanavalin A and appear to be virus free; tests for mycoplasmas were negative. BHK-B4 cells also show the parameters of malignancy: random growth, oncogenicity and agglutinability by lectins.

### *Labelling of cell cultures*

The medium in sparsely seeded cultures was replaced with fresh medium containing labelled precursors, and the culture was used for harvesting the material 2 days later. The cells were differentially labelled with D-[1-$^{14}$C]glucosamine, D-[6-$^{3}$H]glucosamine, L-[G-$^{3}$H]fucose, L-[1-$^{14}$C]fucose, L-[G-$^{3}$H]amino acid mixture, L-[U-$^{14}$C]amino acid mixture, inorganic [$^{35}$S]sulphate, [$^{14}$C]choline.

Radiochemicals ware supplied by New England Nuclear Co. Frankfurt/Main, West Germany. Specific radioactivity was in the range of 1–2 Ci/mmole or of 5–10 Ci/mole for $^{3}$H or $^{14}$C precursors, respectively.

### *Macromolecular turnover of incorporated labels*

Monolayers of the three cell lines were detached with EDTA and incubated for 10 min at 37 °C in 0.01% trypsin (Difco 1:250). After 3 washes in phosphate-buffered saline containing 10% serum, replicate 1-ml cultures were set up with $5 \cdot 10^5$ cells in minimal essential medium with 10% calf serum and with 1 $\mu$Ci D-[1-$^{14}$C]-glucosamine and 4 $\mu$Ci L-[G-$^{3}$H]amino acid mixture. At various intervals of incubation, cell activity was stopped by chilling, and incorporated labels were measured separately in the medium *plus* the first phosphate-buffered saline wash and in washed cells: other cultures were doubly labelled for 20 h, then the cells were washed as above and either reincubated for 24 h in fresh, unlabelled medium, or treated with trypsin (EC 3.4.4.4, deoxycholate treated, Sigma Chemical Co., Miss., U.S.A.) at a final concentration of 1 $\mu$g/ml for 10 min. Both cell-bound and released incorporated labels were counted after precipitation. All points were assayed in duplicate. An analysis of variance was performed on the log metameters of cpm values in order to test the significance of relevant comparisons.

*Cell coat removal for biochemical analyses*

Medium was poured off the culture bottles and 2 ml of 0.01% EDTA in phosphate-buffered saline were added: the bottles were incubated for 15 min in order to detach the cells from the glass. The cells were sedimented at $3000 \times g$ for 30 min and the supernatant was collected. This fluid (indicated as EDTA wash) was found to contain a large amount of radioactive macromolecules highly labelled by glucosamine. Presumably this material is of cell coat origin, as EDTA treatment has been shown to remove this material from the surface of the cell[7].

*Characterization of wash-released material*

*1. Sephadex G-200 and Sepharose 6B chromatography*

The wash was concentrated 30-fold and was applied on a 1.5 cm × 100 cm column packed with a pressure head of 15 cm in 150 mM Tris–HCl buffer (pH 7.2). The elution was carried out with the same buffer at a flow rate of 15 ml per h. Fractions (3 ml) were collected, 100 $\mu$g of albumin were added as carrier and then precipitation carried out with 10% trichloroacetic acid or 60% ethanol. The precipitate was collected on a Whatman GF/C filter, and counted with standard toluene scintillation mixture in an MK-1 Nuclear Chicago scintillation spectrometer under conditions appropriate for dual-labelled material.

*2. DEAE-cellulose chromatography*

The cellulose was pre-cycled with 0.5 M HCl and 0.5 M NaOH. A 1 cm × 30 cm column was packed with a pressure of about 10 lb/inch$^2$ with a flow rate of 30 ml/h in 0.15 M phosphate buffer (pH 7.4). The material was applied to the column and was developed with a linear gradient of NaCl in 0.15 M phosphate buffer (pH 7.5), ranging from 0 to 0.5 M. Fractions of 3 ml were collected and processed for the detection of the radioactivity as indicated above.

*3. Acrylamide electrophoresis*

Samples of lyophilized proteins were subjected to acrylamide electrophoresis at pH 4.5 in 8 M urea according to Reisfeld *et al.*[8], and at pH 7.2 in 0.1% sodium dodecyl sulphate according to Dunker and Ruekert[9]. Acrylamide gels were used at low percentage (from 3 to 5%) owing to the large molecular weight of the material to be examined. The gels were sectioned with a Savant Autogel divider and fractions were counted according to the method of Maizel[10]. The recovery of input radioactivity was about 60%. Some gels were stained with a protein stain (Coomassie brilliant blue) and with a carbohydrate stain (periodic acid–Schiff reagent reaction). Densitometry of protein and carbohydrate-positive bands was carried out with a Saitron-Phoroanalyzer.

