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# Effect of tunicamycin on epidermal glycoprotein and glycosaminoglycan synthesis *in vitro*

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1. When pig ear skin slices were cultured for 18h in the presence of 1 $\mu$g of tunicamycin/ml the incorporation of D-[$^3$H]glucosamine into the epidermis, solubilized with 8 M-urea/5% (w/v) sodium dodecyl sulphate, was inhibited by 45–55%. This degree of inhibition was not increased by using up to 5 $\mu$g of tunicamycin/ml or by treating the skin slices with tunicamycin for up to 8 days. The incorporation of (U-$^{14}$C)-labelled L-amino acids under these conditions was not affected by tunicamycin. Polyacrylamide-gel electrophoresis indicated that the labelling of the major glycosaminoglycan peak with D-[$^3$H]glucosamine was unaffected, whereas that of the faster migrating glycoprotein components was considerably decreased in the presence of tunicamycin. 2. Subcellular fractionation indicated that tunicamycin specifically inhibited the incorporation of D-[$^3$H]glucosamine but not of (U-$^{14}$C)-labelled L-amino acids into particulate (mainly plasma-membrane) glycoproteins by about 70%. The labelling of soluble glycoproteins was hardly affected. Polyacrylamide-gel electrophoresis of the plasma-membrane fraction showed decreased D-[$^3$H]glucosamine incorporation into all glycoprotein components, indicating that the plasma-membrane glycoproteins contained mainly *N*-asparagine-linked oligosaccharides. 3. Cellulose acetate electrophoresis of both cellular and extracellular glycosaminoglycans showed that tunicamycin had no significant effect on the synthesis of the major component, hyaluronic acid. However, the incorporation of both D-[$^3$H]glucosamine and $^{35}SO_4^{2-}$ into sulphated glycosaminoglycans was inhibited by about 50%. This inhibition was partially overcome, at least in the cellular fraction, by 2mM-*p*-nitrophenyl $\beta$-D-xyloside indicating that tunicamycin-treated epidermis retained the ability to synthesize sulphated glycosaminoglycan chains. Tunicamycin may affect the synthesis and/or degradation of proteoglycan core proteins or the xylosyltransferase. 4. Electron-microscopic examination of epidermis treated with tunicamycin for up to 4 days revealed no significant changes in cell-surface morphology or in epidermal-cell adhesion. Either *N*-asparagine-linked carbohydrates play little role in epidermal-cell adhesion or more probably there is little turnover of these components in epidermal adhesive structures such as desmosomes and hemidesmosomes during organ culture.

Tunicamycin is an antibiotic isolated from *Streptomyces lysosuperificus* that inhibits the formation of lipid-linked sugar intermediates involved in the synthesis of complex carbohydrates. It has proved useful for studying the role of such intermediates in glycosylation reactions in many systems, including bacteria (Takatsuki *et al.*, 1972; Ward, 1977), fungi (Speake *et al.*, 1979), insects (Butters & Hughes, 1980) and mammalian tissues (Struck *et al.*, 1978; Speake & White, 1979).

In eukaryotic cells tunicamycin inhibits the synthesis of dolichyl *N*-acetylglucosamine diphosphate (Takatsuki *et al.*, 1975; Tkacz & Lampen, 1975; Lehle & Tanner, 1976), which is essential for the assembly of oligosaccharide chains and their subsequent transfer to asparagine residues in proteins (Waechter & Lennarz, 1976). The glycosylation of glycoproteins containing *O*-glycosidic linkages is not sensitive to tunicamycin. Tunicamycin has therefore been used as a specific probe of the function of the *N*-glycosidically linked oligosaccharide moieties of glycoproteins in a variety of processes, such as the assembly, release and infectivity of viruses (Gibson *et al.*, 1978), the intracellular



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transport and secretion of cell-surface-associated (Olden *et al.*, 1978; Damsky *et al.*, 1979) and secreted proteins (Hickman & Kornfeld, 1978), the maintenance of normal cell-surface morphology (Pratt *et al.*, 1979) and of normal cell adhesion (Duksin *et al.*, 1978).

In previous work we examined the synthesis of cell-surface-associated carbohydrates in the epidermis when pig skin slices were cultured in the presence of D-[$^3$H]glucosamine (King *et al.*, 1980). We showed that the epidermis synthesized large amounts of hyaluronic acid, most of which was extracellular (King & Tabiowo, 1980, 1981). Smaller amounts of sulphated glycosaminoglycans (mainly heparan sulphate) were also made *in vitro* (King, 1981). In addition, D-[$^3$H]glucosamine was incorporated into particulate glycoproteins, which were found to be enriched in the plasma-membrane fraction, and also into soluble glycoproteins (King *et al.*, 1980).

In the present paper we have investigated the effect of tunicamycin on the synthesis of these epidermal cell-surface-associated glycoconjugates with a view to (*a*) elucidating the role of lipid-linked intermediates in their synthesis, and (*b*) determining the consequences of decreased glycosylation for the epidermal cell surface.

