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# CELL SURFACE AND METABOLIC LABELLING OF THE PROTEINS OF NORMAL AND TRANSFORMED CHICKEN CELLS

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## SUMMARY

We have studied the surface proteins of normal and transformed chick cells using four-labelling techniques with different specificities, (a) lactoperoxidase catalysed iodination (b) galactose oxidase/$B^3H_4$ (c) pyridoxal phosphate/$B^3H_4$ and (d) periodate/$B^3H_4$. All methods labelled a large external transformation-sensitive (LETS) protein, in agreement with previous studies. In addition, using galactose oxidase and periodate labelling techniques, we present evidence which suggests that the transformed cell surface glycoproteins are more sialylated.

The LETS protein was also labelled with [$^{14}$C]glucosamine and after trypsinization a small band of identical molecular weight to LETS remained, possibly representing an internal pool of the protein. In contrast LETS protein labelled with [$^3$H]fucose was completely removed by trypsin, suggesting that the internal pool of the protein is incompletely glycosylated. Evidence is also presented to show that although the level of the protein is drastically reduced at the transformed cell surface, it is still synthesised and shed into the medium.

## INTRODUCTION

Lactoperoxidase-catalysed iodination of a variety of animal fibroblasts grown in culture labels a surface protein with apparent mol. wt. 250000 [1–4]. The protein which is maximally exposed during the $G_1$ phase of the cell cycle [5], is a glycoprotein [6–8], markedly sensitive to trypsin and absent or much reduced on cells transformed by tumour viruses. It has been termed the LETS protein i.e. large, external, transformation-sensitive [5].

Other cell surface proteins are less well labelled by iodination, making identification of additional possible transformation sensitive changes in membrane architec-

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Abbreviation: LETS protein, large external transformation-sensitive protein.

ture more difficult. The development of other cell surface labelling methods with different specificities [8–10] made it important to re-examine the proteins of normal and transformed fibroblasts.

In this paper we have chosen to look at transformation of chicken embryo fibroblasts by Rous sarcoma virus because of the availability of viral mutants temperature sensitive for maintenance of the transformed state [11]. Surface proteins labelled by lactoperoxidase catalysed iodination have been compared with those proteins labelled by borotritiide reduction after exposure to (a) galactose oxidase (b) pyridoxal phosphate and (c) periodate.

## METHODS

*Cell culture.* Secondary chicken embryo fibroblast cultures of C/E phenotype were infected with either wild-type Prague strain Rous sarcoma virus subgroup A, or a temperature-sensitive mutant obtained from this virus LA 24 [11]. Cells were cultured at 35 °C, the permissive or 41 °C the non-permissive temperature as described in detail elsewhere [4, 11].

To maximise the number of transformed cells in an infected population, some cultures were obtained by pooling colonies of transformed cells isolated from semi-solid agar medium.

*Surface-labelling techniques.* Dense cultures (9-cm dishes) were labelled in situ after washing twice with phosphate-buffered saline pH 7.4. Tyrosine residues on cell surface proteins were labelled using lactoperoxidase catalysed iodination [1, 12] and galactose and galactosamine residues in glycoproteins by the galactose oxidase/ brotritiide method [7, 8] as previously described [1, 7].

Amino groups in cell surface proteins were labelled with tritium by first incubating with 1 ml of 0.5–1 mM pyridoxal phosphate [9] in phosphate-buffered saline pH 8.0 for 20 min. Excess reagent was removed by washing twice with buffer pH 8.0 prior to reduction in monolayer by adding 0.5 mCi of borotritiide (in 50 $\mu$l of cold 0.01 M NaOH) to cells overlayed with 1 ml of phosphate-buffered saline pH 7.4.

An attempt was also made to label specifically sialoglycoproteins [10] by incubating cells with 1 mM periodate in 1 ml of phosphate-buffered saline ($Ca^{2+}$, $Mg^{2+}$ free) pH 7.0. The reaction was stopped after 10 min at room temperature with equimolar glycerol prior to reduction with borotritiide. All buffers contained 2 mM phenylmethylsulphonylfluoride (Sigma), a protease inhibitor.

Cells were dissolved by boiling in 2 % sodium dodecyl sulphate (SDS) containing dithiothreitol, the labelled proteins separated in SDS-polyacrylamide vertical slab gels [13] and radioactivity detected by autoradiography or fluorography as previously described [7, 14].

*Metabolic labelling.* Cells were labelled for 24 h with [U-$^{14}$C]glucosamine (2 $\mu$Ci/ml, 318 Ci/mol) in Dulbecco's modification of Eagle's medium containing one-third the normal concentration of glucose. A similar procedure was used for labelling with L-[1-$^{3}$H]fucose (10 $\mu$Ci/ml, 2.82 Ci/mol). Total cellular or viral proteins were labelled using L-[U-$^{14}$C]leucine (10 $\mu$Ci/ml, 270 Ci/mol) in medium containing one-tenth the normal concentration of amino acids.

*Virus purification.* Virus-containing culture fluids were harvested at 3–6-h intervals and purified by the method of Duesberg et al. [15]. Virus precipitated from

clarified medium with ammonium sulphate was first concentrated at a 20/65 % sucrose interface and then banded in a 20–50 % linear sucrose gradient.

*Materials.* All isotopes were obtained from the Radiochemical Centre, Amersham, U.K. $KB^3H_4$ (3.3–14.6 Ci/mmol) supplied in 100-mCi batches was dissolved in 10 ml of ice-cold 0.01 M NaOH and 5-mCi aliquots were stored at −70 °C.

Hyaluronidase, 3400 units/mg, was supplied by Worthington Biochemicals and assayed using an acid/albumin turbidity method described in the Worthington Enzymes Handbook. Neuraminidase obtained from Behringwerke was virtually free of trypsin-like activity as judged by its failure to remove iodinated LETS protein (band 1 in figures) which is completely removed from NIL2 hamster fibroblasts in 10 min by 5 μg/ml trypsin.

Protease contamination of galactose oxidase proved to be a much greater problem. Sigma type III enzyme (now discontinued) from *Dactylium dendroides* contained very low levels of protease. Later preparations from *Polyporus circinatus* were heavily contaminated but could be totally freed of the activity using the galactose polymer Sepharose 4B as an affinity column for the galactose oxidase [16]. Recoveries in our hands were however < 50 %. Both Worthington and Kabi enzymes contained far less protease activity and effected the removal of < 10 % of LETS protein over a 90-min period when used at 2 units/ml, a concentration of enzyme which although marginally sub-optimal, is adequate for labelling.

## RESULTS

### *Galactose oxidase/borotritiide labelling*

After incubation with galactose oxidase alone, labelling of normal chick embryo fibroblasts grown at 35 °C was characterised by a high molecular weight glycoprotein ($M_r$ approx. 250 000) of similar mobility to that seen in other fibroblasts (Fig. 1a). Other labelled proteins showed no such obvious correlation with those seen in cells such as NIL2 hamster fibroblasts where the pattern is somewhat less complex [7]. Clearly many of the bands are heterogeneous, but the major bands have been labelled 1 to 7 for ease of reference.