*4. Sensitivity to hydrolytic enzymes*

The coat material purified on Sephadex G-200 was subjected to the action of hydrolytic enzymes. 10 mg of material were incubated with 1 mg of bovine testis hyaluronidase (EC 3.2.1.35, Sigma Chem. Co.) for 1 h at 37 °C in 2 ml of 100 mM acetate buffer (pH 4.5); after centrifugation the hydrolysate was chromatographed on Sephadex G-200. Pronase (B-Grade, Calbiochem, Los Angeles, U.S.A.) digestion of the same material was carried out essentially as described by Wu *et al.*[11] and the hydrolysate was analysed as above.

*5. Analysis of amino acid composition*

2 mg of Sephadex-purified and lyophilized material were placed in an ampoule

with 1 ml of 6 M HCl, the ampoule was sealed under nitrogen pressure and the hydrolysis was accomplished at 110 °C for 24 h. The hydrolysate was lyophilized and then amino acid analysis carried out in a Beckman Unichrom amino acid analyser.

*6. Analysis of sugar composition*

2 mg of purified material were subjected to methanolysis as described by Clamp *et al.*[12]. Sugar recovery and trimethylsilylation were carried out following the same procedure using fresh redistilled and carefully anhydrous methanol and pyridine. The silylated sugar mixture was then analysed in an F and M gas–liquid chromatography apparatus using an SE-30 column. Molar adjustment factors for peak areas were estimated experimentally against mannitol.

*7. Lipids analysis*

Cell-wash material labelled with [$^{14}$C]choline and [$^{3}$H]glucosamine was chromatographed on Sephadex G-200 and the excluded peak was collected. This was extracted with 19 vol. of chloroform/methanol (2:1, by vol.) in order to remove neutral, phospho- and glycolipids. The extract was dried and analysed on silica gel plates (Silica gel $HF_{254}$, Merck), developed with chloroform–methanol–water (65:25:4, by vol.) by ascending chromatography. The plates were dried and sprayed with $H_2SO_4$ to reveal the various lipid classes. The gel was removed from the plates in the areas corresponding to the stained spots and the radioactivity of the removed material was measured. Staining and radioactivity patterns were then compared.

## RESULTS

*1. Labelling pattern with glucosamine and amino acids*

Trypsinized cells transferred to complete medium and incubated in the presence of differentially labelled precursors incorporate label steadily into acid- or ethanol-precipitable material, at least for the first 20 h. Considering total counts per culture (Fig. 1, A and B), there are differences in the rate of incorporation both between labels (glucosamine and amino acids) and between cells. With all three cell lines, incorporated amino acid label is increasingly cell associated after longer labelling periods, as the cell-bound counts increase at a much faster rate than counts released into the medium and phosphate-buffered saline washes. On the contrary, the incorporated glucosamine counts are only transiently cell associated; they are readily released and accumulate extracellularly to such an extent that, after a 20-h labelling period, more than half of the total acid-precipitable glucosamine counts are found either in the medium or very loosely bound to the cells. Fig. 1 (C and B) shows graphically the diverging patterns of distribution of the two labels in the three cell lines: there are minor differences between normal and transformed cells in the proportion of the released glucosamine counts after short labelling periods, during which the cells presumably repair their coat, initially removed by trypsin: it follows that the ratio of glucosamine to amino acid-derived precipitable counts also differs between normal and transformed cells, and between cell-bound and cell-released material. This glycosylation index of cell-associated material (Fig. 1, C) is initially high in the case of normal $BHK_{21}$, then it drops regularly after longer labelling, and it reaches a plateau which is still significantly higher than the corresponding values for transformed cells. Conversely, the glycosylation index of released material becomes higher and higher as more and more surface material is released after labelling. (Fig. 1, D).