## Experimental

### *Materials*

Tunicamycin (lot T-11-5) was a gift from Professor G. Tamura, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. D-[6-$^3$H]Glucosamine hydrochloride (sp. radioactivity >10 Ci/mmol), $^{35}SO_4^{2-}$ and (U-$^{14}$C)-labelled L-amino-acid mixture (>45 Ci/g-atom of carbon) were from The Radiochemical Centre, Amersham, Bucks., U.K. Reagents for gel electrophoresis and for tissue culture were obtained as described previously (King *et al.*, 1980). Papain (type III; twice-crystallized) and *p*-nitrophenyl β-D-xylopyranoside were from Sigma. Dispase (neutral proteinase from *Bacillus polymyxa*) was from Boehringer. Hyaluronic acid and hyaluronidase from *Streptomyces hyalurolyticus* were from Miles Laboratories. Cellulose acetate sheets were from Whatman.

### *Organ culture*

Organ culture of keratotomed slices of pig ear skin, 1 cm square, was as described previously (King *et al.*, 1980). Tunicamycin (2 mg/ml in 25 mM-NaOH) was added to the culture medium to give the appropriate final concentration. Control cultures received the same volume of 25 mM-NaOH. The medium also contained D-[$^3$H]glucosamine (10 or 25 μCi/ml) and in some experiments either (U-$^{14}$C)-labelled L-amino-acid mixture (1 or 2 μCi/ml) or $^{35}SO_4^{2-}$ (50 μCi/ml). The culture time was usually 18 h. When the long-term effect of tunicamycin was examined skin slices were cultured in medium containing tunicamycin but no radioactive precursors for up to 8 days with a change of medium every 2 days. At appropriate times the slices were transferred to medium containing tunicamycin, D-[$^3$H]glucosamine and (U-$^{14}$C)-labelled L-amino-acid mixture for 18 h. When the reversibility of tunicamycin inhibition was examined skin slices were cultured with or without tunicamycin (1 μg/ml) for 18 h, were washed in phosphate-buffered saline (King *et al.*, 1980), pH 7.3, sterilized by Millipore filtration and were transferred to fresh medium containing no tunicamycin for 6 h. After further washing the skin slices were floated on medium containing D-[$^3$H]glucosamine (10 μCi/ml) and (U-$^{14}$C)-labelled L-amino-acid mixture (1 μCi/ml) but no tunicamycin for 18 h. In some experiments when the effect of tunicamycin on the synthesis of sulphated glycosaminoglycans was being examined *p*-nitrophenyl β-D-xylopyranoside (0.5, 2 or 5 mM) was included in the culture medium.

### *Total epidermal glycoconjugates*

Skin slices labelled with D-[$^3$H]glucosamine (10 μCi/ml) and (U-$^{14}$C)-labelled L-amino-acid mixture (1 μCi/ml) were washed in phosphate-buffered saline, the epidermis was separated from dermis using 1 M-$CaCl_2$ and the epidermis was solubilized using 8 M-urea, 5% (w/v) sodium dodecyl sulphate and 10 mM-2-mercaptoethanol at 100°C (King *et al.*, 1980). Samples were analysed by electrophoresis in 7% (w/v) polyacrylamide gels, which were washed in 10% (w/v) trichloroacetic acid, sliced into 2 mm sections and solubilized (King *et al.*, 1980) before measurement of radioactivity.

### *Isolation of subcellular epidermal fractions*

Skin slices labelled with D-[$^3$H]glucosamine (25 μCi/ml) and in some experiments with (U-$^{14}$C)-labelled L-amino-acid mixture (2 μCi/ml) were minced together with 1 g of unlabelled pig skin slices, were homogenized in 5 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose and the filtered homogenate was fractionated by differential centrifugation at 376 $g_{av.}$ and 113 700 $g_{av.}$. The 113 700 $g_{av.}$ pellet was further fractionated by centrifugation on discontinuous sucrose gradients (King *et al.*, 1980). Specific radioactivities were measured after precipitation with 10% (w/v) trichloroacetic acid as described previously. Membrane fractions were dissolved in 2% (w/v) sodium dodecyl sulphate/10 mM-2-mercaptoethanol at 100°C and were analysed by electrophoresis in 7% (w/v) polyacrylamide gels (King *et al.*, 1980), which were sliced into 1 mm sections before measurement of radioactivity.