Pretreatment of the cells with neuraminidase greatly enhanced the labelling of many of the glycoproteins although the high molecular weight glycoproteins in bands 1 and 2 were less affected (Fig. 1a, b, c). No new major glycoprotein bands were detected after neuraminidase, but those in which labelling was enhanced had a slightly greater mobility than those seen after incubation with galactose oxidase alone (e.g. bands 5 and 6). This may represent labelling of totally new proteins within a heterogeneous array of glycoproteins or may be due to a slight change in mobility of proteins after removal of sialic acid. There was little labelling of proteins in the absence of galactose oxidase and most of this was concentrated in the low molecular weight region of the gel.

Cells transformed by wild-type Rous sarcoma virus and grown at 35 °C were barely labelled after incubation with galactose oxidase alone in contrast to normal cells (Fig. 1a, d). Pre-treatment of cells with neuraminidase greatly enhanced labelling mainly in the regions of bands 5, 6 and 7, molecular weight range about 90–170 000, (Fig. 1e). As was the case with normal cells, glycoproteins in the region of bands 5, 6

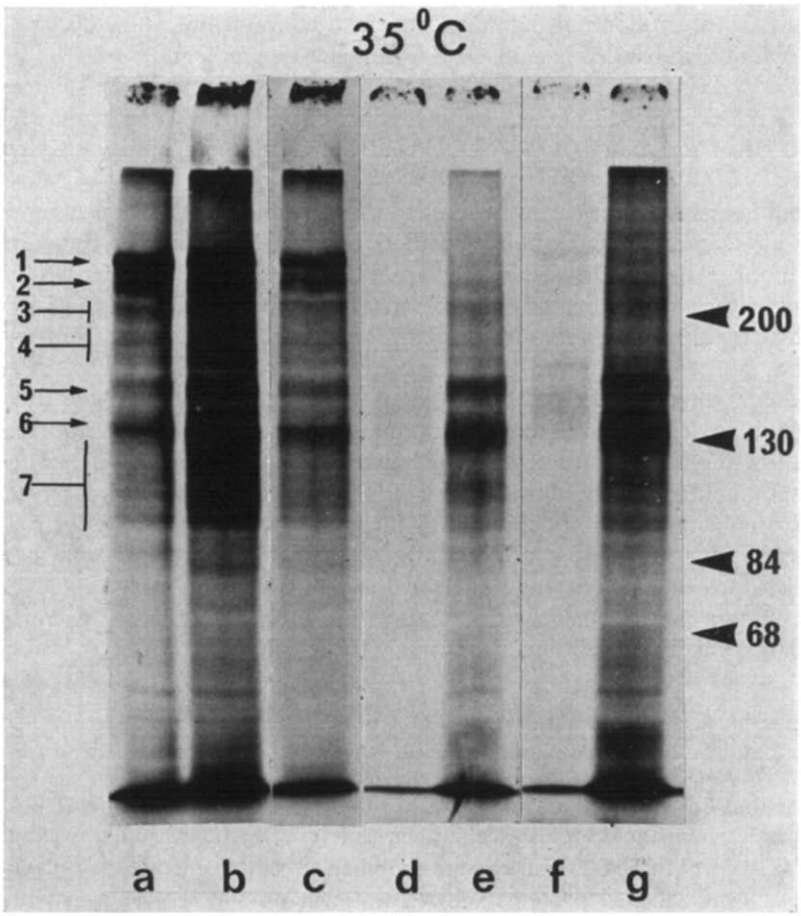


Fig. 1. For legend see opposite page.

and 7 whose labelling was enhanced by neuraminidase pre-treatment did not exactly correspond with proteins seen after galactose oxidase treatment alone. This slight change in mobility was noticeably greater in the transformed compared with normal cells (most clearly seen in Fig. 1k, l).

The most obvious difference in labelling pattern between normal and transformed cells was the virtual absence of the major high molecular weight glycoprotein (band 1) from transformed cells, (Fig. 1c, e). Possibly related to this observation was the absence of the triplet of proteins (band 2) consistently seen in normal cells. Whether these molecules are truly characteristic of the surface of normal cells or are degradation fragments of band 1 is uncertain. In addition, although the molecular weight distribution within bands 3, 4, 5 and 6 was similar in both normal and transformed cells, band 7 was rather more heterogeneous in normal than transformed cells where two predominant bands were observed (Fig. 1c, e). However, the complexity of the labelling pattern in this region of the gel makes it impossible to be certain of slight differences.

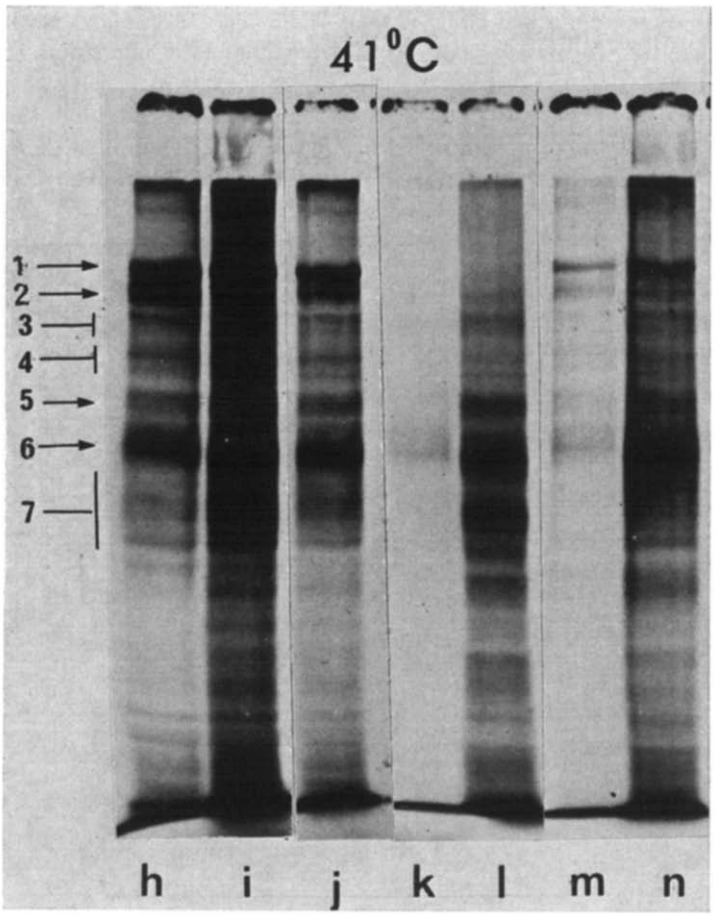


Fig. 1. Galactose oxidase borotritiide labelling of chick embryo fibroblasts and cells infected with wild-type Rous sarcoma virus or *LA*24 virus, a temperature sensitive viral mutant. Cells were grown at 35 °C (a–g) or 41 °C (h–n). (a) Chick embryo fibroblasts incubated with galactose oxidase alone, then labelled with $B^3H_4$. (b) Chick embryo fibroblasts incubated with neuraminidase then galactose oxidase. (c) Lighter exposure of (b). (d) Wild-type transformed cells incubated with galactose oxidase alone. (e) Wild-type transformed cells incubated with neuraminidase then galactose oxidase. (f) *LA*24 infected cells incubated with galactose oxidase alone. (g) *LA*24 infected cells incubated with neuraminidase then galactose oxidase. (h–n) Protocol as for cells grown at 35 °C. All cells were labelled when dense. Proteins were separated on a 7 % SDS-polyacrylamide gel with a 4 % stacking gel. Gel tracks are normalised for protein. Numbers 1–7 on the left of the figure refer to protein bands consistently labelled in normal cells. Molecular weight markers used (see right hand side of gel) were rat myoblast myosin (200 000), $\beta$-galactosidase (130 000), lactoperoxidase (84 000), bovine serum albumin (68 000).