A mild trypsin treatment is very effective in releasing surface labelled material, in particular as far as the glucosamine label is concerned. It releases a good percentage of the total incorporated radioactivity (Fig. 2), with a glucosamine/amino acid ratio

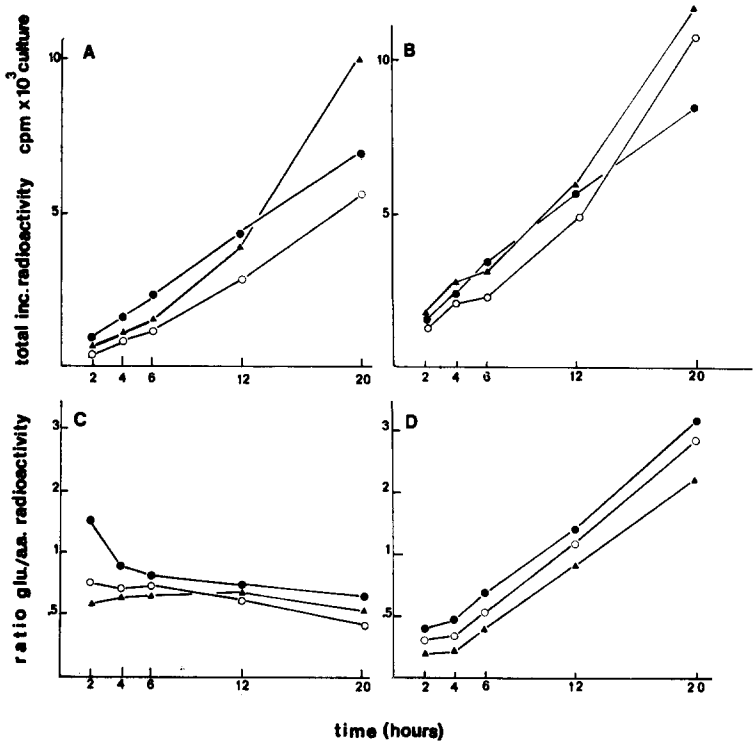


Fig. 1. Total (cell-bound+released) acid-precipitable counts from $^{14}C$ (A) and $^{3}H$ (B) after different periods of double labelling. Ratio of glucosamine to amino acid-derived radioactivity in cell-bound counts (C), and in released acid-precipitable material (D). ●—●, normal BHK; ○—○, BHK-Py; ▲—▲, BHK Rous sarcoma virus.

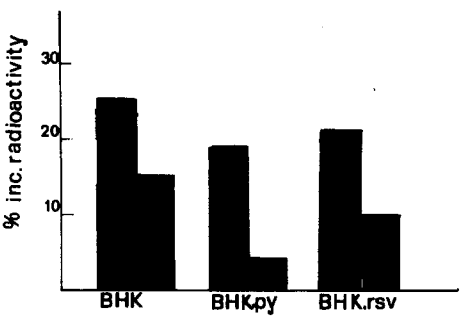


Fig. 2. Distribution of the glucosamine and amino acid-derived radioactivity in the material released with trypsin from the three cell lines. Cells were labelled for 20 h with radioactive glucosamine and amino acids; after gentle washing they were treated with trypsin (1 $\mu$g/ml) for 30 min at 36 °C. Released acid-precipitable counts are expressed as percent of total (bound+released) counts. Striped, glucosamine; stippled, amino acids. RSV, Rous sarcoma virus.

higher than that in whole cells. In addition, differences in the glycosylation index of the trypsin-released material were detected; transformed cells (Py-transformed in particular) exhibited a higher index as compared to normal cells.

No consistent differences between cell lines have been found by following the overall pattern of incorporation of radioactive precursors and the spontaneous release of radioactive molecules. Considerable variations in the labelling patterns have been traced to culture conditions. In particular, cell density strongly affects the breakdown of surface macromolecules: at low cell density a 24-h chase does not affect the total acid-precipitable radioactivity, and the whole amount lost from the cells is found in the medium and in the first wash. On the contrary, at high cell density, the cell-bound radioactivity decreases to a considerable extent through both release and breakdown to non-precipitable radioactivity.

Experiments like those reported above may only give a very rough idea about the cellular turnover of glucosamine and amino acid-containing cell components, because of the loose operational criteria used to distinguish "released" from "cell-bound" radioactivity, and because of the uncertainty about the chemical nature of the precipitable material. We found it particularly hard to find a simple and clear-cut precipitation method for distinguishing radioactivity incorporated into macromolecules from non-incorporated label, and we could not validate any single precipitant as the right criterion for different labels in different materials. Table I reports the mean percentage radioactivity precipitated by different treatments from washes of cultures grown for 48 h in the presence of some radioactive precursors. There are obvious and significant differences between the variously labelled materials in their overall response to precipitation with various treatments: the amino acid label is the most readily precipitated, followed by glucosamine, fucose and inorganic [$^{35}$S]-sulphate. Overall differences between treatments are also significant: ethanol is by