*Isolation of cellular and extracellular epidermal glycosaminoglycans*

Skin slices labelled with D-[$^3$H]glucosamine (10$\mu$Ci/ml) and $^{35}SO_4^{2-}$ (50$\mu$Ci/ml) were treated with Dispase (0.1%, w/v, in phosphate-buffered saline) for 30min at 37°C. The separated epidermis was treated with trypsin (0.2%, w/v, in phosphate-buffered saline) for 15min at 37°C and was shaken vigorously. The stratum corneum was removed and the cell suspension was centrifuged (King *et al.*, 1980). The pellet of trypsin-treated cells, and the supernatant containing material released from the cell surface and extracellular space, were mixed with a stock solution to give final concentrations of 5mM-EDTA, 5mM-cysteine and 0.1M-sodium acetate, pH6.0. Fractions were treated with papain (0.5mg/ml) for 18h at 60°C. Hyaluronic acid and dermatan sulphate (100$\mu$g of each/ml) were added as carrier and after precipitation with a final concentration of 5% (w/v) trichloroacetic acid glycosaminoglycans were isolated from the acid-soluble fraction by precipitation with 3vol. of 5% (w/v) potassium acetate in ethanol (King & Tabiowo, 1981) and were dissolved in 100$\mu$l of water. In experiments to examine the effect of tunicamycin on the synthesis specifically of sulphated glycosaminoglycans, samples were treated with hyaluronidase from *Streptomyces hyalurolyticus* (40 units/ml) in 0.0375M-sodium acetate/0.0375M-NaCl, pH5.4, for 18h at 37°C. The enzyme was inactivated by heating for 2min in a boiling-water bath. Glycosaminoglycans were analysed by electrophoresis on cellulose acetate and were detected by staining with Alcian Blue. The sheets were cut into 1cm strips, which were dissolved using 80% (v/v) acetic acid (King & Tabiowo, 1981) before measurement of radioactivity.

*Protein and radioactivity determination*

Protein was measured by the Hartree (1972) modification of the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Radioactivity was measured by liquid-scintillation counting with PCS scintillation fluid.

*Electron microscopy*

Skin slices (about 2mm square) were cultured for a total of 18, 45 or 96h without or with 2$\mu$g of tunicamycin/ml. The tissue was fixed in glutaraldehyde, postfixed in $OsO_4$, dehydrated, embedded in Spurr's resin and sections were stained with lead citrate and uranyl acetate as described previously (King *et al.*, 1980).

## Results

*Whole epidermis*

The total incorporation of D-[$^3$H]glucosamine into the epidermis of pig skin slices cultured for 18h was inhibited by increasing concentrations of tunicamycin (Fig. 1*a*). Maximum inhibition of 45–55% was obtained with 1$\mu$g of tunicamycin/ml. This degree of inhibition was not increased when skin slices were treated with tunicamycin for up to 8 days (Fig. 1*b*). The effect of tunicamycin on epidermal D-[$^3$H]glucosamine incorporation was reversible. Thus when skin slices that had been treated with 1$\mu$g of tunicamycin/ml for 18h were depleted of the drug by an intermediate incubation in tunicamycin-free medium and then labelled for 18h, the incorporation of D-[$^3$H]glucosamine by the epidermis returned to 85–90% of the value found in untreated tissue.

Sodium dodecyl sulphate/polyacylamide-gel electrophoresis of the solubilized epidermis (Fig. 2) revealed that the incorporation of D-[$^3$H]glucosamine into the major peak near the top of the gel was not significantly affected by tunicamycin. This peak was previously shown to contain glycosaminoglycans (King *et al.*, 1980). However, the labelling of all the faster-migrating glycoproteins was decreased by up to 70% in tunicamycin-treated epidermis. The electrophoretic profile of proteins labelled with (U-$^{14}$C)-labelled L-amino acids showed a slight increase in the labelling of higher-molecular-weight components in the presence of tunicamycin (Fig. 2).

*Epidermal glycoproteins*

To determine whether tunicamycin affected the synthesis of particulate and soluble glycoproteins to the same extent control and tunicamycin-treated skin slices that had been labelled with D-[$^3$H]glucosamine and (U-$^{14}$C)-labelled L-amino acids were diluted with unlabelled skin slices, homogenized and fractionated by a combination of differential and sucrose-density-gradient centrifugation. The labelling of the membrane fractions with D-[$^3$H]-glucosamine was inhibited 65–70%, whereas that of the soluble fraction was not significantly altered (Table 1). The decreased incorporation of D-[$^3$H]-glucosamine into membrane fractions was due to decreased glycosylation since their incorporation of (U-$^{14}$C)-labelled L-amino acids was unchanged (Table 1). The membrane fraction banding at the 0.5M-/1.0M-sucrose interface was enriched in plasma membranes and also in [$^3$H]glycoproteins in untreated epidermis (King *et al.*, 1980). Polyacrylamide-gel electrophoresis of the plasma-membrane-enriched fraction from tunicamycin-treated epidermis showed decreased labelling of all glycoprotein components with D-[$^3$H]glucosamine (Fig. 3). This suggests that the glycoproteins in epidermal plasma membranes contain mainly *N*-glycosidically linked oligosaccharide chains. The electrophoretic mobility of the major group of plasma-membrane glycoproteins was apparently unchanged.

