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EXPOSURE OF TRYPSIN-REMOVABLE SULPHATED POLYANIONS ON THE SURFACE OF NORMAL AND VIRALLY TRANSFORMED $BHK^{21/C13}$ CELLS

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SUMMARY

1. Surface materials have been removed by chelant or by trypsin treatment from normal $BHK_{21/C13}$ cells and from the same cells transformed by polyoma and Rous sarcoma viruses.

2. These materials, labelled by various radioactive precursors, have been analysed by chromatographic and electrophoretic techniques.

3. The results suggest that the material removed from the cell surface is a mixture of glycopeptides and glycosaminoglycans.

4. A sulphated glycosaminoglycan component (tentatively identified as heparan sulphate) is removed from the transformed cells in a significantly lower relative amount as compared with the control untransformed cells.

5. A possible role of this surface polyanionic component as a negative control element of cell growth is suggested and discussed.

INTRODUCTION

Polyanions have been focused as possible control elements in the cell cycle. Heparin has been found to prevent the increase in viscosity during mitosis, the so-called 'mitotic gelation' which precedes the appearance of the spindle and of the cleavage furrow. In addition, a periodic release of free heparin has been found in synchrony with the cell cycle, with a rapid decrease during mitosis and an increase in the G^1 period. On this basis an 'heparin cycle' has been proposed [1] in the fertilized sea urchin egg. It was supposed that the jelly coat or the cortical granules [2] may be the source of the polyanion in the fertilized egg.

The effect of N-sulphated polyanions on tissue growth in vivo and in vitro has been recognized since 1932 [3]. The growth inhibitory effect of polyanions of the heparin group has been shown in fibroblast cultures [4–6]. Other sulphated polyanions either naturally occurring or artificial have also been shown to inhibit cell growth in vitro, but the most effective is the N-sulphated group (heparin and heparan sulphate). Polyanions may also play a role in cell adhesion. For example,

cells treated with trypsin do not adhere to negatively charged surfaces [7], and it has been demonstrated that trypsin removes a mucopeptide population which includes heparan sulphate from cell surfaces [8]. It is possible that Ca^{2+} or other divalent cations increase the cell adhesion by acting as counterions to the polyanions thus forming a complex at the cell surface which may be involved in both cell adhesion and cell growth control. Although non-sulphated mucopolysaccharides (as hyaluronic acid) have also been detected at the cell surface [9], they have not been shown to interfere negatively with cell growth unless they have been artificially sulphated [10]. Kraemer [11] has examined six different cell lines of both connective and epithelial origin and found heparan sulphate to be a surface component in all six of these. The widespread presence of this component may indicate that is serves as a basic control element of cellular replication, independent of differentiation events.

These considerations stimulate general interest in the surface-coat polyanions in connection with malignant transformation. Following this approach studies were made on the ultrastructure of the cell-coat material in normal and transformed cells stained by rutenium red [12, 14], and on the biochemical properties of the surface material removed by trypsin, including molecular size, crude carbohydrate composition, reactivity to lectins, electric charge density and differences between normal and transformed cells [15–18]. Thorough investigations are, however, still required on the properties of the material exposed at the cell surface, and its involvement in contact-mediated phenomena and in the control mechanisms of cellular replication. Although both sulphated mucopolysaccharides and mucoproteins have been recognized as exposed molecules on the cell surface very little is now known about their structural properties and molecular assembly.

This paper reports on the high resolution chromatography of surface material from cultured hamster cells, comparing the materials removed with chelants with those removed with trypsin, from both normal- and virus-transformed cells.

MATERIALS AND METHODS

Cells and viruses

The BHK-py cell line was derived from $BHK_{21/C13}$ after transformation with the LP strain of polyoma virus; the BHK-B4 cell line was kindly supplied by Dr J. Machperson after transformation of the same $BHK_{21/C13}$ cell line with the Carl Brian strain Rous sarcoma virus (RSV).

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; they have a more globular form and a decreased adhesivenesss.

Labelling of cell cultures

The medium in sparsely-seeded cultures was replaced with fresh medium containing labelled precursors and incubation was continued for 48–60 h before harvest

when the cultures appeared as confluent monolayers. 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, G-^{3}H-labelled L-amino acid mixture, (U-^{14}C)-labelled L-amino acid mixture and ^{35}S-labelled inorganic sulphate.

