The Effect of Tunicamycin on the Glycosylation of Lactating-Rabbit Mammary Glycoproteins

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1. Tunicamycin inhibited the incorporation of D-[2-³H]mannose into dolichol-linked oligosaccharide and glycoprotein of lactating-rabbit mammary explants by approximately the same extent (approx. 30% of control value), suggesting that lipid-linked intermediates are involved in the mannosylation of mammary glycoproteins. 2. The incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into dolichol-linked oligosaccharide was inhibited by tunicamycin to 32% of the control value, whereas the incorporation of the radiolabel into glycoprotein was only inhibited to 72% of the control value. 3. Considerable redistribution of label from *N*-acetylglucosamine to *N*-acetylgalactosamine was found to occur in the explants. In the presence of tunicamycin approx. 76% of the radioactivity incorporated into glycoprotein from *N*-acetyl-D-[1-¹⁴C]glucosamine was present as *N*-acetylgalactosamine, compared with approx. 61% in the absence of the inhibitor. Thus tunicamycin selectively inhibits the incorporation of *N*-acetylglucosamine into glycoprotein. 4. Radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine was incorporated into a glycoprotein that was identified as casein by the use of a casein-specific antiserum, and also into a group of glycopolypeptides with apparent mol.wts. ranging between 40000 and 80000. *N*-Acetylgalactosamine was the only radioactive sugar released on strong-acid hydrolysis of the immunoprecipitated casein, whereas *N*-acetylglucosamine was the major radioactive residue present in the non-casein glycoproteins. Glucosamine and galactosamine were the only radiolabelled sugars detected by paper chromatography of the strong-acid hydrolysate of the protein fraction. 5. Tunicamycin inhibited the incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into the glycopolypeptides with mol.wts. between 40000 and 80000 as described by polyacrylamide-gel electrophoresis, but did not affect the incorporation of label into casein. It appears that tunicamycin inhibits the incorporation of mannose and *N*-acetylglucosamine into a number of mammary glycoproteins by inhibiting the formation of lipid-linked intermediates, but does not inhibit the incorporation of *N*-acetylgalactosamine into casein.

The glycosylation of glycoproteins containing an asparagine-linked carbohydrate moiety is thought to involve the pre-assembly of an oligosaccharide chain on a lipid carrier (dolichol pyrophosphate), followed by transfer of the oligosaccharide from the lipid to protein [see Waechter & Lennarz (1976) and Hemming (1977) for reviews]. Further processing of the oligosaccharide attached to protein may occur after transfer from the lipid carrier (Tabas *et al.*, 1978).

The involvement of lipid-linked sugars as intermediates in glycoprotein biosynthesis in lactating rabbit mammary gland has been demonstrated in cell-free preparations (White, 1978), and a lipid-linked oligosaccharide containing mannose, *N*-acetylglucosamine (Speake & White, 1978*a*) and glucose (Speake & White, 1978*b*) has been isolated from explants of lactating mammary tissue. Radio-

activity from D-[1-¹⁴C]mannose was largely incorporated by mammary explants into glycopolypeptides of mol.wts. between 40000 and 80000, whereas radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine was incorporated into a polypeptide with an electrophoretic mobility identical with that of rabbit casein (mol.wt. 32000) as well as into polypeptides of higher molecular weight (Speake & White, 1978*a*). Direct evidence for the transfer of sugar to protein via the lipid-linked oligosaccharide was not, however, obtained in these studies. It did not, therefore, prove possible to determine which polypeptides served as acceptors for the lipid-linked oligosaccharide and which were glycosylated directly from nucleotide sugars.

The present paper describes the use of tunicamycin, an *N*-acetylglucosamine-containing antibiotic from

Streptomyces lysosuperificus (Takatsuki *et al.*, 1971; Takatsuki & Tamura, 1971), in evaluating the importance of lipid-linked sugars in the glycosylation of mammary glycoproteins. Tunicamycin inhibits the synthesis of lipid-linked oligosaccharide by blocking the transfer of *N*-acetylglucosamine phosphate from UDP-*N*-acetylglucosamine to dolichol phosphate (Tkacz & Lampen, 1975; Takatsuki *et al.*, 1975; Lehle & Tanner, 1976). Struck & Lennarz (1977) reported that tunicamycin prevents the incorporation of *N*-acetylglucosamine and mannose into ovalbumin by hen oviduct slices, suggesting a role for lipid-linked oligosaccharide in the glycosylation of this protein.