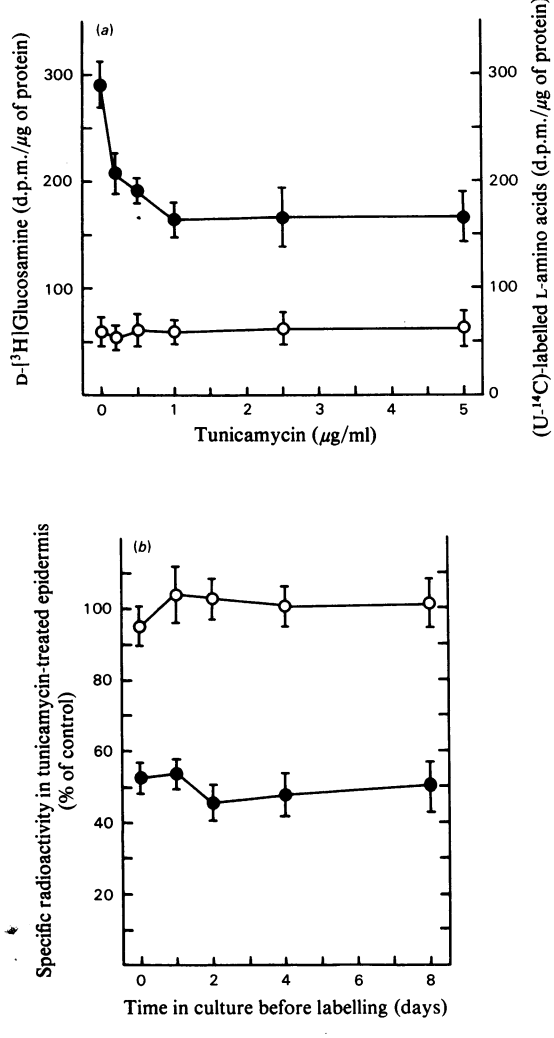


Fig. 1. *Effect of tunicamycin on incorporation of* D-[$^3H$]*glucosamine and* (U-$^{14}C$)*-labelled* L*-amino acids into pig epidermis*

Skin slices were treated with (*a*) increasing concentrations of tunicamycin for 18h in the presence of D-[$^3$H]glucosamine (10$\mu$Ci/ml) and (U-$^{14}$C)-labelled L-amino acids (1$\mu$Ci/ml) and (*b*) 2$\mu$g of tunicamycin/ml for up to 8 days in medium containing no radioactive precursors. In (*b*) the medium was changed at 2 day intervals and at appropriate times skin slices were transferred to fresh medium containing 2$\mu$g of tunicamycin/ml and the radioactive precursors for 18h labelling. The skin slices were washed, separated using 1M-$CaCl_2$ and the epidermis was solubilized using 8M-urea/5% (w/v) sodium dodecyl sulphate at 100°C. The radioactivity and protein content of the solubilized epidermis was measured. Values are means for three separate skin slices ± S.E.M. ●, D-[$^3$H]Glucosamine; ○, (U-$^{14}$C)-labelled L-amino acids.

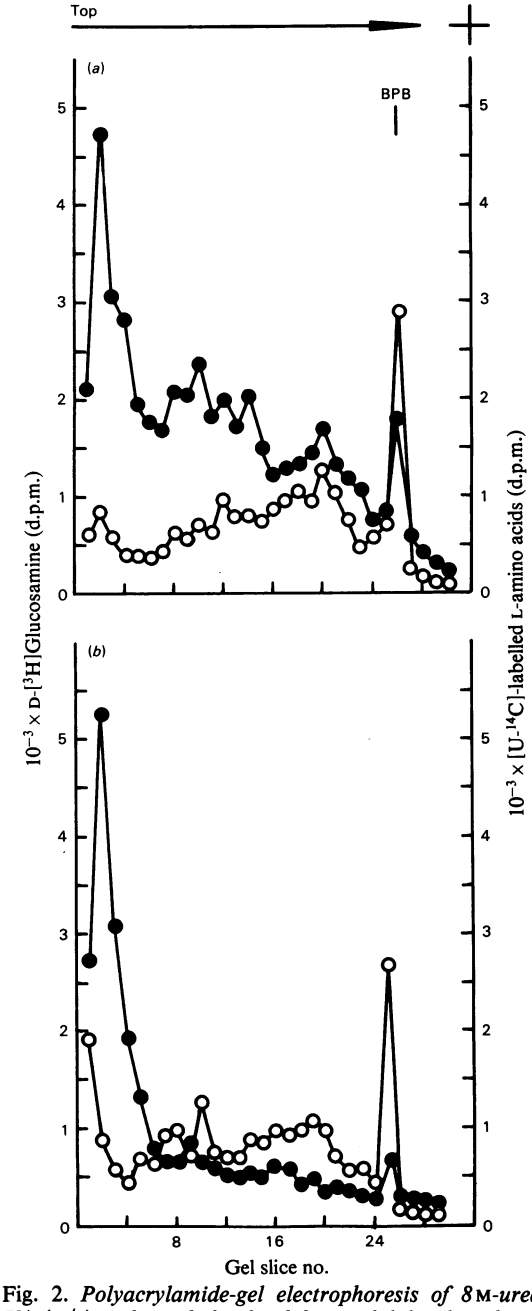


Fig. 2. *Polyacrylamide-gel electrophoresis of* 8M*-urea/5% (w/v) sodium dodecyl sulphate-solubilized epidermis from control and tunicamycin-treated skin slices*

