The Sequence of Addition of Terminal Sugars to an Immunoglobulin A Myeloma Protein

By N. J. Cowan* and G. B. Robinson
*Department of Biochemistry, University of Oxford,
South Parks Road, Oxford OX1 3QU, U.K.*

Numerous studies support the theory that glycoprotein synthesis proceeds by sequential addition of monosaccharides to a polypeptide core while the protein is transported from the membrane-bound polyribosomes to the cell exterior (Spiro, 1970). Oligosaccharide side chains from some mammalian glycoproteins show common structural features in that they consist of a branched core of *N*-acetylglucosamine and mannose residues; galactose residues serve to link this core to terminal residues of sialic acid or fucose. These structural features may reflect both the specificity of the glycosyltransferases and the topological organization of the enzymes along the intracellular transport path. That spatial organization of the transferases does occur is indicated by results obtained from studies with plasmacytoma cells (Melchers, 1971; Choi *et al.*, 1971).

It is not known whether the terminal sugars of the oligosaccharides, fucose and sialic acid, are attached concurrently or sequentially. This possibility has been investigated in the present study by using MPC-1 plasmacytoma cells, which secrete an IgA† myeloma protein. The kinetics of incorporation of both [³H]fucose and [¹⁴C]sialic acid into myeloma protein were followed, [¹⁴C]sialic acid being derived from precursor [¹⁴C]glucosamine by intracellular metabolism. Experiments were also conducted with [¹⁴C]lysine.

Solid plasmacytoma tumours were maintained in Balb/C mice; 18-day tumour tissue was dispersed by teasing or by gently squeezing tissue through gauze. The cells were washed twice in Eagle's salt solution (Eagle, 1959) and then suspended in incubation medium (Eagle's medium, without vitamins or glucose, containing 50mM-sodium pyruvate). Isotopically labelled compounds were added to the suspension and equal portions were separately incubated for various times at 37°C with CO_2+O_2 (5:95). Incubations were terminated by separating the cells from the medium by centrifugation and cooling in ice.

Total protein-bound radioactivity was measured on 5%-(w/v)-trichloroacetic acid precipitates washed thrice with 5% trichloroacetic acid and thrice with ether–chloroform–methanol (1:1:2, by vol.). Pellets

* Present address: Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

† Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G.

were dissolved in 0.5M-NaOH for scintillation counting of radioactivity and protein determination (Lowry *et al.*, 1951).

Immunoprecipitable radioactivity was isolated by using antisera to myeloma protein α-chain. Myeloma IgA (monomer) was purified from the sera of tumour-bearing mice by $(NH_4)_2SO_4$ precipitation, starch-block electrophoresis (Stelos, 1967) and gel filtration on Sephadex G-200. H(α)- and L-chains were prepared from the purified myeloma IgA (Abel & Grey, 1968). Antisera against α-chain were raised in rabbits and showed no cross-reaction against any serum protein other than myeloma protein; no reaction was obtained against mouse IgG or IgG L-chains.

Immunoprecipitations were conducted by allowing antisera to react against either incubation media or cell homogenates. The homogenates were prepared by disrupting cells in 0.25M-sucrose in a Potter–Elvehjem homogenizer and adding sodium deoxycholate (10%, w/v) to a final concentration of 1% (w/v). The suspensions were treated with deoxyribonuclease (10μg/ml) (at 37°C for 30min) and centrifuged (at $1.5 \times 10^5 g_{av.}$ for 60min), and the supernatants were used for precipitations. Unlabelled myeloma IgA and equivalent amounts of antisera were added to media or homogenates, and the immunoprecipitates were recovered by centrifugation after incubation at 37°C (for 30min) and 4°C (for 18h) and washed thrice in 150mM-NaCl.

Insignificant amounts of radioactivity were recovered on repeated precipitation of the supernatants. The results were corrected for non-specific radioactivity in control immunoprecipitations with human IgG and rabbit antisera.