These differences between putative cell surface glycoproteins of normal and transformed cells were all found to be temperature sensitive in cells infected with the viral mutant *LA* 24. Thus cells grown at 35 °C, the permissive temperature were poorly labelled without prior neuraminidase treatment, and at 41 °C labelling was increased (Fig. 1f, m). The neuraminidase induced mobility change in bands 5 and 6 was also

temperature sensitive being more marked at 35 °C than 41 °C (Fig. 1f, g, m, n). Both these results suggest that transformation leads to a higher degree of sialylation of some cellular glycoproteins.

In addition, labelling of band 1 was markedly increased at 41 °C although the extent of labelling was never quite as great as seen in normal cells. In contrast there were no obvious temperature-sensitive differences in normal cells or cells transformed by wild-type virus.

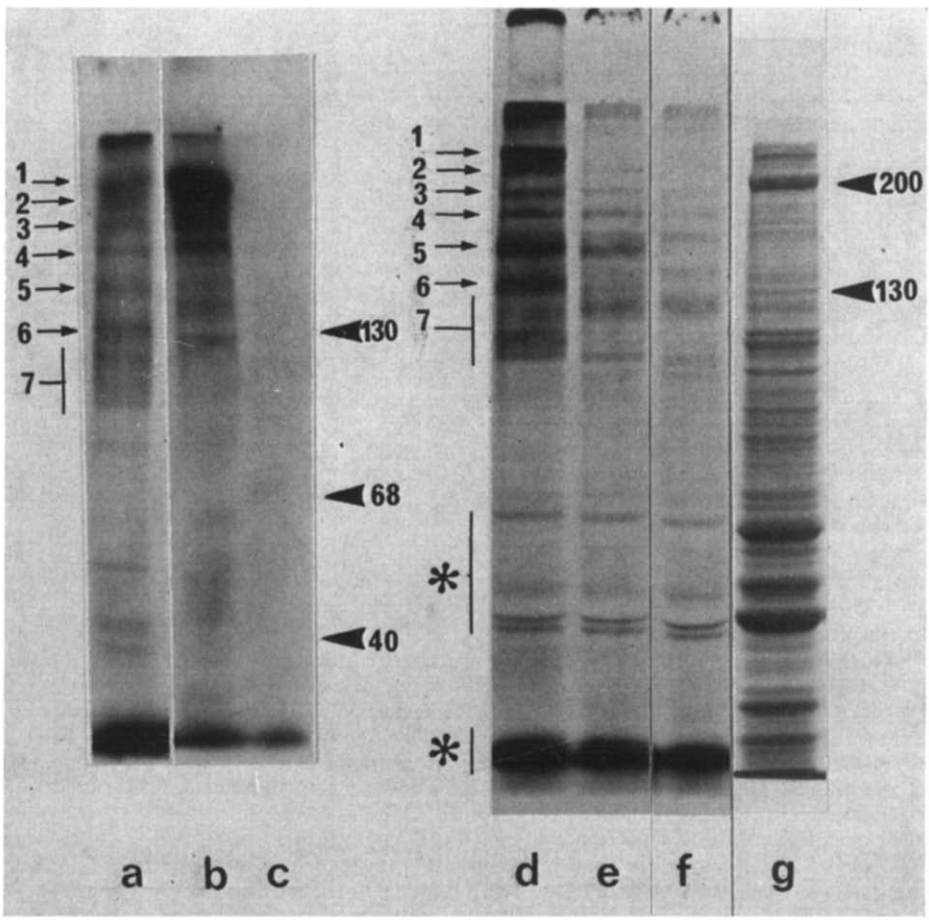


Fig. 2. Comparison of proteins of chick embryo fibroblasts labelled by galactose oxidase/borotritiide, and lactoperoxidase catalysed iodination; protease sensitivity of label. (a) Cells labelled with borotritiide after neuraminidase and galactose oxidase treatment. (b) Iodinated cells. (c) Iodinated cells treated with trypsin (50 $\mu$g/ml, 10 min). (d) Cells labelled after neuraminidase and galactose oxidase treatment. (e) Cells labelled as in (d) then treated with 50 $\mu$g/ml trypsin. (f) 2000 $\mu$g/ml trypsin. (g) Coomassie stained gel equivalent to track (d) is shown for comparison with labelled proteins. Proteins were separated on 7% SDS-polyacrylamide gels. Asterisks represent areas of labelling with borotritiide not dependent on galactose oxidase. Molecular weight markers as in Fig. 1 except creatine kinase (40 000). Samples a–c were normalised to a different protein value than d–f.

*Comparison of galactose oxidase/borotritiide, and lactoperoxidase-catalysed iodination labelling patterns. Trypsin sensitivity of labelled proteins.*

The high molecular weight protein seen by the galactose oxidase/borotritiide method has an identical mobility to the LETS protein previously detected by lactoperoxidase-catalysed iodination of chick cells, (Fig. 2a, b) [4]. Some of the other iodinated proteins have a similar molecular weight range (90–220 000) to those seen by the galactose oxidase method although there is no exact correspondence between the bands labelled. The labelling intensities of the various bands produced by the two methods are also quite different.

Using either labelling method, band 1 was completely removed by exposing the labelled cells to 50 μg/ml trypsin for 10 min (Fig. 2b, c, d, e), although many of the other proteins were less sensitive to trypsin. Band 2 was also completely removed by this concentration of enzyme, and bands 3 and 4 were reduced to some extent, although even 2 mg/ml trypsin for 10 min failed to effect their complete removal; (Fig. 2d–f). Bands 5, 6 and 7 showed evidence of altered mobility after 50 μg/ml trypsin and some new bands of lower molecular weight were observed. An overall reduction in label remaining was produced by 2 mg/ml trypsin but a considerable amount of label remained even after this rather more rigorous treatment. Similar results were obtained using pronase (not shown).

The sensitivity of the proteins labelled to mild proteolysis suggests that the galactose oxidase method is specific for cell surface molecules. The specificity of the procedure is shown if one compares the labelling pattern with total cellular proteins stained by Coomassie blue, (Fig. 2d, g). Proteins labelled in the absence of galactose oxidase had molecular weights of less than 55 000.