TABLE 1

PRECIPITABILITY OF VARIOUSLY LABELLED WASH-RELEASED MACROMOLECULES

Acids and cetylpyridinium chloride were used at the final concentration of 10%, ammonium sulphate at 2/3 saturation and ethanol at 60%. The precipitate was filtered through a Whatman GF/C filter, washed with absolute ethanol and ether, dried and counted.

| *Precipitating agent* | *Total counts in material labelled with* | | | |
|---|---|---|---|---|
| | *Glucosamine* 68 000 | *Amino acids* 85 000 | *Fucose* 34 000 | *[$^{35}$S]sulphate* 22 000 |
| | *Percent precipitated* | | | |
| Ethanol | 38.1 | 92.35 | 62.90 | 74.60 |
| Sulphosalycilic acid | 30.40 | 99.90 | 29.20 | 8.33 |
| Phosphotungstic acid | 58.40 | 66.55 | 24.86 | 6.06 |
| Perchloric acid | 33.80 | 42.63 | 31.71 | 7.25 |
| Trichloroacetic acid | 45.70 | 79.65 | 57.61 | 3.26 |
| Cetylpyridinium chloride | 32.31 | 33.92 | 39.62 | 17.20 |
| Ammonium sulphate | 71.63 | 95.00 | 56.50 | 5.63 |

far the most effective, followed by ammonium sulphate, sulphosalycilic acid, cetylpyridinium chloride, trichloroacetic, phosphotungstic and perchloric acids. There is a significant interaction between materials and treatments so that the rank order of effectiveness varies with the label being tested. It is clear, however, that it is not possible to precipitate selectively mucopolysaccharides, glycoproteins or simple prote ns.

As shown above, large amounts of labelled macromolecules are released by cells and accumulate in the medium after long periods of incubation with radioactive precursors. This material has not been analysed in any great detail because, although it derives from the cell coat, it may not represent its original composition; besides, it would need to be separated from the large excess of calf serum proteins present in the medium. In contrast, most of the material in the EDTA wash may be considered to be of immediate cell-coat origin, and its contamination with medium components to be comparatively slight. We have purposely avoided the use of trypsin or other enzymes to release materials to be analysed, because we aimed at a characterization of the cell-coat materials in their native state.

*2. Characterization of the EDTA wash*

Concentrated EDTA washes were first processed by Sephadex G-200 chromatography; this consistently resolved three discrete peaks as monitored by absorbance at 280 nm (Fig. 3). Peaks 1 and 3 eluted along with the blue dextran (excluded volume) and the phenol red (included volume) marker dyes, respectively, the intermediate Peak 2 was broader and represented polydisperse material. The pattern of radioactivity varied widely according to the label used: glucosamine-derived radioactivity was mostly associated with excluded molecules, and very little with peaks 2 and 3. The amino acid label was about equally distributed in the three Peaks; the fucose label was also represented in the three peaks, while the inorganic sulphate label was mainly present in the excluded peak. Unlabelled, adsorbed calf serum proteins were detected by immunoprecipitation in Peaks 2 and 3, but they were absent from Peak 1 where, in general, the specific radioactivity tended to be higher.

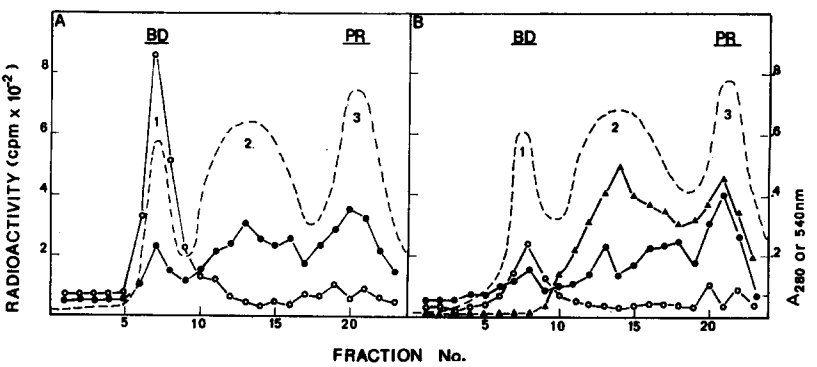


Fig. 3. Sephadex G-200 chromatography of EDTA washes from BHK$_{21/C13}$ cells labelled with different precursors. (A) – – –, absorbance profile at 280 nm; ○—○, glucosamine label; ●—●, amino acid label. (B) – – –, absorbance profile at 280 nm; ●—●, fucose label; ○—○, inorganic [$^{35}$S]sulphate; ▲—▲, amount of immune precipitate with anti-calf serum (biuret reaction: absorbance at 540 nm). BD, blue dextran; PR, phenol red.