The experiments described in the present paper indicate that lipid-linked sugars are not involved in the glycosylation of casein, but do serve as intermediates in the glycosylation of a group of mammary polypeptides with mol. wts. in the range 40000–80000. Some of this work has been published in preliminary form (Speake & White, 1977).

Materials and Methods

Animals and materials

The animals and materials used have been described (Speake & White, 1978a). Tunicamycin was kindly provided by Professor G. Tamura (Department of Microbiology, University of Tokyo, Tokyo, Japan). Purified rabbit casein (Al-Sarraj *et al.*, 1978a) and adsorbed antiserum to rabbit casein (Al-Sarraj *et al.*, 1978b) were gifts from Dr. K. Al-Sarraj of this Department.

Preparation of mammary explants

Explants of lobuloalveolar mammary tissue were prepared as described by Speake *et al.* (1975).

Measurement of the incorporation of D-[2-³H]mannose, N-acetyl-D-[1-¹⁴C]glucosamine and L-[4,5-³H]leucine into endogenous acceptors of mammary explants

Duplicate groups of 20 explants were incubated in Medium 199 (Morgan *et al.*, 1950) for various periods of time, as described by Speake & White (1978a), in the presence or absence of tunicamycin (20 µg/ml). The incubation medium contained 10 µCi of D-[2-³H]mannose (sp. radioactivity 2 Ci/mmol), 2.0 µCi of *N*-acetyl-D-[1-¹⁴C]glucosamine (sp. radioactivity 52 mCi/mmol) or 5.0 µCi of L-[4,5-³H]leucine (sp. radioactivity 53 Ci/mmol). Only a single radioactive substrate was added to each incubation vessel. The explants were preincubated in Medium 199 for 2 h in the presence or absence of tunicamycin before the addition of radioactive substrate.

At the end of the incubation period, each group of explants was washed with Medium 199 and homo-

genized in chloroform/methanol (2:1, v/v; 3 ml). Dolichol-linked monosaccharide, dolichol-linked oligosaccharide and protein fractions were isolated and the radioactivity in each fraction was determined as described by Speake & White (1978a).

A highly labelled protein fraction was prepared for polyacrylamide-gel electrophoresis by including larger amounts of radioactivity in the incubation: 50 µCi of [³H]mannose or 20 µCi of *N*-acetyl[¹⁴C]-glucosamine. The explants were incubated for 2 h in the presence of radiolabel.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Speake & White (1978a).

Chromatographic methods

Ascending paper chromatography was performed on sodium tetraborate-treated Whatman 3MM paper in solvent A [butan-1-ol/pyridine/water (4:6:3, by vol.)].

Descending paper chromatography was carried out on Whatman 3MM paper in solvent B [pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.)].

Alkaline AgNO₃ (Trevelyan *et al.*, 1959) was used for the detection of sugars. Radioactive zones were detected by liquid-scintillation counting of 1 cm strips of the chromatogram (White & Waechter, 1975).

Treatment with acid

Strong-acid hydrolysis of protein was performed in 4M-HCl (1 ml) at 100°C for 4 h. The hydrolysate was dried under N₂.

Acetylation

N-Acetylation of hexosamines derived from strong-acid hydrolyses of protein was carried out in acetic anhydride/methanol (1:5, v/v; 0.6 ml) as described by Clamp *et al.* (1972).

Immunochemical isolation of radiolabelled casein from mammary explant extracts

Duplicate groups of 20 explants were incubated for 2 h in Medium 199 as described by Speake & White (1978a). The incubation medium contained 20 µCi of *N*-acetyl-D-[1-¹⁴C]glucosamine. At the end of the incubation period, each group of explants was washed with Medium 199 and homogenized in 1 ml of water. Explant supernatant fractions were obtained by centrifugation (6 × 10⁵ g·min) of the homogenates.

Portions (0.4 ml) of the resultant supernatant fractions were incubated with 3 ml of antiserum to rabbit casein at 25°C for 30 min and then at 4°C for 24 h. The antiserum had previously been purified by

immunoabsorption with casein coupled to Sepharose 2B (Al-Sarraj *et al.*, 1978b); 1 ml of adsorbed anti-serum precipitated 25 μ g of rabbit casein at equivalence (K. Al-Sarraj, personal communication).