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

Cell-coat removal for biochemical analyses: EDTA and trypsin treatment

Medium was poured off the culture bottles and 2 ml of 0.01% EDTA in phosphate saline were added; after 15 min the detached cells were collected with phosphate-buffered saline and sedimented at $3000 \times g$ for 30 min. The supernatant (indicated as EDTA wash) was found to contain a large amount of radioactive macromolecules. The sedimented cells were resuspended and treated with crystalline trypsin (EC 3.4.4.4., diphenylcarbonyl chloride treated, Sigma chemical Co. Miss. U.S.A.) at a final concentration of 1 μg/ml for 30 min at 37 °C. The cells, still viable, were sedimented as above and the supernatant designated as 'cell trypsinate'.

Chromatography on Sephadex G-25

Washes and trypsinates were processed by Sephadex G-25 chromatography in order to separate the molecules with smaller size (mol. wt $< 10\,000$) from the larger aggregates. The chromatography was carried out either at pH 5.0 in pyridine–acetic acid buffer (0.2 M) or at pH 7.4 in Tris–HCl buffer (0.2 M). The column (1 cm $\times$ 30 cm) was eluted with a pressure head of 15 cm and a correspondent flow rate of 20 ml/h. Aliquots of each fraction were dried in scintillation vials counted in a standard toluene scintillation mixture in an ISOCAP-300 Nuclear Chicago scintillation spectrometer.

AGI X2 chromatography

The Sephadex fractions corresponding to molecular weights from 10 000 to 1000 were pooled, lyophilized and stored. The lyophilized material was dissolved in 3% pyridine at pH 8.5. Appropriate samples of inputs of radioactivity were loaded on an AG1 X2 column (0.9 cm $\times$ 60 cm). The column was developed with a concave gradient of pyridine– acetic acid buffer ranging from 0.3 to 2 M with a correspondent pH decrease from 8.5 to 5.0. The column pressure was 10 atm and the flow rate was 30 ml/h. Fractions of 3 ml were collected, dried and counted.

High voltage electrophoresis

High voltage electrophoresis was carried out either with total mucopeptide mixtures or with single peaks from the AGI X2 chromatography. Electrophoresis has been carried out on Whatman 3 MM paper (50 cm $\times$ 45 cm) in pyridine–acetic acid buffer (0.2 M, pH 3.5) at 3800 volts for 15 min.

Affinity chromatography

Cells labelled with glucosamine were first treated with trypsin and the removed molecules harvested as indicated above. The same cells were then incubated for 24 h at 37 °C in PBS saline in the presence of 1 μg/ml of pronase (B grade, Calbiochem,

U.S.A.). The mixture was centrifuged at 30 000 $\times g$ for 1 h and the supernatant was harvested. Trypsin and pronase supernatants were purified on Sephadex G-25 and the included materials were used for affinity chromatography.

Affinity chromatography was carried out on a column of concanavalin A-Sepharose 4B (Pharmacia Chem., Upsala, Sweden) by elution with 0.1 M acetate buffer pH 6 containing 1 M NaCl and 1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ followed by a linear gradient of α-methylglycoside (α-methyl-D-glucose).

Tests for the identification of the heparan sulphate

Trypsinates labelled with ^{35}S-labelled inorganic sulphate were chromatographed on Sephadex G-25. The included material (eluted as a sharp peak with a mol. wt around 12 000) was collected and lyophilized. The material was dissolved in 0.1 ml of 1.5 M H_2SO_4, 0.5 ml of 5.5 M $NaNO_2$ was added and the deaminative cleavage was allowed to proceed for 10 min at room temperature. The reaction mixture was neutralized by 0.2 ml of 2 M sodium carbonate and rechromatographed on Sephadex G-25.

The same material was incubated for 1 h at 37 °C with 1 mg of bovine testis hyaluronidase in 1 ml of 100 mM acetate buffer (pH 5.0). After enzymatic treatment the mixture was rechromatographed on Sephadex G-25.