Consideration of the latter findings led us to analyse further the glucosamine-labelled material from the first peak, which was rechromatographed on Sepharose 6B (Fig. 4A). Several ill-resolved peaks eluted in the region of retarded molecules, while the highest peak was clearly excluded from the gel and behaved like material with molecular weight in excess of 2000000. The same material was run on DEAE-cellulose (Fig. 4B), where it eluted as a sharp peak at 0.28 M NaCl, thus showing homogeneity

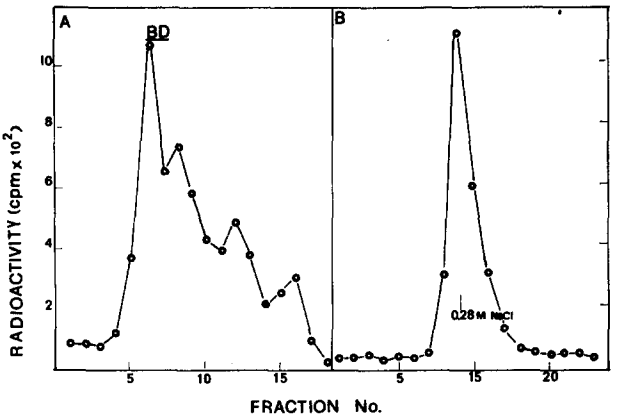


Fig. 4. Chromatography on Sepharose 6B (A) and on DEAE-cellulose (B) of Peak 1 material from a previous Sephadex G-200 separation of glucosamine-labelled EDTA wash from $BHK_{21/C13}$ cells. BD, blue dextran.

of electric charge density. A further characterization of the material excluded from Sephadex G-200 was attempted by sodium dodecyl sulphate–acrylamide electrophoresis: fractions of the first peak were pooled, concentrated by lyophilization and treated with 1% sodium dodecyl sulphate at 60° for 30 min, then were charged on gels of high porosity. Even in these conditions, some material failed to enter the gel, but it was possible to analyse from 50 to 60% of the input. Fig. 5 shows the appearance of four groups of bands, with positions corresponding to molecular weights in the range from 200000 to 10000, three bands were consistently periodic acid–Schiff reagent positive.

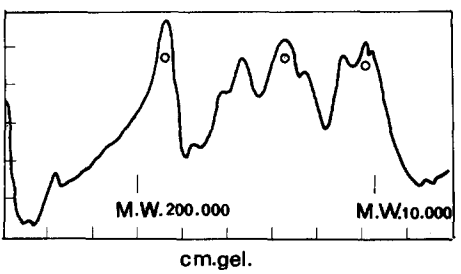


Fig. 5. Acrylamide electropherogram of Sephadex G-200 peak 1 material from $BHK_{21/C13}$ cells. Wash material purified on Sephadex G-200 (excluded peak) has been lyophilized, treated with sodium dodecyl sulphate and then subjected to acrylamide electrophoresis. Densitometry tracings were obtained after staining with Coomassie brilliant blue of by the periodic acid–Schiff reagent reaction. Periodic acid–Schiff reagent positive bands are indicated with an open circle. M.W.= molecular weight.

The sensitivity to enzymatic breakdown of the high molecular weight glucosamine-labelled material recovered in the first Sephadex peak was next explored by digesting it with pronase and/or hyaluronidase (Fig. 6). After a second chromatography the pronase- and hyaluronidase-treated material showed a complete shift

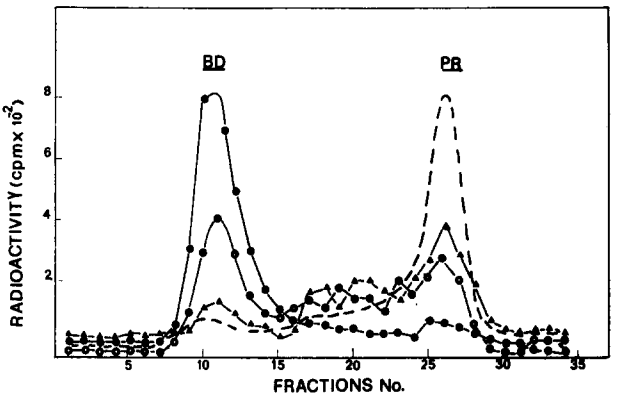


Fig. 6. Sensitivity to hydrolytic enzymes of glucosamine-labelled material recovered in the peak 1 in a previous Sephadex G-200 chromatography. The material has been treated with enzymes and then re-chromatographed in the same way. ●—●, untreated; ○—○, treated with pronase; ▲—▲, treated with hyaluronidase; – – – – –, treated with both.

of the label distribution from the blue dextran peak to the phenol red one. The amount of label still eluting in the excluded volume was reduced by 60 to 70% after hyaluronidase treatment, by 30 to 40% after a pronase treatment.