The solubilized epidermis from skin slices cultured without (*a*) or with (*b*) 2$\mu$g of tunicamycin/ml for 18h was analysed by electrophoresis on 7% (w/v) polyacrylamide gels, which were washed, sliced into 2mm sections and dissolved as described in the Experimental section. Epidermal protein (700$\mu$g) was applied to each gel. The arrow indicates the direction of electrophoretic migration. BPB indicates the position of the Bromophenol Blue tracking dye. ●, D-[$^3$H]Glucosamine; ○, (U-$^{14}$C)-labelled L-amino acids.

Table 1. *Subcellular fractionation of control and tunicamycin-treated epidermis*

Skin slices were cultured for 18h in the presence of D-[$^3$H]glucosamine and (U-$^{14}$C)-labelled-L-amino acid mixture with or without tunicamycin (2$\mu$g/ml). Each slice was added to 1g of unlabelled pig skin, which was homogenized, filtered and fractionated by differential centrifugation. The 113700 $g_{av.}$ pellet was further fractionated by centrifugation on sucrose-density gradients. Fractions were precipitated with 10% (w/v) trichloroacetic acid before measurement of protein and radioactivity. Values are means for three different skin slices ± S.E.M.

| | Specific radioactivity of tunicamycin-treated fraction (% of control value) | |
|---|---|---|
| | D-[$^3$H]-Glucosamine | $^{14}$C-labelled L-amino acids |
| Homogenate | 57 ± 2 | 112 ± 7 |
| 376 $g_{av.}$ pellet | 40 ± 1 | 99 ± 15 |
| 113700 $g_{av.}$ pellet | 31 ± 2 | 107 ± 14 |
| 0.5M/1.0M-Sucrose | 32 ± 1 | 92 ± 10 |
| 1.0M/1.5M-Sucrose | 32 ± 2 | 104 ± 2 |
| 1.5M/2.0M-Sucrose | 33 ± 3 | 99 ± 10 |
| 113700 $g_{av.}$ supernatant | 102 ± 2 | 123 ± 8 |

*Epidermal glycosaminoglycans*

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of whole epidermis (Fig. 2) indicated that tunicamycin had little effect on the synthesis of epidermal glycosaminoglycans. To study this in more detail, glycosaminoglycans were labelled by culturing skin slices in the presence of D-[$^3$H]glucosamine and $^{35}SO_4^{2-}$. Cellular and extracellular glycosaminoglycans were isolated from the pellet and supernatant obtained when the epidermis was trypsin-treated, shaken and centrifuged. Cellulose acetate electrophoresis revealed that the labelling of hyaluronic acid in both cellular and extracellular fractions was not significantly affected by tunicamycin (Fig. 4 and Table 2). The labelling of the minor, sulphated glycosaminoglycans with $^{35}SO_4^{2-}$ was significantly decreased in both the cellular and extracellular fractions from tunicamycin-treated epidermis (Fig. 4). Because of the presence of very large amounts of hyaluronic acid it was not possible to determine whether the labelling of sulphated glycosaminoglycans with D-[$^3$H]glucosamine was also affected by tunicamycin. Samples were therefore digested extensively with *Streptomyces* hyaluronidase to specifically degrade hyaluronic acid before analysis by cellulose acetate electrophoresis (Table 2). The labelling of sulphated glycosaminoglycans with both D-[$^3$H]glucosamine and $^{35}SO_4^{2-}$ was inhibited approx. 45–50% by tunicamycin in both cellular and extracellular fractions. This suggests that tunicamycin genuinely inhibits the synthesis of sulphated glycosaminoglycans *de novo* rather than their sulphation.