The same lyophilized material was used again for the test of elution molarity from Dowex 1. This was applied on a column of Dowex 1 $\times$ 2 (0.2 cm $\times$ 5 cm) equilibrated at pH 8.5 in 3% pyridine. Stepwise elutions were carried out with NaCl solutions ranging from 0.5 to 2 M.

RESULTS

1. *pH-dependent aggregation*

Fig. 1 shows the pattern of elution from Sephadex G-25 of the cell wash (A) and of the cell trypsinate (B) of normal BHK cells labelled with glucosamine: the two patterns are very similar, as are those of materials labelled by ^{35}S, fucose, or amino acids. A previous analysis of the EDTA wash labelled with glucosamine [16], showed that most of the material was excluded from a Sephadex G-200 column run with a pH 7.2 buffer. The finding that the largest part of the material was included in Sephadex G-25, thus exhibiting a molecular weight smaller than 15 000, led us to examine the influence of the elution buffer on the behaviour of our material with the gel sieves. The results are reported in Figs 1C and D: the same wash from BHK cells labelled with glucosamine was subjected to chromatography at pH 7.4: most of it was excluded from the gel while at pH 5.0 it eluted mostly in the included volume. When the latter included material was collected, lyophilized and rerun on Sephadex G-25 it appeared in the void volume in a large amount (Fig. 1E). This shows that the prominent first peak observed in the analysis of the cell coat at physiologic pH is made up of large aggregates of much smaller molecules. We selected the material which is included at pH 5.0 for further analyses, as it should be clean from serum proteins and trypsin.

2. *Affinity chromatography of trypsin and pronase-removed materials*

The radioactive material labelled by glucosamine, obtained in the supernatant from trypsin treatment of either normal or transformed cells, gave two peaks on

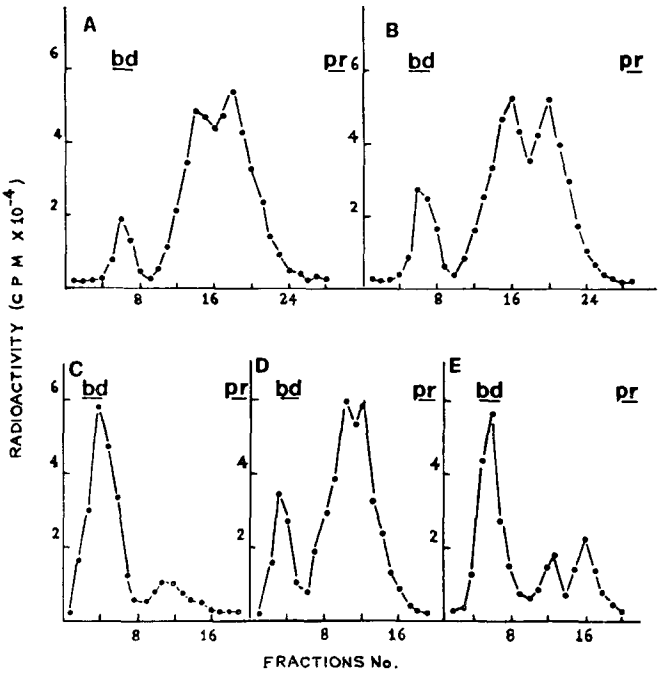

Fig. 1. Effect of the buffer on the elution profile of EDTA wash on Sephadex G-25. Glucosamine-labelled material removed by EDTA (A) or by trypsin (B) eluted in pyridine–acetic acid buffer (0.2 M, pH 5.0). C shows the pattern of elution of a wash material eluted in Tris–HCl buffer (0.2 M, pH 7.5), D shows the pattern of the same material in pyridine–acetic acid buffer (pH 5.0) and E a second chromatography as in (C) of fractions 8/16 from D. Markers: bd, blue dextran; pr, phenol red.

Sephadex G-25 as shown in Fig. 2A. The included material was analyzed by affinity chromatography on concanavalin A-Sepharose 4B column in order to investigate whether or not this material contained concanavalin A receptors. The material appeared as a single peak in the first elution (in the absence of the competing ligand) and no significant radioactivity could then be eluted with a linear gradient of α-methyl-glycoside. This shows that our trypsin treatment is unable to release the concanavalin A receptors from the cell surface of either normal or transformed cells. Indeed, as shown in Fig. 2C, concanavalin A receptors are released upon pronase digestion of previously trypsinized cells.