Finally, the material recovered in the first Sephadex peak was analysed in term of its sugar and amino acid components. Table II shows its sugar composition, as determined by gas–liquid chromatography. *N*-Acetylglucosamine and glucuronic

TABLE II

CARBOHYDRATE COMPOSITION OF SEPHADEX G-200-EXCLUDED MATERIAL FROM EDTA WASH OF $BHK_{21/C13}$ CELLS AS DETERMINED BY GAS–LIQUID CHROMATOGRAPHY

The carbohydrate content of the examined material was in the range of 60 to 50%.

| | *% of total carbohydrate present* |
|---|---|
| Fucose | 5.68 |
| Xylose | 1.76 |
| Mannose | 18.34 |
| Galactose | 6.72 |
| Glucuronic acid | 21.33 |
| Glucose | 3.06 |
| *N*-Acetylglucosamine | 24.90 |
| *N*-Acetylgalactosamine | 12.84 |
| *N*-Acetylneuraminic | 5.28 |

TABLE III

AMINO ACID COMPOSITION OF THE SEPHADEX G-200-EXCLUDED MATERIAL FROM EDTA WASH OF $BHK_{21/C13}$ CELLS AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

The amino acid content of the examined material was about 40%.

| | *% of total amino-acid present* |
|---|---|
| Aspartic acid | 9.37 |
| Threonine | 5.70 |
| Serine | 9.10 |
| Glutamic acid | 13.10 |
| Proline | 1.49 |
| Glycine | 7.99 |
| Alanine | 7.50 |
| Cysteine | 0.68 |
| Valine | 8.20 |
| Methionine | 1.38 |
| Isoleucine | 4.35 |
| Leucine | 10.00 |
| Tyrosine | 3.19 |
| Phenylalanine | 4.11 |
| Lysine | 9.14 |
| Histidine | 1.54 |
| Arginine | 3.11 |

acid, the repetitive components of mucopolysaccharides, were the major components, followed by other sugars, characteristically present in surface glycoproteins; these were found in greater amounts than fucose and sialic acid, which are the best studied sugar components of plasma membranes, and which appeared to be minor components. Results of the amino acid analyses are reported in Table III: glutamic acid, leucine and lysine are the best represented amino acids.

Lipid analysis revealed the presence of neutral, phospho- and glycolipids in this material. Both the glycolipids, which were poorly represented, and the phospholipids exhibited very high labelling with radioactive precursors (see Fig. 7), thus indicating cellular origin.

*3. Comparison of cell-coat material in normal and transformed cells*

EDTA-released material from normal and transformed cells was comparatively analysed: no significant and reproducible differences were observed for biochemical and chromatographic properties. The material from the three sources considered exhibited the same elution properties from Sephadex G-200 when labelled with any of the four different labels, and nearly the same composition of sugars, amino acids and phospholipid classes.

As glucosamine was by far the most effective label for the Sephadex G-200 excluded material, the latter was prepared from normal and transformed cells differently labelled with both [$^3$H]- and [$^{14}$C]precursors, and then comparatively analysed in sodium dodecyl sulphate–acrylamide gels. Owing to large differences in molecular