*Effect of hyaluronidase on labelling of transformed cells*

Although the low degree of labelling of transformed cells by galactose oxidase may be explained by increased sialylation of their glycoproteins, we also noted that they are iodinated less well than the normal controls (Table I). It had previously been reported that transformed chicken embryo fibroblasts produce greatly increased levels of hyaluronic acid, and Burger and Martin [17] showed that this could indeed

TABLE I

EFFECTS OF NEURAMINIDASE AND HYALURONIDASE PRETREATMENT ON IODINATION OF NORMAL AND VIRUS-TRANSFORMED CHICKEN CELLS

Pretreatments were for 2.5 h in phosphate-buffered saline, pH 7.4 at room temperature. Neuraminidase, 50 μg/ml; Hyaluronidase, 100 μg/ml.

| Cell type | Pretreatment | Specific activity (cpm/μg) |
|---|---|---|
| Normal | – | 1510 |
| | neuraminidase | 1426 |
| | hyaluronidase | 1199 |
| Transformed | – | 193 |
| | neuraminidase | 180 |
| | hyaluronidase | 145 |

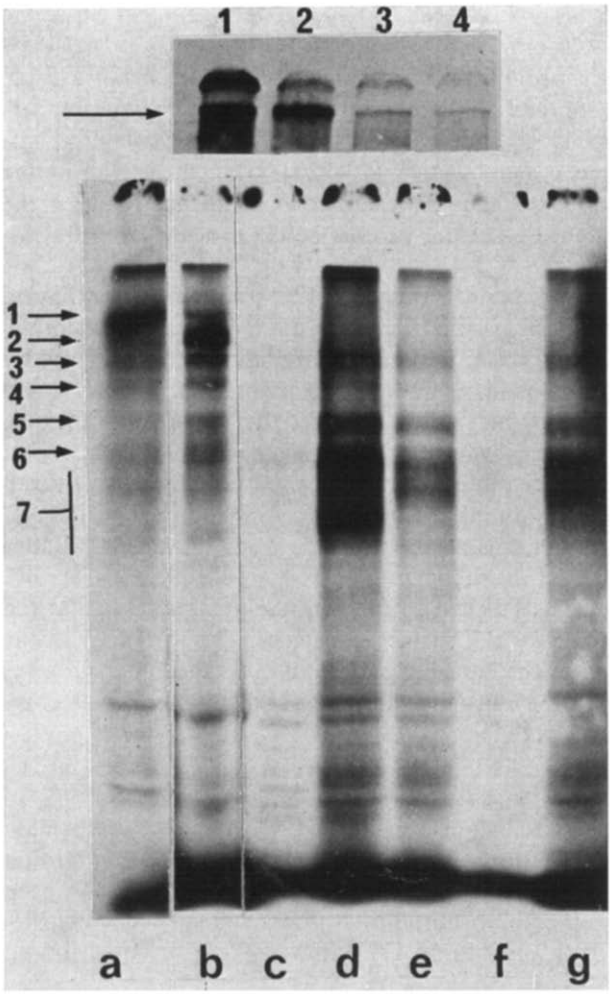


Fig. 3. Effect of hyaluronidase on galactose oxidase labelling patterns. Normal chick embryo fibroblasts were labelled after (a) treatment with galactose oxidase alone (b) treatment with hyaluronidase 100 $\mu$g/ml 60 min then galactose oxidase. Transformed chick embryo fibroblasts were also labelled after treatment with: (c) Galactose oxidase alone. (d) Neuraminidase then galactose oxidase. (e) Trypsin 50 $\mu$g/ml, then neuraminidase and galactose oxidase. (f) Hyaluronidase (100 $\mu$g/ml) then galactose oxidase. (g) Hyaluronidase, neuraminidase and galactose oxidase. Proteins were separated on 7 % SDS-polyacrylamide gels. Gel tracks were normalised for protein. Insert shows a stacking gel from cells metabolically labelled with [$^{14}$C]glucosamine and treated with (1) phosphate-buffered saline 60 min; (2) 100 $\mu$g/ml trypsin 30 min; (3) hyaluronidase 100 $\mu$g/ml for 30 min and (4) hyaluronidase for 60 min.

affect a comparison of the surface properties of these cells. We therefore tested the effect of hyaluronidase on the labelling pattern of normal and transformed cells by both techniques. Hyaluronidase failed to increase the labelling of transformed cells by galactose oxidase alone, and no new proteins were detected if neuraminidase was also included (Fig. 3c, f, g). The enzyme preparation appears to have some proteolytic

activity as evidenced by a reduction in labelling of band 1 in normal cells, with a concomitant increase in label in band 2 region (Fig. 3a, b).

To check that indeed hyaluronic acid was being removed, transformed cells metabolically labelled with [$^{14}$C]glucosamine were treated with either trypsin, hyaluronidase, or buffer as a control. As seen in Fig. 3 (insert 1–4), the intensity of a band running in the stacking gel was markedly reduced by incubating the cells with hyaluronidase (100 $\mu$g/ml) for 30 or 60 min but was little affected by incubation with 100 $\mu$g/ml trypsin for 30 min, or buffer. Labelled proteins in the running gel were not affected by hyaluronidase.

Trypsin was also tested to see if it might remove a surface component from transformed cells which would result in improved labelling with galactose oxidase. Incubation with 50 or 500 $\mu$g/ml trypsin failed to increase labelling (not shown) and no new bands were detected if cells were first incubated with trypsin then neuraminidase/galactose oxidase (Fig. 3e). In contrast to these negative results with hyaluronidase and trypsin, neuraminidase was again found to dramatically enhance labelling of transformed cells by the galactose oxidase/borotritiide method (Fig. 3c, d). However, Table I shows that neither neuraminidase nor hyaluronidase improved iodination of transformed cells. Clearly poor iodination of transformed cells cannot be explained on the same basis as that for galactose oxidase labelling.

*Pyridoxal phosphate and periodate labelling*

In an attempt to probe further a possible transformation dependent difference in membrane architecture two other labelling techniques were employed. Initially cells were labelled with a pyridoxal phosphate/borotritiide reaction previously used to label exposed proteins on the surface of Rous sarcoma virus [9]. In contrast to iodination which labels exposed tyrosine residues, pyridoxal phosphate reacts with free amino groups, the Schiff's base formed being stabilised by reduction with borotritiide. However, as with iodination, the major protein labelled in normal chicken embryo fibroblasts was a high molecular weight protein of apparent $M_r$ 250 000. (Fig. 4a). The protein was markedly sensitive to trypsin as were less well labelled proteins suggesting that the method does indeed label surface proteins (Fig. 4b). As expected from previous results, band 1 protein was not detected on cells infected with the temperature sensitive Rous sarcoma virus mutant *LA* 24 and grown at 35 °C, but reappeared on cells grown at 41 °C, (Fig. 4c, e).