3. *High resolution chromatography of normal cell trypsinates*

Sephadex G-25 included fractions were obtained from normal BHK cells labelled with different precursors and analysed by ion-exchange chromatography on AGI X2 columns. This basic resin was much superior to cationic exchangers for resolving discrete peaks from our material, thus suggesting that these included fractions contain some strongly acidic molecules. The elution patterns obtained with our chromatographic procedure were very reproducible. The amino acid label typically eluted in seven well-resolved peaks, as shown in Fig. 3A; the fucose label eluted similarly (Fig. 3B) but it was lacking in peak 7. Peak 7 on the other hand was the most prominent

in the elution pattern of the glucosamine label (Fig. 3C), and the only one present in the pattern of the inorganic ^{35}S label (Fig. 3D). It was concluded that the cell trypsinate contains at least two basic components (peaks 1 and 2) and three increasingly acidic ones (peaks 3, 4 and 5) which presumably represent glycoprotein fragments containing amino acids, fucose and glucosamine. The material in peak 7 is

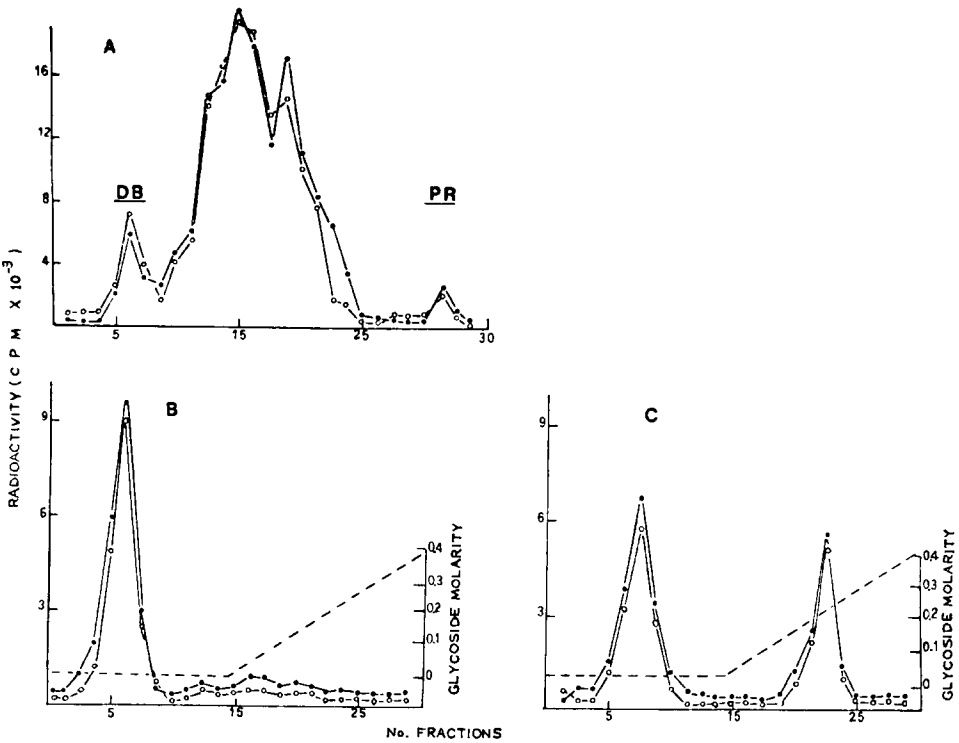

Fig. 2. A. Cochromatography on Sephadex G-25 of trypsinates from amino acid-labelled normal ([^{14}C],●) and polyoma-transformed ([^{3}H], ○) BHK cells. BD and PR· blue dextran and phenol red markers. Elution with pyridine–acetic acid buffer (0.2 M, pH 5.0). B. Affinity cochromatography of trypsin-removed surface glycopeptides on Sepharose 4B bound to concanavalin A. Glucosamine-labelled glycopeptides were separated on Sephadex G-25 at pH 5.0. The included fractions were pooled and run on a concanavalin A-Sepharose 4B column. Symbols as above. C. Affinity cochromatography of glucosamine-labelled glycopeptides removed from previously trypsinized cells by pronase digestion. Symbols as above.

strongly acidic, does not contain fucose, shows only minor labelling with amino acids, and it is heavily labelled with glucosamine and inorganic sulphate. On this basis the material in peak 7 was tentatively identified as a sulphated mucopolysaccharide. The glucosamine labelling was the most effective for showing the complete spectrum of components and was adopted for further analyses.