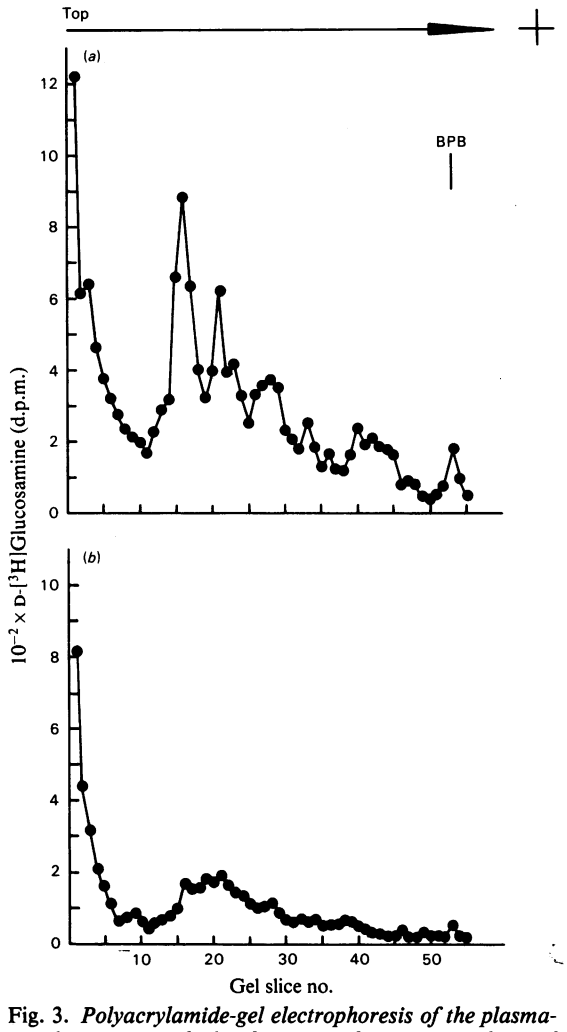


Fig. 3. *Polyacrylamide-gel electrophoresis of the plasma-membrane enriched fraction from control and tunicamycin-treated epidermis*

The membranes banding at the 0.5M-/1.0M-sucrose interface during sucrose-density centrifugation of the crude particulate fraction from (*a*) control and (*b*) tunicamycin-treated epidermis labelled with D-[$^3$H]glucosamine (Table 2) were dissolved in 2% (w/v) sodium dodecyl sulphate/10mM-2-mercaptoethanol at 100°C. Samples containing 500$\mu$g of protein were analysed by electrophoresis on 7% (w/v) polyacrylamide gels, which were sliced into 1mm sections and dissolved before measurement of radioactivity. The arrow indicates the direction of electrophoretic migration. BPB indicates the position of the Bromophenol Blue tracking dye.

To determine whether this inhibition was due to

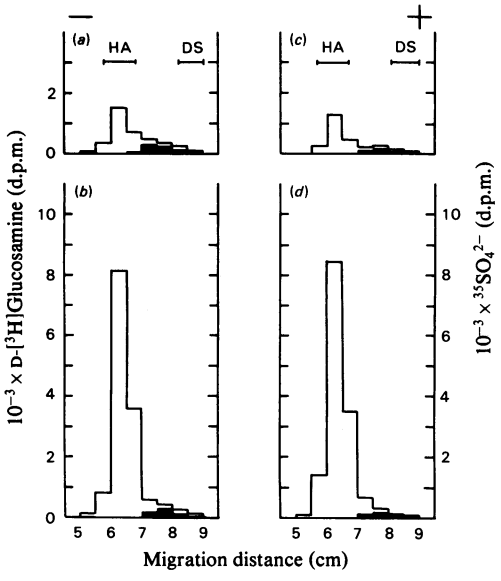


Fig. 4. *Cellulose acetate electrophoresis of epidermal glycosaminoglycans synthesized in the absence and presence of tunicamycin*

Skin slices were cultured without (*a* and *b*) or with (*c* and *d*) tunicamycin (2$\mu$g/ml) for 18h in the presence of D-[$^3$H]glucosamine (10$\mu$Ci/ml) and $^{35}SO_4^{2-}$ (50$\mu$Ci/ml). The epidermis was separated from dermis using Dispase, trypsin-treated and cellular (*a* and *c*) and extracellular (*b* and *d*) glycosaminoglycans were isolated from the pellet and supernatant obtained after centrifugation of the epidermal-cell suspension. Samples were analysed by electrophoresis on cellulose acetate sheets that were stained to detect carrier glycosaminoglycans and cut into 0.5 cm strips before measurement of radioactivity. Open columns, D-[$^3$H]glucosamine; filled columns, $^{35}SO_4^{2-}$. HA and DS indicate the position of hyaluronic acid and dermatan sulphate standards respectively.