An attempt was also made to introduce label specifically into sialic acid residues by first exposing the cells to a mild periodate oxidation followed by reduction with borotritiide. The major protein labelled by this technique was again a protein of apparent $M_r$ 250 000, (Fig. 4g) exactly corresponding in mobility with the iodinated protein, and totally removed by mild proteolysis (not shown). Other proteins labelled, ranging in molecular weight from 85–200 000 did not exactly correspond to those seen by the galactose oxidase method. Prior incubation of the cell with neuraminidase markedly reduced this labelling supporting the idea that sialic acid residues are labelled by this method, (Fig. 4i). However, band 1 was still strongly labelled after a neuraminidase pretreatment, and other evidence suggests that this protein contains little sialic acid [7, 18]. Other linkages sensitive to mild periodate treatment must be responsible for labelling of this molecule. As by the other methods, labelling of band 1 behaved in the predicted fashion in cells infected with the *LA* 24 mutant, (Fig. 4f, g,

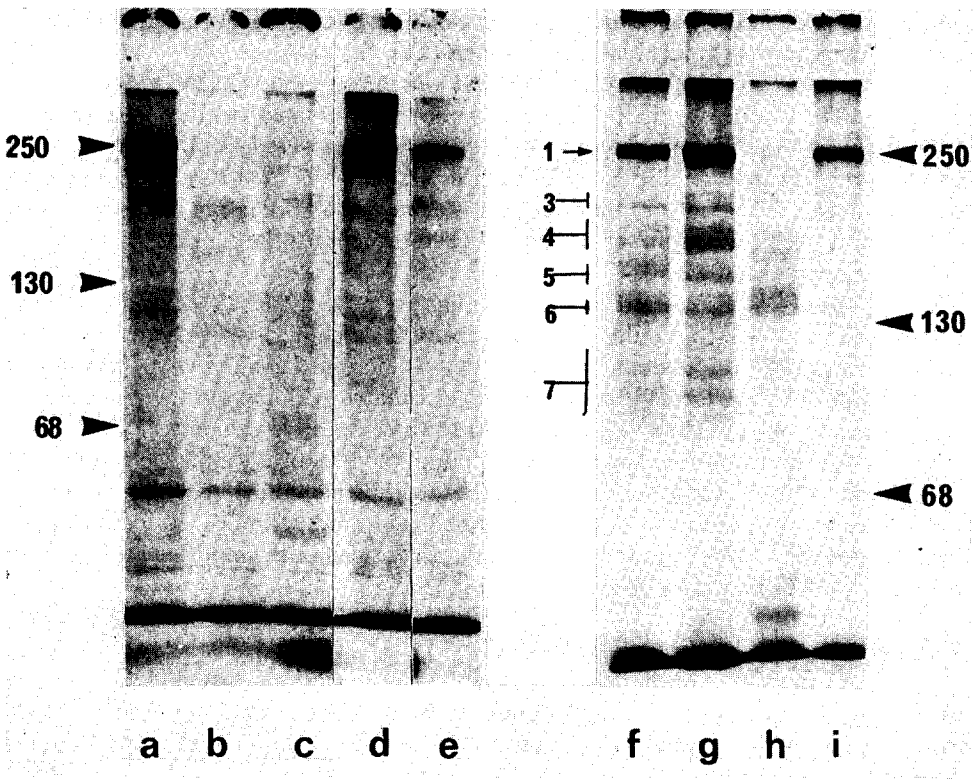


Fig. 4. Borotritiide labelling pattern produced after reaction with pyridoxal phosphate, and periodate. Key to gel: Pyridoxal phosphate labelling (a–e). (a) Chick embryo fibroblasts grown at 35 °C. (b) Chick embryo fibroblasts, 35 °C treated with trypsin (50 $\mu$g/ml) after labelling. (c) *LA*24 infected chick embryo fibroblasts grown at 35 °C. (d) Chick embryo fibroblasts, 41 °C. (e) *LA*24 infected chick embryo fibroblasts, 41 °C. Periodate labelling (f–i) (f) *LA*24 infected chick embryo fibroblasts grown at 41 °C. (g) Chick embryo fibroblasts, 41 °C. (h) *LA*24 infected chick embryo fibroblasts, 35 °C. (i) Chick embryo fibroblasts 41 °C, pretreated with neuraminidase before reaction with periodate. Proteins were separated on 7 % SDS-polyacrylamide gels. The approximate positions of bands 1–7 labelled by galactose oxidase are also shown. Samples a–c were normalised to a different protein value than f–i.

h). Comparison of the profile of the lower molecular weight proteins in this system showed considerable differences between the virus infected cells grown at 35 °C or 41 °C again suggesting that transformation leads to an alteration in cellular sialoglycoproteins.

*Metabolic labelling of cellular glycoproteins*

To compare the overall pattern of glycoprotein synthesis in these cells with those proteins labelled by surface-labelling techniques, subconfluent cultures were labelled for 20 h with either [$^3$H]fucose or [$^{14}$C]glucosamine. The transformed cells were frequently better labelled than the normal cells, possibly because the rate of cell division slows when normal cells reach confluence. Fig. 5a, shows that band 1 was the major protein labelled by [$^{14}$C]glucosamine during a 20-h period in normal cells. The

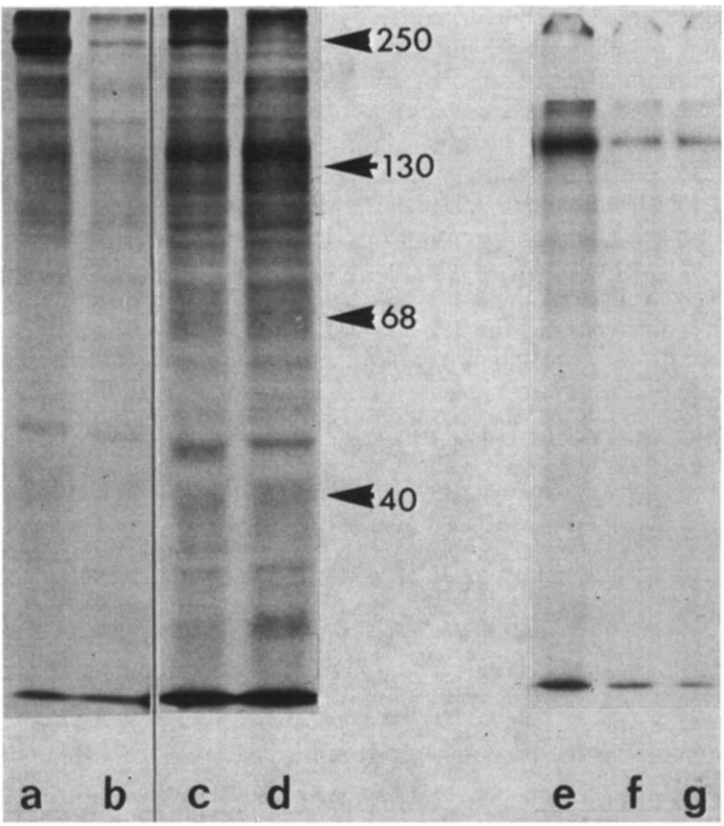


Fig. 5. Comparison of [$^{14}$C]glucosamine labelling of normal and transformed chick embryo fibroblasts with the cell surface iodination pattern. [$^{14}$C]glucosamine labelling profiles, (a–d). (a) Chick embryo fibroblasts. (b) Chick embryo fibroblasts labelled and susbequently trypsinized (50 $\mu$g/ml for 10 min). (c) "Colony purified" transformed cells. (d) "Colony purified" transformed cells labelled then trypsinized. Lactoperoxidase-catalysed iodination patterns, (e–g). (e) Chick embryo fibroblasts. (f) Wild-type transformed cells. (g) "Colony purified" transformed cells. Proteins were separated on 7 % SDS-poylacrylamide gels. Samples a–d were normalised to a different protein value than e–g.

label was nearly completely removed by trypsin but a small band consistently remained possibly representing an internal pool of band 1, (Fig. 5b). Labelling of transformed cells showed that a glycoprotein of identical molecular weight to band 1 was synthesised, but it represented only a small percentage of the total label (not shown).