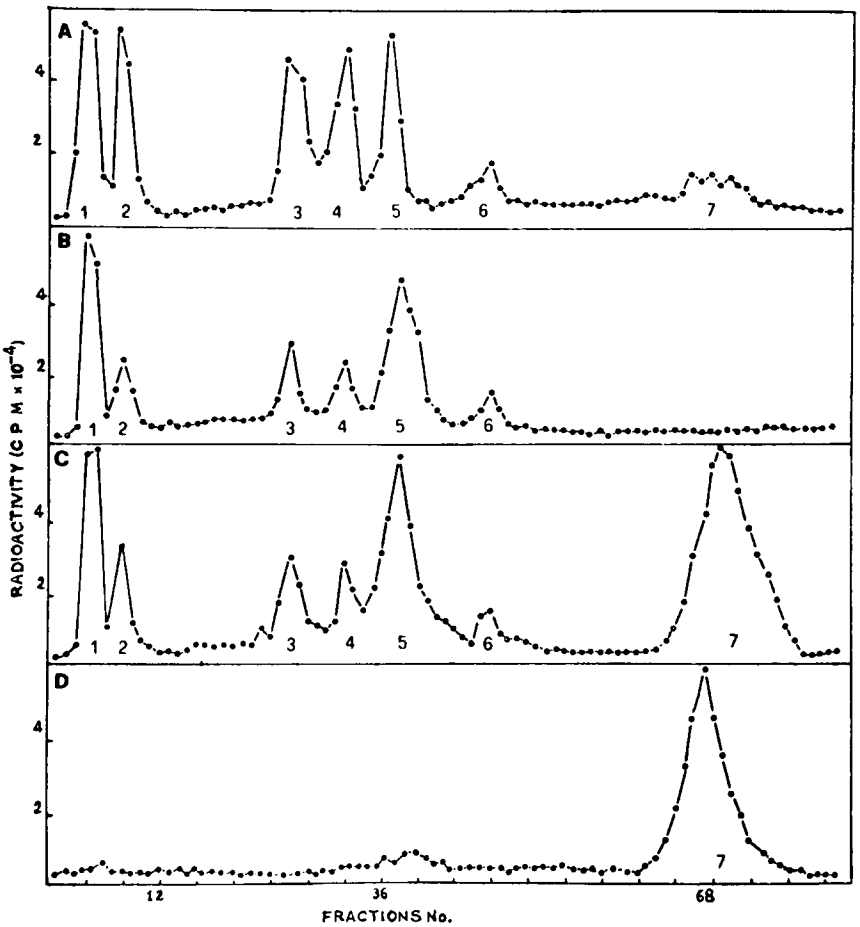

Fig. 3. Labellings pattern by various radioactive precursors as revealed by AGI X2 chromatography. A, ^{14}C-labelled amino acids; B, [^{14}C]fucose; C, [^{14}C] glucosamine; D, ^{35}S- labelled inorganic sulphate.

4. *Comparison of glucosamine-labelled trypsinates from normal- and virus-transformed cells*

Trypsinate fractions from virus-transformed BHK cells growing in the presence of labelled glucosamine were chromatographed on AGI X2 resin as detailed above, their elution patterns were generally similar to that described for the corresponding material from normal cells, again showing 7 discrete peaks of radioactivity; the last peak was, however, consistently lower in the material from transformed cells. In 8 comparative runs the mean ratio of radioactivity in peak 5 to radioactivity in peak 7 was 0.4 ± 0.06 for normal BHK cells, 2.13 ± 0.26 for polyoma-transformed BHK and 5.58 ± 1.52 for RSV-transformed BHK cells. Differences of the same order were obtained when comparing the areas under peak 7, considered as a fraction of the total pattern. The relative decrease of peak 7 in transformed cells was confirmed by co-chromatography experiments: appropriate amounts of radioactive material

from normal cells were mixed with differently labelled material from transformed cells and subjected to chromatography as shown in Fig. 4.