decreased ability to add sugar residues to xylose residues on the proteoglycan core protein the effect of *p*-nitrophenyl $\beta$-D-xyloside on tunicamycin-induced inhibition of sulphated glycosaminoglycan synthesis was examined. $\beta$-D-Xylosides have been shown to stimulate the synthesis of sulphated glycosaminoglycans in several other systems (Schwartz, 1977; Hart & Lennarz, 1978; Pratt *et al.*, 1979). In the epidermis glycosaminoglycan synthesis was not significantly affected by 0.5mM-$\beta$-D-xyloside whether tunicamycin was absent or present (results not shown). $\beta$-D-Xyloside (2mM) stimulated the total synthesis of sulphated glycosaminoglycans approx. 75%, but had little effect on the synthesis of hyaluronic acid when tunicamycin was absent (Table 2). In the presence of tunicamycin, 2mM-$\beta$-D-xyloside also stimulated the total synthesis of sulphated glycosaminoglycans by the epidermis. The synthesis of sulphated glycosaminoglycans in the cellular fraction approached the value found in the absence of both tunicamycin and $\beta$-D-xyloside. However, the tunicamycin-induced inhibition of the synthesis of sulphated glycosaminoglycans found in the extracellular fraction was not overcome by 2mM-$\beta$-D-xyloside (Table 2). It was therefore of interest to find that in the presence, but not the absence, of tunicamycin $\beta$-D-xyloside stimulated the synthesis of extracellular hyaluronic acid. $\beta$-D-Xyloside (5mM) appeared to be toxic and even in the absence of tunicamycin epidermal synthesis of glycosaminoglycans was decreased by 75–80%.

*Epidermal cell-surface morphology*

To determine the consequences of decreased glycoprotein and sulphated glycosaminoglycan synthesis for the ultrastructure of the epidermal cell surface, control and tunicamycin-treated skin slices were examined by transmission electron microscopy. Even after 4 days treatment with tunicamycin, desmosomes, hemidesmosomes and surface microvilli appeared normal both in number and structure. There was no significant change in the size of the intercellular spaces. Intracellularly dilation of the Golgi apparatus was often observed and after 4 days tunicamycin-treated cells were highly vacuolated.

## Discussion

Tunicamycin had little effect on the synthesis of proteins in the epidermis when pig skin slices were maintained in organ culture. However, it did affect the synthesis of specific epidermal glycoconjugates. The glycosylation of particulate glycoproteins was considerably decreased, whereas that of soluble glycoproteins was hardly affected. In addition, tunicamycin inhibited the synthesis of sulphated glycosaminoglycans but not of hyaluronic acid.

The glycosylation of the glycoproteins present in epidermal membrane fractions was inhibited by about 70%, whereas the synthesis of their polypeptide chains was unaffected by tunicamycin (Table 1). These glycoproteins were normally present in the plasma membranes (King *et al.*, 1980). This would suggest that the plasma-membrane-bound glycoproteins contain mainly *N*-asparagine-linked carbohydrate chains and are synthesized using lipid-linked intermediates. The failure of tunicamycin to completely inhibit glycosylation of these components may be due to the failure of the drug to penetrate completely into cells in the higher levels of the epidermis where glycoprotein synthesis is known to occur (King *et al.*, 1980). It is unlikely to represent the addition of preformed oligosaccharides since the degree of

Table 2. *Effect of tunicamycin and p-nitrophenyl β-D-xyloside on epidermal glycosaminoglycan synthesis*
Pig skin slices were labelled with D-[$^3$H]glucosamine and $^{35}SO_4^{2-}$ for 18h in the absence or presence of tunicamycin (2μg/ml). Half of the cultures also received 2mM-*p*-nitrophenyl β-D-xyloside. Skin slices were separated using Dispase, the epidermis was trypsin-treated and cellular and extracellular glycosaminoglycans were isolated as described in the Experimental section. Hyaluronic acid labelling was determined by cellulose acetate electrophoresis of intact samples, whereas labelling of sulphated glycosaminoglycans was determined after digestion with hyaluronidase. Values are means for three skin slices ± S.E.M.

| | | | $10^{-4}$ × Total labelling of epidermal glycosaminoglycan (d.p.m.) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Hyaluronic acid | | | Sulphated glycosaminoglycans | | |
| | | | Cellular | Extracellular | Total | Cellular | Extracellular | Total |
| Control | −Tunicamycin | [$^3$H]GlcN | 2.9 ± 0.5 | 14.1 ± 0.8 | 17.0 ± 0.4 | 1.8 ± 0.3 | 1.6 ± 0.1 | 3.4 ± 0.4 |
| | | $^{35}SO_4^{2-}$ | — | — | — | 0.9 ± 0.1 | 0.8 ± 0.05 | 1.7 ± 0.2 |
| | +Tunicamycin | [$^3$H]GlcN | 2.2 ± 0.4 | 16.4 ± 1.0 | 18.6 ± 1.0 | 0.9 ± 0.2 | 1.0 ± 0.1 | 1.9 ± 0.2 |
| | | $^{35}SO_4^{2-}$ | — | — | — | 0.4 ± 0.1 | 0.5 ± 0.03 | 0.9 ± 0.1 |
| +β-D-Xyloside | −Tunicamycin | [$^3$H]GlcN | 3.1 ± 0.1 | 13.4 ± 1.7 | 16.5 ± 1.8 | 3.6 ± 0.5 | 2.5 ± 0.2 | 6.1 ± 0.8 |
| | | $^{35}SO_4^{2-}$ | — | — | — | 1.6 ± 0.1 | 1.2 ± 0.03 | 2.8 ± 0.2 |
| | +Tunicamycin | [$^3$H]GlcN | 3.0 ± 0.2 | 21.4 ± 0.8 | 24.4 ± 1.0 | 1.6 ± 0.05 | 1.0 ± 0.1 | 2.6 ± 0.1 |
| | | $^{35}SO_4^{2-}$ | — | — | — | 0.7 ± 0.02 | 0.4 ± 0.03 | 1.1 ± 0.05 |