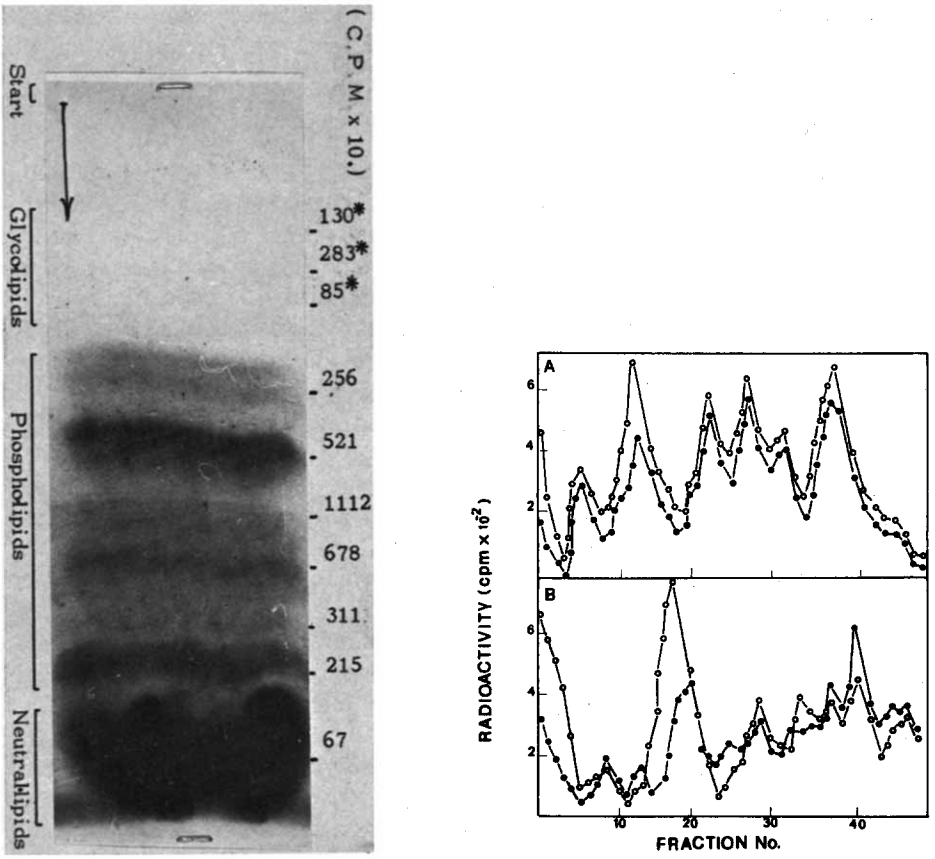


Fig. 7. Silica gel chromatography of cell-coat lipids labelled with [$^{14}$C]choline and [$^{3}$H]glucosamine. Lipid classes were revealed by sulphuric acid spray and by measuring the radioactivity of the stained spots. Glycolipids, due to their small amount, were revealed poorly by sulphuric acid and do not appear in the photograph; however, they were revealed very clearly as glucosamine-derived radioactivity. The cpm of [$^{14}$C]choline-derived radioactivity and [$^{3}$H]glucosamine-derived radioactivity are given; the latter are indicated by an asterisk.

Fig. 8. Acrylamide electrophoresis of [$^{14}$C]- and [$^{3}$H]glucosamine-labelled material (Sephadex G-200 Peak 1) from normal and polyoma-transformed $BHK_{21/C13}$ cells. The Sephadex-excluded fractions (fractions 5–11 as indicated in Fig. 3) were divided into two pools and analysed separately due to differences in molecular size. (A) Fractions 5–7 analysed in 3% gel. (B) Fractions 8–11 analysed in 5% gel. ●—●, normal; ○—○, transformed.

weight, Fractions 5–7 (see Fig. 3) and Fractions 8–10 of the blue dextran peak were compared separately, after sodium dodecyl sulphate treatment, using 3 and 5% gels, respectively. As shown in Fig. 8A, the electrophoretic patterns of the earlier (larger molecular weight) fractions of the excluded material failed to exhibit significant differences between extracts from normal and polyoma-transformed cells. Similar coincident patterns were obtained when comparing materials from normal and Rous sarcoma virus-transformed cells. A minor but reproducible difference was detected (Fig. 8B) by comparison of Sephadex Fractions 8–10 of normal and Py-transformed cells; a component of molecular weight around 130000 appeared more highly glyco-

sylated in transformed cells. A similar difference was also found by comparing normal and Rous sarcoma virus-transformed cells; however, this was less evident and reproducible.

## DISCUSSION

The surface of tumour cells is presently attracting a great deal of attention, and several groups are looking for molecular alterations in the surface of transformed cells. What should be considered as the cell surface has, however, still to be defined. Indeed, cells conventionally harvested with trypsin or chelating agents are deprived of a large part of their surface coat[7], and further manipulations for the isolation of plasma membranes lead to preparations which, at best, represent only the insoluble backbone of the cytoplasmic membrane. On the other hand, the cell coat, which is completely lost from such preparations, might well be considered the best candidate for study, as its presence is required in order to establish contact-dependent inhibition of growth.

Although extensively studied morphologically[6,7], the cell-coat turnover and composition are still largely unknown. The present studies were aimed at a preliminary characterization of the material which is readily released from surfaces of normal BHK cells and of the same after transformations with polyoma or Rous sarcoma viruses.