If the high molecular weight glycoprotein synthesised by a transformed cell population is indeed band 1, the possibility remained that it was due to the presence of a small number of untransformed cells. Transformed cell colonies were therefore isolated from semi-solid agar medium, a procedure which should select for a population of fully transformed cells. These cells were then compared with normal cells for (a) incorporation of [$^{14}$C]glucosamine into a high molecular weight protein (b) their surface labelling profile. The colony-purified transformed cells still incorporated significant quantities of [$^{14}$C]glucosamine into a protein with identical mobility to

band 1, and the label was significantly reduced by trypsin, (Fig. 5c, d). By surface labelling, small amounts of iodinatable band 1 protein were detected on both the uncloned and the cloned population of cells, (Fig. 5e–g).

The synthesis of a high molecular weight protein by both normal and transformed cells was also demonstrated by metabolic labelling with [$^3$H]fucose (Fig. 6). Interestingly, whereas a band in the same position as band 1 can still be detected after trypsinization of [$^{14}$C]glucosamine labelled cells (Fig. 5a–d), little or no label was detected in this position after trypsinization of [$^3$H]fucose-labelled cells, (Fig. 6a–d). Glycoproteins in the molecular weight range 80–170 000 labelled by [$^3$H]-fucose were qualitatively similar to those labelled by [$^{14}$C]glucosamine although there were considerable differences in the 190–220 000 region which remain to be evaluated. Comparison of these metabolically labelled glycoproteins with those

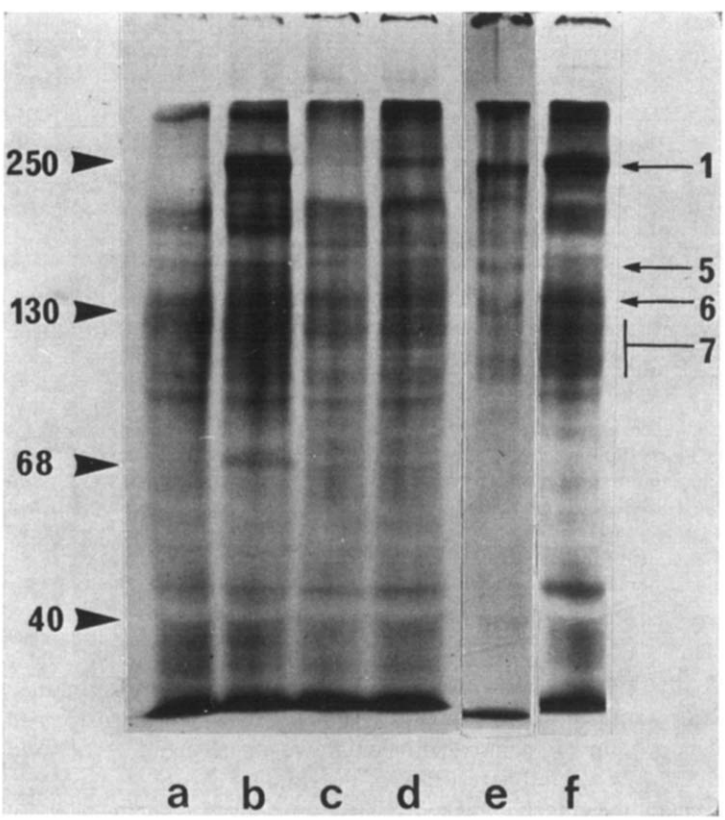


Fig. 6. Comparison of [$^3$H]fucose and [$^{14}$C]glucosamine labelling patterns with surface proteins labelled by the galactose oxidase/borotritiide method. Key to gel: (a) Chick embryo fibroblasts labelled with [$^3$H]fucose then trypsinized (50 $\mu$g/ml for 10 min). (b) Chick embryo fibroblasts labelled with [$^3$H]fucose; untreated. (c) Wild-type transformed cells labelled with [$^3$H]fucose then trypsinized. (d) Transformed cells labelled with [$^3$H]fucose; untreated. (e) Chick embryo fibroblasts labelled by the galactose oxidase/B$^3$H$_4$ method. (f) Chick embryo fibroblasts labelled with [$^{14}$C]glucosamine Proteins were separated on 7 % SDS-polyacrylamide gels. Numbers 1, 5, 6, 7 relate to bands routinely seen after galactose oxidase/B$^3$H$_4$ labelling; see track (e). Gel tracks a–d were normalised to protein.