The immunoprecipitates were collected by centrifugation at 1000g for 30 min at room temperature and washed twice with 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl (2 ml).

Bovine serum albumin (fatty acid-poor, 250 μ g) was added as carrier to the supernatant fraction which remained after immunoprecipitation, and the protein was precipitated with trichloroacetic acid (final concn. 5%, w/v). The precipitates were washed five times by resuspension in 1 ml of 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, followed by reprecipitation with trichloroacetic acid (final concn. 5%, w/v).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Effect of tunicamycin on the incorporation of radioactive precursors into protein and dolichol-linked oligosaccharide fractions

The time course of incorporation of D-[2-³H]-mannose, N-acetyl-D-[1-¹⁴C]glucosamine and L-[4,5-³H]leucine into protein of mammary explants incubated in the presence and absence of tunicamycin is shown in Fig. 1. Tunicamycin markedly inhibits the incorporation of radioactivity from D-[2-³H]-mannose into protein. The amount of radioactivity incorporated into protein in the presence of tunica-

Fig. 1. Effect of tunicamycin on the incorporation of (a) D-[2-³H]mannose, (b) N-acetyl-D-[1-¹⁴C]glucosamine and (c) L-[4,5-³H]-leucine into protein by mammary explants

Duplicate groups of 20 explants were preincubated for 2 h at 37°C under O₂/CO₂ (19:1) in 1 ml of Medium 199 (glucose-free) in the presence (●) or absence (○) of tunicamycin (20 μ g/ml); 10 μ Ci of D-[2-³H]mannose, 2.0 μ Ci of N-acetyl-D-[1-¹⁴C]glucosamine or 5.0 μ Ci of L-[4,5-³H]leucine was then added to the incubation vessel and the incubations were allowed to continue for the times indicated. At the end of the incubation period, each group of explants was homogenized in chloroform/methanol (2:1, v/v; 3 ml). A lipid-free protein fraction was prepared as described by Speake & White (1978a), and the radioactivity in this fraction was determined.

mycin was approx. 27% of the control value after 3 h of incubation. The inhibition by tunicamycin of the incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into protein was less marked. In this case, the amount of radioactivity incorporated into protein after 3 h incubation in the presence of tunicamycin was approx. 72% of the control value. The rate of protein synthesis, as measured by the incorporation of L-[4,5-³H]leucine into protein, was only slightly inhibited by tunicamycin.

The effect of tunicamycin on the incorporation of radiolabelled sugars into a dolichol-linked oligosaccharide fraction is shown in Fig. 2. The incorporation of D-[2-³H]mannose into dolichol-linked oligosaccharide, in the presence or absence of tunicamycin, was linear with time for 90 min and then reached a plateau. Explants incubated in the presence of tunicamycin exhibited a progressive inhibition of the incorporation of radioactivity into dolichol-linked oligosaccharide. After 3 h of incubation in the presence of tunicamycin, the incorporation of D-[2-³H]mannose into dolichol-linked oligosaccharide was approx. 32% of the control value. The incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into dolichol-linked oligosaccharide was also inhibited by tunicamycin. In this case, the extent of the inhibition was constant throughout the 3 h incubation period (approx. 33% of the control value),

in contrast with the inhibition of incorporation of D-[2-³H]mannose, which only became marked after approx. 60 min of incubation.

Effect of tunicamycin on the relative incorporation of N-acetylglucosamine and N-acetylgalactosamine into mammary explant protein

The nature of the radioactive residues present in the protein fraction labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine was investigated after hydrolysis of the protein in strong acid. Paper chromatography of the hydrolysate in solvent B, or of the re-acetylated hydrolysate in solvent A, indicated that in the absence of tunicamycin considerable redistribution of label from *N*-acetylglucosamine to *N*-acetylgalactosamine had taken place (Fig. 3a). Approx. 39% of the total radioactivity was present as *N*-acetylglucosamine and approx. 61% was present as *N*-acetylgalactosamine. In the presence of tunicamycin, the proportion of the total radioactivity incorporated into protein as *N*-acetylgalactosamine was increased to approx. 76% (Fig. 3b). It appears that tunicamycin does not significantly inhibit the incorporation of *N*-acetylgalactosamine into protein, but markedly inhibits the incorporation of *N*-acetylglucosamine. In the presence of tunicamycin, the amount of radioactivity incorporated into protein as *N*-acetylglucosamine was approx. 39% of the control value.