The relative decrease of peak 7 in transformed relative to normal cells is statistically significant in both the cell lines examined. Also significant is the difference between polyoma- and RSV-transformed cell lines, with the latter showing the most pronounced change, perhaps reflecting the extreme phenotypic expression of transformation of the BL4 cell line.

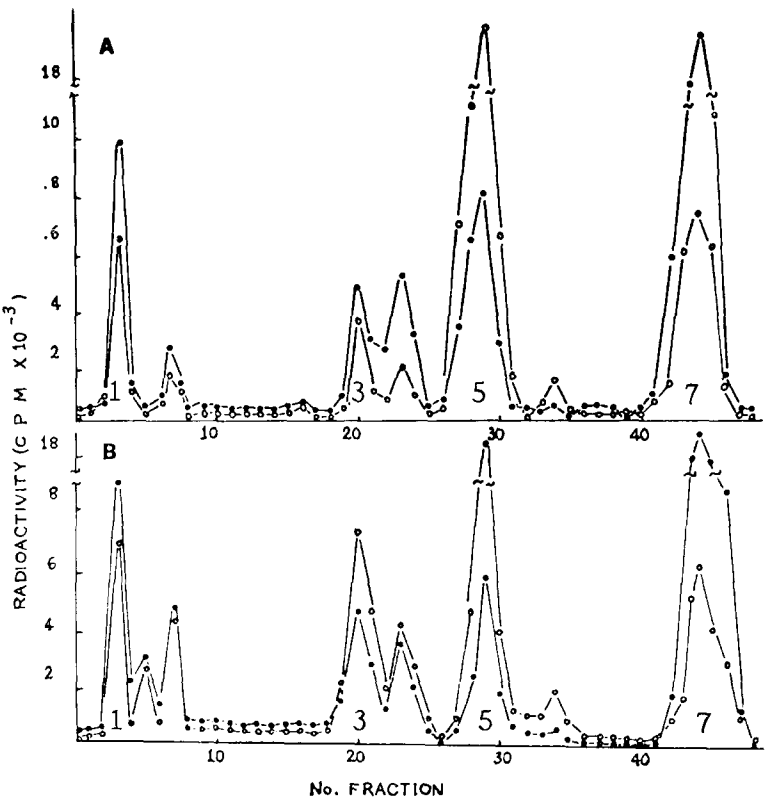

Fig. 4. Cochromatography on AGI X2 resin of glycopeptides removed by trypsin from normal and transformed cells and purified by Sephadex G-25 chromatography. A. Glycopeptides obtained from normal cells labelled w.th [^{14}C]-glucosamine (●) and from polyoma virus-transformed cells labelled with [^{3}H]glucosamine (○). B. Glycopeptides obtained from normal cells labelled with [^{14}C]glucosamine (●) and from Rous sarcoma virus-transformed cells labelled with [^{3}H]glucosamine (○).

5. *Comparison of EDTA washes and trypsinates*

EDTA washes and trypsinates from the three cell lines were compared by cochromatography on AGI X2. The material for these analyses was obtained from cells grown in the presence of differently labelled glucosamine and was first purified on Sephadex G-25. In the case of normal cells, the EDTA wash material eluted exactly along the trypsinate material (Fig. 5A).