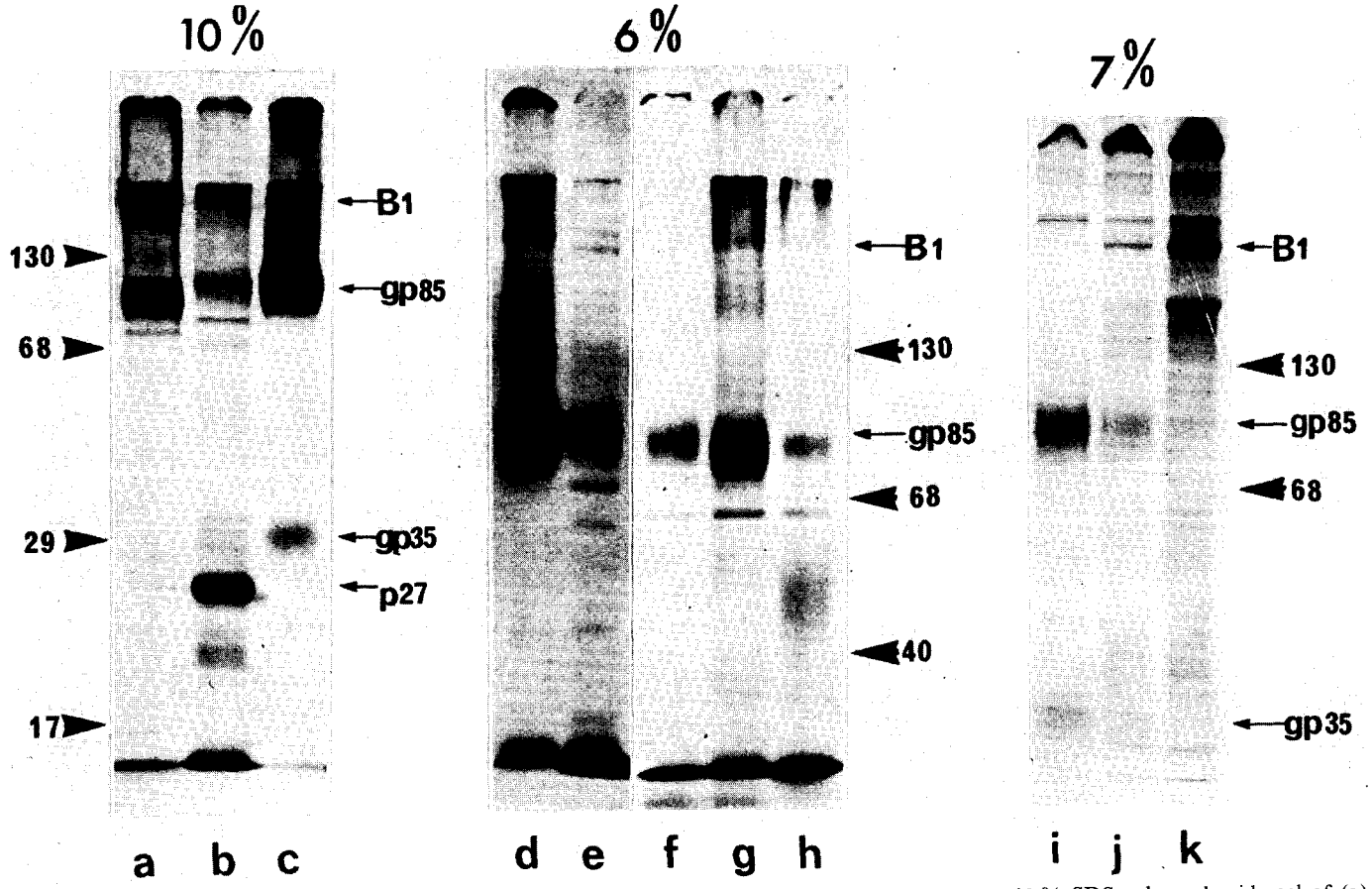


Fig. 7. Metabolic and surface labelling patterns of Rous sarcoma virus preparations. Key to figure: a–c, 10% SDS-polyacrylamide gel of (a) virus labelled with borotritiide after neuraminidase and galactose oxidase. (b) [$^{14}$C]leucine and (c) [$^{14}$C]glucosamine labelled virus. (d–h) 6% SDS-polyacrylamide gels of (d) [$^{14}$C]glucosamine and (e) [$^{14}$C]leucine labelled virus. (f) Virus labelled after galactose oxidase treatment alone. (g) After neuraminidase and galactose oxidase. (h) as (g) followed by 50 $\mu$g/ml trypsin 10 min. (i–k) 7% SDS-polyacrylamide gel. Profile of [$^{14}$C]glucosamine labelled virus concentrated at a 20/65% sucrose interface. Fractions were collected dropwise from the bottom of the tube, and material in track (i) was the first to be collected. Major viral components are identified by the recently proposed nomenclature [19]. B1 represents the mobility of the 250 000 mol. wt. protein characteristic of untransformed fibroblast as determined from samples simultaneously run but not shown. Additional molecular weight markers not previously used, carbonic anhydrase (29 000) and myoglobin (17 000).

labelled by the neuraminidase galactose oxidase technique showed that the majority of the glycoproteins synthesised by the cell are in the same molecular weight range as those detected by a surface-labelling method, (Fig. 6b, e, f). However no precise comparisons were possible except in the case of band 1.

*Band 1 glycoprotein in preparations of Rous sarcoma virus*

Incidental to attempts to detect viral proteins and tumour specific antigens in transformed cells using surface labelling techniques we found that Rous sarcoma virus preparations purified by standard methods frequently contained a protein of identical mobility to band 1 protein. Fig. 7b, c shows [$^{14}$C]leucine- and glucosamine-labelled virus run on 10 % SDS-polyacrylamide gels. The major viral proteins are tentatively identified and labelled according to the recently published nomenclature [19]. Virus preparations showed one major band when labelled with galactose oxidase-borotritiide approximately corresponding to the major viral glycoprotein (gp 85), (Fig. 7d, e, f). Labelling was markedly enhanced in virus pretreated with neuraminidase, (Fig. 7g) although the mobility of the major protein labelled was now significantly different from gp 85, presumably due to removal of sialic acid from the molecule (Fig. 7a, c, d, f, g). In addition a protein of similar mobility to band 1 was consistently present. Unlike the cell-associated protein, the band was not completely removed by 50 $\mu$g/ml trypsin although this treatment dramatically reduced the level of label in gp 85, yielding a fragment of broad molecular weight range (43–58 000). (Fig. 7h).

A more detailed examination of the virus purification procedure showed that a diffuse band of material was present just above the 20/65 % sucrose interface at which the virus is first concentrated before subsequent banding on a linear gradient. A gel profile of these fractions labelled with [$^{14}$C]glucosamine is shown in Fig. 7i–k. The material in the 20 % sucrose was enriched in a protein of identical mobility to band 1, a glycoprotein of apparent $M_r$ 180 000, and other heterogenous glycoproteins including small amounts of gp 85. In contrast, the material at the interface contained only trace amounts of band 1 but was enriched in gp 85 and gp 35. The profile of labelling was uniform throughout the virus, which subsequently gave a homogenous band at a density of 1.16 on a 20–50 % linear sucrose gradient. Identical results were obtained with virus harvested from the medium of colony-purified transformed cells substantiating the idea that the band 1 protein is still synthesised after viral transformation.

## DISCUSSION

The results reported here show the advantage of using several labelling methods with different specificities when studying cell surface proteins. While iodination and pyridoxal phosphate labelling reactions emphasize a 250 000 mol. wt. protein on the surface of chick embryo fibroblasts, the galactose oxidase and periodate methods show it to be only one of many other surface glycoproteins.

The idea that galactose oxidase labels only cell surface components is partially confirmed by the sensitivity of the labelled proteins to low concentrations of trypsin. All of the proteins labelled (except LETS [18]) are also found in an isolated plasma membrane fraction (unpublished results). Interestingly whereas the 250 000 mol. wt. glycoprotein is totally removed by incubation with 50 $\mu$g/ml trypsin for 10 min, only

the mobility of the other molecules labelled is altered. The major glycoprotein (gp 85) of Rous sarcoma virus showed a similar resistance to trypsin, a carbohydrate-containing fragment ($M_r$ approx. 50 000) remaining bound to the virus even after extensive washing. Assuming that the C-terminal regions are buried in the lipid bilayer, the results suggest that only short regions of the N-terminal end of the molecules are sensitive or available to trypsin.

Evidence for the pyridoxal phosphate borotritiide method specifically labelling surface components largely rests on the work of Rifkin et al. [9] who showed that the nucleocapsid protein of influenza virus was not labelled unless virus was made leaky. Similarly, we have found that major internal cell proteins are not labelled by this method and the label is sensitive to brief exposure to low concentrations of trypsin. The similarity between iodination and pyridoxal phosphate labelling patterns suggests that (a) band 1 protein was not iodinated to the exclusion of other surface proteins because it might be unique in having a large number of tyrosine residues; (b) the polypeptide portion of the molecule must be relatively exposed; the fact that other membrane glycoproteins are poorly labelled suggests that their polypeptide chains are inaccessible either in the lipid bilayer or buried under an umbrella of carbohydrate residues.