Fig. 2. Effect of tunicamycin on the incorporation of (a) D-[2-³H]mannose and (b) *N*-acetyl-D-[1-¹⁴C]glucosamine into dolichol-linked oligosaccharide by mammary explants

Explants were incubated with D-[2-³H]mannose or *N*-acetyl-D-[1-¹⁴C]glucosamine in the presence (●) and absence (○) of tunicamycin as described in the legend for Fig. 1. At the end of the incubation period, each group of explants was homogenized in chloroform/methanol (2:1, v/v; 3 ml). The dolichol-linked oligosaccharide fraction was prepared as described by Speake & White (1978a) and the radioactivity in this fraction was determined.

Fig. 3. Paper chromatography of the hydrolysate of the protein labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine after incubation in the absence (a) or presence (b) of tunicamycin

Protein fractions were obtained from explants that had been incubated for 2 h with *N*-acetyl-D-[1-¹⁴C]glucosamine in the presence or absence of tunicamycin, as described in the Materials and Methods section. A portion (approx. 400 µg of protein) of each fraction was hydrolysed in 4 M-HCl (1 ml) at 100°C for 4 h. The hydrolysate was re-acetylated before ascending chromatography in solvent A for 20 h. The chromatogram was cut into segments 1 cm in length and the radioactivity in each segment was determined. The positions of migration of standard *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) are shown.

Polyacrylamide-gel electrophoresis of the glycoproteins labelled from D-[2-³H]mannose and N-acetyl-D-[1-¹⁴C]glucosamine in the presence and absence of tunicamycin

In the absence of tunicamycin, D-[2-³H]mannose is incorporated into a group of polypeptides with mol.wts. greater than 40000 as determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Speake & White, 1978a).

A major peak of radioactivity corresponding to a polypeptide with an approximate mol.wt. of 70000 was observed on the gel (Fig. 4a). Tunicamycin inhibits the incorporation of D-[2-³H]mannose into this group of polypeptides with mol.wts. greater than 40000 (Fig. 4b). In the presence of tunicamycin, the incorporation of radioactivity into the polypeptide with an approximate mol.wt. of 70000 was approx. 50% of the control value.

Label from *N*-acetyl-D-[1-¹⁴C]glucosamine was incorporated in the absence of tunicamycin into polypeptides with mol.wts. greater than 40000 and also into a polypeptide with an electrophoretic mobility identical with that of rabbit casein (Fig. 4c). In the presence of tunicamycin, the incorporation of radioactivity into the polypeptides with mol.wts. greater than 40000 was decreased to approx. 38% of the control value, whereas the incorporation into the polypeptide that co-migrates with rabbit casein was relatively unaffected (Fig. 4d).

Immunochemical identification of radiolabelled casein

Label from *N*-acetyl-D-[1-¹⁴C]glucosamine is incorporated by mammary explants into a polypeptide that co-migrates with rabbit casein during polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 4c). In order to obtain more direct evidence for the identity of this polypeptide, an extract of mammary explants labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine was incubated with an excess of antisera specific for rabbit casein.

Analysis of the immunoprecipitate by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed the presence of a single peak of radioactivity with a mobility identical with that of a rabbit casein (Fig. 5). The amount of radioactivity in the immunoprecipitate was identical with that which co-migrated with rabbit casein during polyacrylamide-gel electrophoresis of an equivalent portion of explant extract that had not been treated with antisera (results not shown). Thus all of the radioactivity incorporated into the polypeptide with an electrophoretic mobility identical with that of rabbit casein is precipitated by antisera specific for rabbit casein.

The nature of the radioactive residues present in the immunoprecipitate and in the supernatant remaining after immunoprecipitation was investigated after hydrolysis of the protein in strong acid. Paper chromatography in solvent system B of the protein hydrolysates indicated that the radioactivity in the immunoprecipitate was present as galactosamine, whereas the radioactivity released from the non-precipitated supernatant protein was present as glucosamine (Fig. 6). Label from *N*-acetyl-D-[1-¹⁴C]glucosamine is, therefore, incorporated into casein as *N*-acetylgalactosamine and into other mammary extract glycoproteins as *N*-acetylglucosamine.