inhibition could not be increased by prolonged exposure to tunicamycin or by exposure to higher concentrations of the drug (Fig. 1). Alternatively, the plasma-membrane glycoproteins may also contain *O*-glycosidically-linked oligosaccharides. It is not clear whether these poorly glycosylated glycoproteins are transported and inserted into the plasma membrane in the normal fashion. In baby-hamster-kidney fibroblasts the transport of plasma-membrane glycoproteins to the cell surface was not affected by tunicamycin (Damsky *et al.*, 1979). However, the organization of the poorly glycosylated glycoproteins in the plasma membrane was not ascertained in these studies. Furthermore there are conflicting data about the role of carbohydrates in the intracellular transport and export of extracellular (Duksin *et al.*, 1978; Olden *et al.*, 1978) and secreted (Hickman & Kornfeld, 1978; Singer *et al.*, 1980) rather than plasma-membrane-bound glycoproteins.

The synthesis of hyaluronic acid in the epidermis was not affected by tunicamycin (Fig. 4 and Table 2). Similar results have been obtained using cultured chick-embryo fibroblasts (Pratt *et al.*, 1979) and in a cell-free fibrosarcoma system (Hopwood & Dorfman, 1977). However, the synthesis of epidermal sulphated glycosaminoglycans was inhibited about 50% by tunicamycin. Previous studies have shown that tunicamycin inhibited the synthesis of sulphated glycosaminoglycans in chick-embryo fibroblasts but not chondrocytes (Pratt *et al.*, 1979) and also in chick cornea (Hart & Lennarz, 1978). The reasons for the inhibition of epidermal sulphated glycosaminoglycan synthesis are not clear. It is generally considered that the carbohydrate side chains of glycosaminoglycans other than keratan sulphate are linked to serine residues in the proteoglycan core protein via a xylose residue (Rodén & Horowitz, 1978). Such *O*-glycosidically linked carbohydrates are not considered to utilize lipid-linked intermediates in their synthesis and would therefore be expected to be insensitive to tunicamycin. In common with other systems (Hart & Lennarz, 1978; Pratt *et al.*, 1979) tunicamycin inhibition was partially overcome by β-D-xyloside (Table 2). This would suggest that tunicamycin-treated epidermis retained the ability to synthesize sulphated glycosaminoglycan chains in the presence of an exogenous initiator. Tunicamycin may affect either the proteoglycan core protein or the xylosyltransferase, which is also a glycoprotein (Schwartz & Dorfman, 1975). As with fibronectin (Olden *et al.*, 1978) the poorly glycosylated forms of the proteoglycan core protein and the xylosyltransferase may be subject to increased proteolytic degradation. Although β-D-xyloside stimulated the synthesis of sulphated glycosaminoglycans in the cellular fraction of tunicamycin-treated epidermis it had little effect on the extracellular fraction (Table 2). It is therefore possible that the transport and/or secretion of extracellular sulphated glycosaminoglycans may require their correct assembly into intact proteoglycans.

Although tunicamycin had marked effects on the synthesis of cell-surface-associated carbohydrates it had no apparent effect on epidermal cell-surface morphology. Hemidesmosomes and desmosomes, which mediate basal-cell–basement-membrane attachment and cell–cell adhesion respectively, were equally numerous in control and tunicamycin-treated

epidermis. No differences in their fine structure could be observed after tunicamycin treatment. Cell-surface microvilli and the intercellular space were similarly unaffected by tunicamycin. These results suggest either that carbohydrates play little role in epidermal cell–cell and cell–substrate adhesion or more probably that there is little turnover of the glycoprotein components of desmosomes and hemidesmosomes in organ culture. Elucidation of the role of surface carbohydrates in epidermal-cell adhesion should result from studies of the effect of tunicamycin on cultures of dispersed epidermal cell rather than the intact tissue used here. Trypsin treatment cleaves epidermal desmosomes and hemidesmosomes and in culture the dispersed epidermal cells rapidly re-synthesize their surface carbohydrate coat (Davies & Trotter, 1981). Studies on other cell types in culture have shown tunicamycin to be a valuable probe of the role of carbohydrates in both cell-surface morphology and cell adhesion (Duksin *et al.*, 1978; Pratt *et al.*, 1979).

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