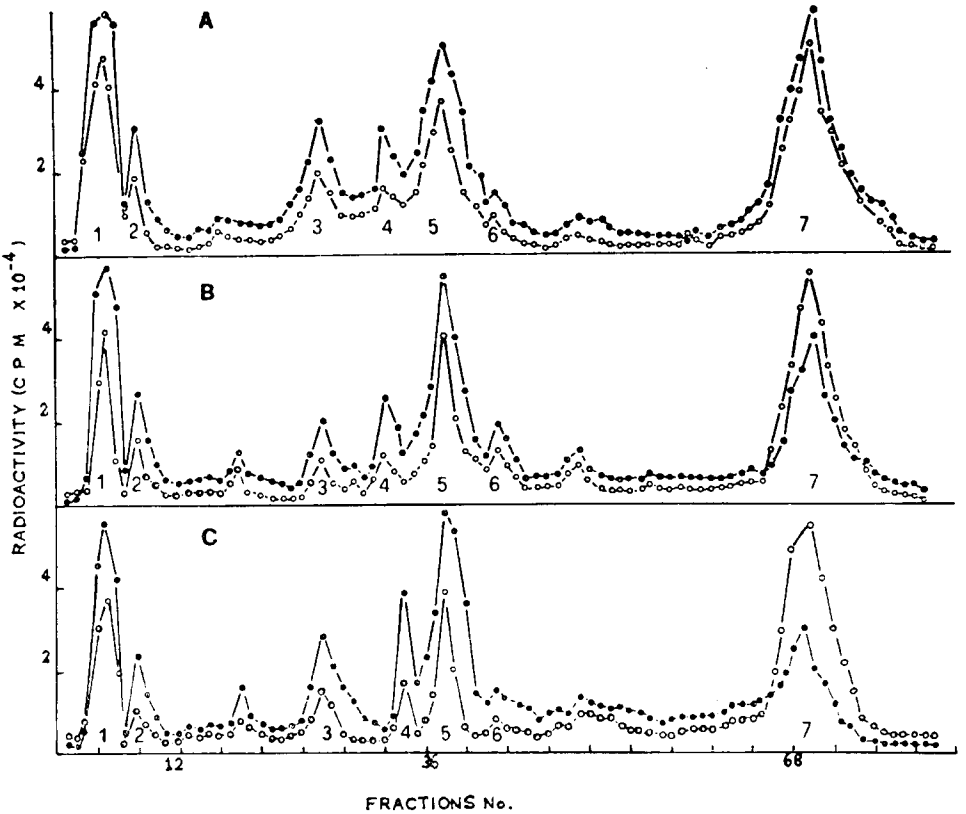

Fig. 5. Comparison of washes and trypsinates from normal and transformed cells by AGI X2 chromatography. Washes and trypsinates differently labelled were mixed as appropriate inputs of radioactivity, purified on Sephadex G-25 and the included material lyophilised and fractionated on AGI X2. A, BHK; B, BHK-py; C, BHKrsv. Open circles indicate the wash, closed circles the trypsinate.

Whereas with transformed cells there was a difference in the relative amount of radioactivity in peak 7, there being less of it in the trypsinates as compared with the EDTA washes (Figs 5 B and C), particularly in the case of RSV-transformed cells. The ratios between the areas of the largest peaks (Peaks 5–7) separated by AGI X2 chromatography have been compared. In the cell trypsinate the ratios for BHK, BHK-py and BHK-rsv were 0.41, 1.10 and 3.02, respectively. In the cell washes, the corresponding ratios were 0.37, 0.40 and 0.51. The notion that transformed cell lines release relatively less of the strongly acidic peak 7 material holds only when considering the trypsinates, as the ratios do not differ grossly in the case of the EDTA-wash material.

6. *High-voltage electrophoresis of glucosamine-labelled mucopeptides in trypsinates from normal and transformed cells*

High-voltage electrophoresis was performed, for checking the homogeneity of the fractions isolated by AGI X2 chromatography and for an independent comparison of the cell trypsinate composition. The results are summarised in Fig. 6, which