Fig. 4. Polyacrylamide-gel electrophoresis of explant protein labelled from D-[2-³H]mannose and N-acetyl-D-[1-¹⁴C]glucosamine in the presence and absence of tunicamycin

Polyacrylamide-gel electrophoresis of the protein fractions labelled from D-[2-³H]mannose in the absence (a) and presence (b) of tunicamycin and from N-acetyl-D-[1-¹⁴C]glucosamine in the absence (c) and presence (d) of tunicamycin was carried out as described by Speake & White (1978a). Approx. 150 μ g of the lipid-free protein pellet was applied to each gel. The gel was cut into slices 0.2 cm in length and the radioactivity in each slice was determined. The mobilities of the tracking dye (T.D.), rabbit casein and protein standards ($10^{-3} \times$ mol.wt.) are indicated by arrows.

Discussion

The role of lipid-linked sugars as intermediates in the glycosylation of proteins has been demonstrated in a variety of tissues and this has been reviewed by Waechter & Lennarz (1976) and Hemming (1977). The proteins that are glycosylated via these intermediates have, however, been identified in only a few cases. Eagon & Heath (1977) have demonstrated that the glycosylation of a κ -type immunoglobulin light chain by mouse myeloma cells requires the participation of lipid-linked intermediates. In addition, the transfer of an oligosaccharide from an oligosaccharide-lipid to specific viral polypeptides has been demonstrated in a number of systems (Sefton, 1977; Robbins *et al.*, 1977; Tabas *et al.*, 1978). The recent

availability of the antibiotic tunicamycin, which prevents the synthesis of lipid-linked oligosaccharide, has enabled the identification of several glycoproteins that are glycosylated via lipid-linked intermediates. Such intermediates have thus been implicated in the glycosylation of immunoglobulin by mouse and rat plasmacytomas (Hickman *et al.*, 1977) and of ovalbumin by hen oviduct slices (Struck & Lennarz, 1977).

Tunicamycin inhibited glycosylation of newly synthesized LETS protein in 3T3 cells and resulted in a reduction in secretion of the protein (Duksin & Bornstein, 1977). Olden *et al.* (1978) demonstrated a similar inhibition of glycosylation of the major surface glycoproteins of chick-embryo fibroblasts, but their secretion on to the cell surface was not blocked.

Fig. 5. Polyacrylamide-gel electrophoresis of the immunoprecipitate obtained after incubation of an explant extract, labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine, with antisera to rabbit casein

The immunoprecipitate was prepared as described in the Materials and Methods section, solubilized in sodium dodecyl sulphate (final concn. 2%, w/v) and 2-mercaptoethanol (final concn. 2%, w/v) and analysed on 10% polyacrylamide gels containing sodium dodecyl sulphate. The gel was cut into slices 0.2 cm in length and the radioactivity in each slice was determined. The mobilities of tracking dye (T.D.) and rabbit casein are indicated by arrows.

Total amounts of this protein were reduced, however, owing to a specific 2–3-fold increase in the degradation of the non-glycosylated protein. Inhibition of glycosylation of sea-urchin-embryo glycoproteins by a brief exposure to tunicamycin prevented differentiation beyond the gastrula stage (Schneider *et al.*, 1978).

The present paper describes the effect of tunicamycin on the incorporation of mannose and *N*-acetylglucosamine into protein and dolichol-linked oligosaccharide by explants of lactating-rabbit mammary gland. Tunicamycin markedly inhibits the incorporation of mannose into both dolichol-linked oligosaccharide and protein (Figs. 1*a* and 2*a*). The incorporation of mannose into dolichol-linked oligosaccharide was not, however, completely inhibited by tunicamycin, in spite of the fact that the antibiotic was present at a concentration much higher than that required for complete inhibition in cell-free systems (Lehle & Tanner, 1976). This possibly reflects the time required for the antibiotic to penetrate into the cells, particularly those in the interior of the explant. In the presence of tunicamycin the incorporation of mannose into protein was inhibited by approximately the same extent as was

Fig. 6. Paper chromatography of the hydrolysate of immunoprecipitated casein (a) and of non-casein proteins (b) isolated from extracts of explants that had been incubated with *N*-acetyl-D-[1-¹⁴C]glucosamine