We have yet to fully characterize the periodate labelling technique although in erythrocyte membranes the label was concentrated in the major sialoglycoprotein as a tritiated seven carbon derivative of sialic acid, [10]. The sensitivity of much of the label in chick embryo fibroblasts to neuraminidase suggests that the method is specifically labelling sialoglycoproteins. However labelling of band 1 was not greatly reduced by neuraminidase. Periodate cleaves C-C bonds between *cis*-hydroxyls, and sialic acid is more sensitive than other sugars because it contains this configuration on a short 3-carbon chain outside the pyranose ring structure. Labelling of band 1 in sugars apart from sialic acid may be due to the presence of a large excess of periodate, and we are presently looking more closely at the concentration dependence of the reaction. An additional problem with periodate is that there is no reason to assume that it will not enter the cell. Labelling with other techniques is thought to be limited to the surface membrane because the cell excludes the enzymes required to catalyze the reactions. Cell membranes are also relatively impermeable to phosphate esters e.g. pyridoxal phosphate. However sensitivity of periodate induced labelling to trypsin and neuraminidase supports a surface localization. The short incubation time and low concentrations of periodate used may help to limit the reaction to surface components.

These surface labelling studies show that the high molecular weight proteins labelled by iodination or galactose oxidase have the same mobility on SDS-polyacrylamide gels. Along with evidence from other systems [7] this suggests that the proteins are identical. Also in agreement with previous studies on chick embryo fibroblasts [4, 20–22] the protein was markedly reduced as a function of transformation, and has been termed the LETS glycoprotein. The molecule was also strongly labelled with [$^{14}$C]glucosamine, but whereas surface labelled material was totally removed by mild proteolysis, a fraction of the metabolic label remained after similar treatment. This may represent a totally separate glycoprotein or an internal pool of LETS glycoprotein. We favour the latter interpretation preliminary experiments suggesting that this pool can be chased over an eight hour period into surface material

of identical molecular weight. The LETS glycoprotein was also labelled by fucose but in contrast we found no internal pool after trypsinization. Fucose is a terminal sugar in many glycoproteins and this may imply that the internal pool of LETS glycoprotein has an incomplete carbohydrate chain fucose being added at a late stage, possibly the plasma membrane. Similar observations have been made concerning glycosylation of IgM molecules [23].

The LETS glycoprotein was still just detectable in wild-type transformed cells labelled with [$^{14}$C]glucosamine, and some of this material was clearly trypsin sensitive, in agreement with the finding that it can be iodinated by the surface-labelling method. However, a pool of [$^{14}$C]glucosamine-labelled material remained as in the normal cell. We have attempted to exclude the possibility that the glycoprotein is detected due to contamination with untransformed cells by isolating colonies of transformed cells from semi-solid agar medium. It remains possible that normal cells could be isolated together with the agar colonies, but the procedure should at least produce a population greatly enriched in transformed cells. Significantly, metabolically labelled colony-purified transformed cells had at least as much LETS glycoprotein as uncloned populations. These results suggest that it is synthesised by transformed cells and are in agreement with the previous observations that cells infected with *LA* 24 at 35 °C and shifted to 41 °C in the presence of cycloheximide regain considerable amounts of the LETS glycoprotein [4].

Synthesis of LETS glycoprotein by transformed cells is also suggested by the finding that virus isolated from the growth medium of either uncloned or colony purified transformed cells contains the molecule as a contaminant. Thus although the levels of LETS glycoprotein detected at the transformed cell surface are much reduced compared to normal, synthesis is not totally shut off, and it is turned over into the medium. At present the results do not differentiate between a number of possibilities discussed in detail elsewhere [4]. For example we have no information on the relative rates of synthesis and degradation of the molecule in normal and transformed cells, or whether the reduced level occurring on transformation is an expression of the lack of a Go phase in these cells. The fact it is found in the medium shows that the molecule can proceed from the intracellular pool to the membrane, but it is possible that a protein normally responsible for its retention is missing. In this context, the demonstration that the actin content of membranes from transformed chick embryo fibroblasts is reduced may be relevant [24]. A further possibility is that turnover in the plasma membrane of the transformed cell is increased due to elevated protease activity, and the protein is rapidly shed into the medium [25–27]. As one of the proteins separated from the medium of transformed cells has an identical mobility on SDS-polyacrylamide gels to that associated with cells, the extent of proteolytic cleavage resulting in turnover must be fairly limited. However, the other major glycoprotein isolated from medium has a molecular weight similar to that of a protein said by Yamada and Weston [28] to be a degradation production of LETS glycoprotein.

An alternative to explain the presence of LETS glycoprotein in medium is that it stems from dead cells. However, the glucosamine profile of the total cell is quite different from that of the particulate fraction isolated from the virus preparation tending to exclude this explanation. The result may be related to the recent report of Graham et al. [18] that a surface coat fraction containing LETS glycoprotein but not

other iodinated membrane proteins can be isolated from NIL2 hamster fibroblasts. The material we have isolated may represent surface coat which has been shed into the medium. The fact that the LETS glycoprotein tends to associate with isolated virus may reflect its ability to re-adsorb to cell surfaces [29].

In addition to these marked change in levels of LETS glycoprotein, we have also noted an increase on transformation in sialylation of a group of lower molecular weight glycoproteins. This conclusion is based on the observation that normal cells are generally better labelled by galactose oxidase alone than transformed cells. Labelling of both cell types is greatly enhanced by neuraminidase fitting in with the general finding that galactose residues are frequently sub-terminal to sialic acid in membrane glycoproteins. Such enhanced labelling could not be reproduced with trypsin or hyaluronidase suggesting that the masking of galactose residues in transformed cells is specifically due to sialic acid, and not to masking perhaps by a thick coat of proteoglycan or hyaluronic acid. The point is substantiated by the finding that whereas the mobility of normal cell glycoproteins labelled by galactose oxidase is barely altered by prior neuraminidase treatment, that of the transformed cell is markedly increased. Marked increases in mobility of proteins after removing sialic acid was also clearly shown when Rous sarcoma virus was treated with neuraminidase (Fig. 7).

The complexity of the galactose oxidase and [$^{14}$C]glucosamine labelling patterns makes it impossible to be sure that the effect is limited to cell proteins and not adsorbed serum proteins. However the fact that the changes in extent of labelling and mobility described above are temperature sensitive in cells infected with *LA* 24 indicates that they relate to changes in sialylation of glycoproteins synthesized by chick embryo fibroblasts. Temperature sensitive changes in periodate labelling of *LA* 24 infected cells also suggest that there is a marked change in sialylation on transformation.

These results are contradictory to those which show transformed cells to contain lower levels of sialic acid than their normal counterpart [30]. They may be related, however to the results of Warren and co-workers [31, 32] who found that the slightly higher molecular weight of a population of glycopeptides derived from the surface of transformed cells could be explained by increased sialylation in these cells compared to normal cells. These results were markedly influenced by the growth state of the cell, glycopeptides from normal growing or mitotic cells being similar to those of transformed cells.

It is interesting that changes in levels of LETS glycoprotein [5], changes in the degree of sialylation of surface glycoproteins [30], and changes in complexity of membrane glycolipids [33] are all growth dependent. For all three parameters, growing normal cells are much like transformed cells. Whether these changes in complex carbohydrates are primary or secondary events in loss of growth control therefore remains to be established.

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