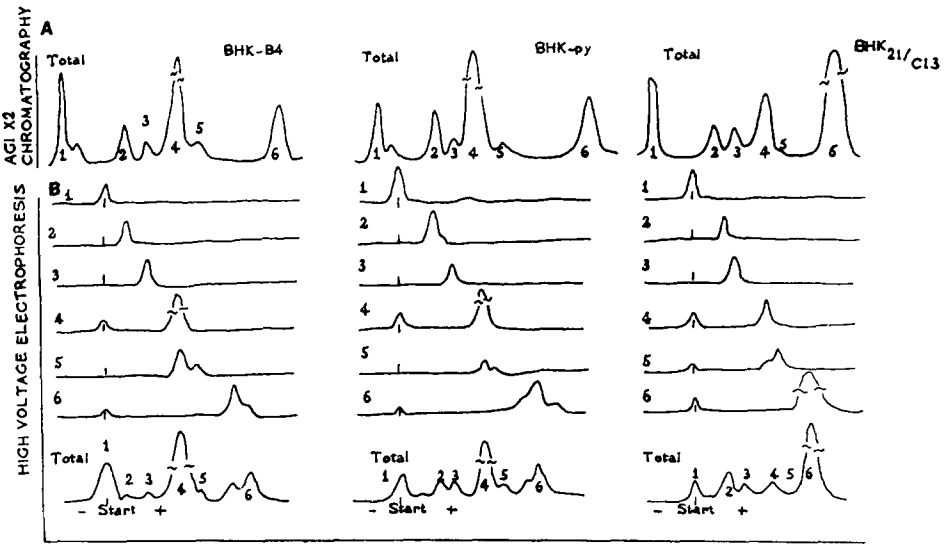

Fig 6. Comparative analysis of glucosamine-labelled trypsinates by AGI chromatography and high-voltage electrophoress. A shows the patterns in AGI X2 of the three total mucopeptides mixture previously purified on Sephadex G-25. B shows the electrophoretic mobility of each peak previously isolated by AGI X2 chromatography and the electrophoretic patterns of the total peptide mixture.

shows an excellent correlation between the anodal mobility of the main isolated fractions and their elution position (i.e. the increasing pyridine molarity and decreasing pH required for their elution). Also evident is the electrophoretic homogeneity of most of the chromatographic peaks, and the general correspondence of the electrophoretic separation patterns with the elution profiles from AGI X2.

7. Preliminary characterization of the sulphated polyanion

Deaminative degradation by nitrous acid is considered to be specific for N-sulphated glycosaminoglycans [19] and it is currently used as an identification procedure for heparan sulphate. A trypsinate from cells grown in the presence of ^{35}S-labelled inorganic sulphate was chromatographed on Sephadex G-25. Fractions from the highest peak (mol. wt around 12 000) were subjected to deaminative treatment with nitrous acid and the material rechromatographed on Sephadex G-25. There was a shift of the elution peak towards the phenol red marker, indicating a partial degradation to material of lower molecular weight.

N-Sulphated glycosaminoglycans are insensitive to hyaluronidase while chondroitin sulphate is sensitive [20]. The experiments with hyaluronidase showed that after treatment with the enzyme the peak labelled by ^{35}S mantained exactly the same molecular weight as revealed by Sephadex G-25 chromatography.

The molarity of NaCl necessary for the elution from Dowex 1 is specific for the various components of the mucopolysaccharide class. Hyaluronic acid, chondroitin sulphate, keratan sulphate, heparan sulphate and heparin are eluted at 0.5, 1.5, 1.3, 1.7 and 2.0 M NaCl, respectively [20]. 1.3 M NaCl was able to elute near 100% of the column input when the ^{35}S-labelled peak was adsorbed to an AGI X2 column.

DISCUSSION

We present evidence that trypsin detaches a complex mixture of glycopeptides and mucopolysaccharides from the surface of cultured hamster fibroblasts which can reproducibly be resolved into discrete fractions on the basis of their ionic behaviour. One of these fractions is a strongly acidic polyanionic material which is not labelled by fucose, is slightly labelled by an amino acid mixture, and is strongly labelled by glucosamine and by inorganic sulphate. This material has been tentatively identified as heparan sulphate.

Interest in this cell surface sulphated polyanion springs from the finding that it is present in lesser amounts in the trypsinates from cells transformed by either polyoma or Rous sarcoma virus as compared with their normal BHK counterparts. All three cell lines also release glycosylated macromolecules upon a mild EDTA treatment, and the materials thus recovered are quite similar in composition among themselves and also to the trypsinate from normal cells. Thus the sulphated polyanion appears to be produced by both normal- and virus-transformed BHK cells, and to be present in similar amounts at the outer, chelant-sensitive, coat; however less of it is covalently attached to the surface of transformed cells in a position accessible to endopeptic cleavage by trypsin.

We have shown that the cell trypsinate does not contain material with affinity for concanavalin A. The fact that trypsin treatment abolishes the difference in agglutinability between transformed and normal cells by making the latter more agglutinable, could be explained by the different amounts of the polyanionic components which are released by trypsin. The increased amounts of the sulphated polyanion which are removed from normal cells by protease could be involved in covering the agglutination sites or somehow interfering with the agglutinability.

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