Kinetic labelling experiments of low density cultures established the pattern of incorporation of glucosamine and amino acids into cell constituents, and their distribution between tightly cell-bound and readily released macromolecules. The latter are thought to derive mainly from the outer cellular layer, and a mechanism has been envisaged by which cell-coat macromolecules are continuously synthesized and, in particular, they are continuously glycosylated by cellular transferases; such newly formed molecules displace the older ones, which are lost from the immediate surroundings of the cell and accumulate in the medium[13]. In crowded conditions the cell-coat turnover by release into the medium is apparently accompanied by breakdown, and this may well be explained by the action of degradative enzymes, located at each cell surface and acting on the closest neighbours of the cell[14]. Interpolating from the kinetic studies one may think of the cell coat at any particular moment as a labile structure made up of molecules which, on the average, are younger than the bulk of the cell itself: these molecules range widely in the strength of their attachment to the cell; some are released spontaneously, some are released by EDTA, some others only by trypsin. The topographical arrangement of such molecules is speculative at present, although consistent models of cell-coat architecture have been proposed[7].

From the analytical point of view, we have first considered those macromolecules which are still cell associated but are most readily released with a mild EDTA treatment. Sephadex G-200 chromatography was found to be a suitable procedure for a crude purification of the released material, in particular from adsorbed serum proteins; the material recovered in the void volume exhibited properties consistent with a cell-coat origin, *i.e.* very high specific radioactivity from glucosamine and absence of cell-adsorbed medium proteins. The distribution of the various labelled precursors in the eluate of the Sephadex chromatography suggests that there is an abundance of mucopolysaccharides in the excluded material, which

were highly labelled by glucosamine and to a limited extent by inorganic sulphate. Probably hyaluronic acid is the main component, but chondroitin sulphate is also present. The enzyme sensitivity demonstrated, however, that mucopolysaccharides are not the only components of this material, in fact neither hyaluronidase nor pronase, when used alone, were able to breakdown completely the glucosamine-labelled material. As the material exhibited a protein content of nearly 40% by weight, and the sugar composition revealed the presence not only of glucuronic acid and acetylglucosamine, but also of other carbohydrates characteristic of glycoproteins, it is reasonable to suggest that this material consists of both mucopolysaccharides and glycoproteins, perhaps associated in giant aggregates with phospholipids. The latter were revealed in the EDTA washes as highly labelled by radioactive choline. As those molecules are generally believed to be present only in the Danielli–Dawson backbone of cell membranes, their presence in the material examined might suggest contamination by pieces of membranes or dead cells. However, neither an electron microscope approach nor assays for membrane marker enzymes (5′-nucleotidase, NADH–cytochrome *c* reductase, NADH diaphorase) were able to reveal membrane structures. If the presence of phospholipids in the cell coat is confirmed in the future, a new molecular model of this structure will be required.

Differences in the cell coat between normal and transformed cells have been reported recently[6,7,15], and are consistent with an increase in the thickness of the coat layers. Our findings suggest that the coat material does not differ grossly in its biochemical properties between normal and transformed cells. We found similar gel filtration properties after labelling with any of the four radioactive precursors, similar protein to carbohydrate ratio, very close composition of carbohydrates, amino acids and phospholipids, almost identical patterns of separation in sodium dodecyl sulphate and in pH 4.5 electrophoresis on acrylamide gel, either by staining of the gels with Coomassie blue or by the periodic acid–Schiff reagent reaction, or by fractionation of dual-labelled radioactive molecules. When slightly gel-retained material has been compared (see Fig. 8B), differences in the degree of glycosylation of a component with a molecular weight close to 130000 were determined. This finding is consistent with a similar difference observed by a comparative analysis made previously in this laboratory by acrylamide fractionation of crude cell wash from normal and Py-transformed BHK cells[5].

Although the cell-coat layer is reported to be present in increased amount in transformed cells[6,7–15], our preliminary biochemical analysis suggests that it does not change much in composition following transformation, without obviously ruling out possible architectural differences.

The different reactive sites to lectins now recognized on transformed cell surfaces are probably more "buried". In keeping with this hypothesis are the recent findings of Burger and Martin[16], who demonstrated that extensive coat removal by hyaluronidase increases the sensitivity of transformed cells to concanavalin A and to wheat germ agglutinin.

ACKNOWLEDGEMENTS

We thank Dr J. Macpherson for kindly supplying us with Rous sarcoma virus-transformed BHK cells; Prof. R. Davoli and Prof. A. Fonnesu for their interest

in this work; Mrs A. M. Fabbroni and Mrs C. Ballotta for their skilful technical assistance.

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