Explant extracts were prepared, incubated with absorbed antiserum to casein and the resulting immunoprecipitate and supernatant were separated and processed as described in the Materials and Methods section. The immunoprecipitate and the supernatant proteins were hydrolysed in strong acid and the hydrolysates were analysed by paper chromatography in solvent system B for 30 h. The chromatogram was cut into segments 1 cm in length and the radioactivity in each segment was determined. The positions of migration of standard galactosamine (GalN) and glucosamine (GlcN) are shown.

the incorporation into dolichol-linked oligosaccharide. This result suggests that a large proportion of the mannose residues present in mammary glycoproteins are attached to the polypeptide either as components of the 'core' oligosaccharide transferred from the lipid carrier or as subsequent additions to the 'core' oligosaccharide present on the protein. Both of the above alternatives require the continuous synthesis of dolichol-linked oligosaccharide in order for incorporation of mannose into protein to occur.

The incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into dolichol-linked oligosaccharide was reduced, in the presence of tunicamycin, to approx. 33% of the control value (Fig. 2*b*). In

contrast, tunicamycin was found to cause a relatively slight inhibition of the incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into protein (Fig. 1b). This is explained by the observations that considerable redistribution of label from *N*-acetylglucosamine to *N*-acetylgalactosamine occurs in the explant, and that the incorporation of *N*-acetylgalactosamine into protein is not affected by tunicamycin (Fig. 3).

Lactating mammary explants incubated in the presence of *N*-acetyl-D-[1-¹⁴C]glucosamine incorporate radioactivity into a polypeptide (or group of polypeptides) with an electrophoretic mobility identical with that of rabbit casein (Fig. 4c). Previous work (Speake & White, 1978a) has shown that explants taken from the mammary gland of a mid-pregnant rabbit do not incorporate label from *N*-acetyl-D-[1-¹⁴C]glucosamine into this polypeptide(s), indicating that the glycosylation and/or synthesis of this product is specific for mammary tissue that is fully differentiated. Electrophoretic co-migration is insufficient evidence for conclusive identification of this polypeptide(s) as casein. Explant extracts labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine were therefore treated with antisera specific for rabbit casein. The polypeptide(s) with an electrophoretic mobility identical with that of rabbit casein was specifically precipitated by the antisera (Fig. 5). This polypeptide(s) is therefore identical with casein, as judged by both electrophoretic and immunological criteria.

Rabbit casein consists of a group of glycopolypeptides, each of which contains *N*-acetylgalactosamine, galactose and sialic acid (Al-Sarraj *et al.*, 1978a). It would thus appear that radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine is incorporated into casein in the form of *N*-acetylgalactosamine. This supposition was confirmed by analysis of the acid hydrolysate of immunoprecipitated casein that had previously been labelled from *N*-acetyl-D-[1-¹⁴C]glucosamine. Paper chromatography of the hydrolysate indicated that *N*-acetylgalactosamine was the sole radioactive sugar present in casein, whereas the non-casein mammary glycoproteins were labelled largely with *N*-acetylglucosamine (Fig. 6). We conclude therefore that the lactating mammary tissue incorporates radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into casein, after the conversion of the initial precursor to *N*-acetylgalactosamine.

The incorporation of mannose and *N*-acetylglucosamine into a group of polypeptides with apparent mol.wts. ranging between 40000 and 80000 was considerably reduced in the presence of tunicamycin (Fig. 4). This result implies that at least some of these glycopolypeptides are of the *N*-glycosidic type and that lipid-linked intermediates are involved in their glycosylation. In contrast, the incorporation of *N*-acetylgalactosamine into casein was unaffected by tunicamycin (Fig. 4d), suggesting that lipid-

linked intermediates are not involved in the glycosylation of this protein. This would be expected, since casein is a glycoprotein in which the carbohydrate moiety is linked to the polypeptide by an *O*-glycosidic bond (Jolles, 1972) and also because *N*-acetylgalactosamine is not a component of the dolichol-linked oligosaccharide synthesized by mammary explants (Speake & White, 1978b).

In conclusion, the results presented in this paper suggest that the pathway of protein glycosylation involving lipid-linked intermediates is of considerable quantitative importance in the biosynthesis of several mammary glycopolypeptides which have mol.wts. between 40000 and 80000, whereas casein, the major glycoprotein synthesized by the mammary gland, is most likely glycosylated by direct sequential transfer of monosaccharides to the protein.

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