In experiments with [¹⁴C]lysine and [³H]fucose the radioactivity of the whole immunoprecipitate was counted after it had been dissolved in 0.5M-NaOH. In experiments with [¹⁴C]glucosamine the precipitates were hydrolysed in 5% trichloroacetic acid (at 80°C for 60min) and sialic acid was isolated by chromatographing the ether-extracted supernatant on Dowex 1 (Cl⁻ form) columns (Molnar *et al.*, 1965). Radioactive material not adhering to the resin was added to the precipitates remaining from the hydrolysis, and the whole was again hydrolysed in 5.0M-HCl (at 100°C for 3h). The hydrolysates were dried and dissolved in water, and the amino sugars were isolated by using Dowex 50 (H⁺ form) columns (Molnar *et al.*, 1965). The total radioactivities of the sialic acid and
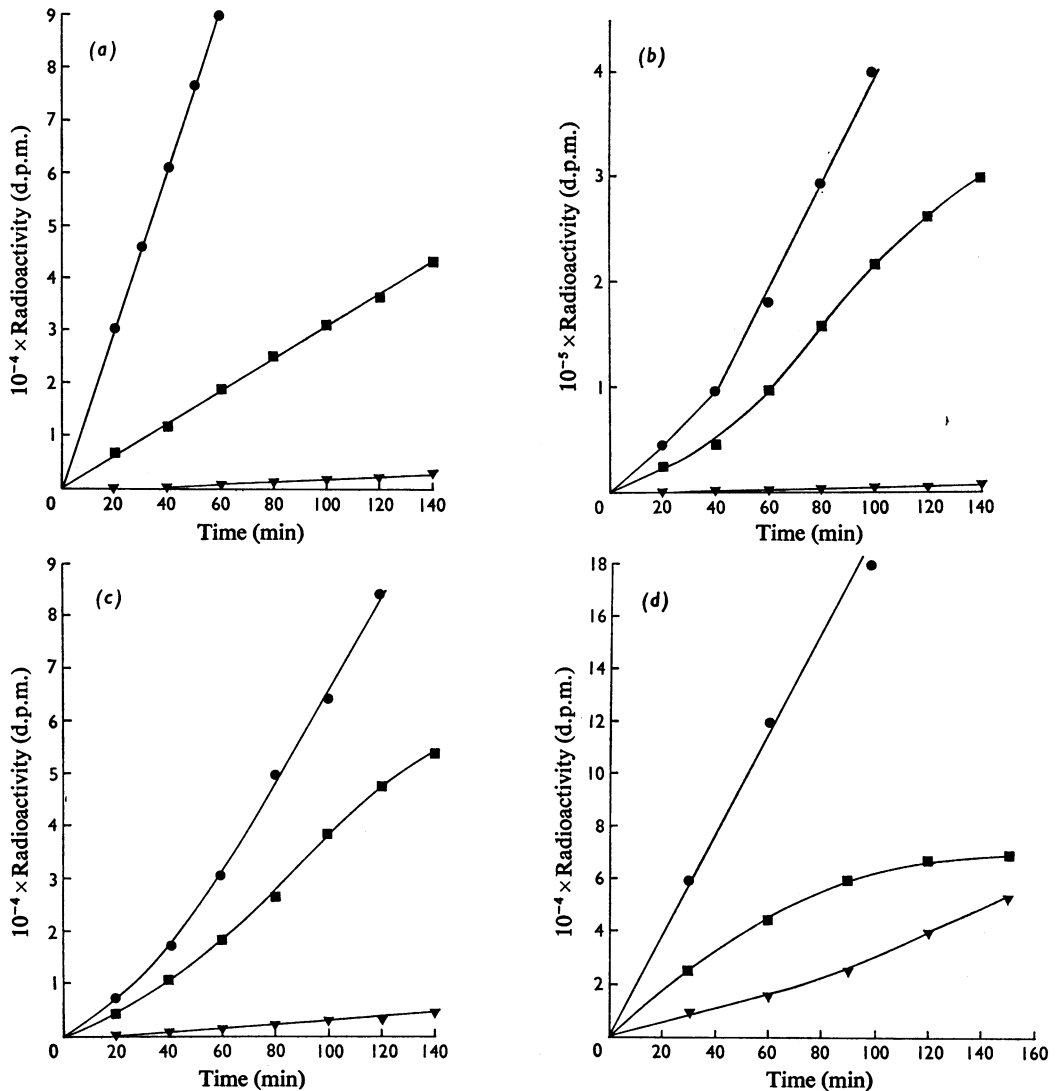
Fig. 1. *Incorporation by MPC-1 cells of lysine, glucosamine, sialic acid and fucose into intracellular and extra-cellular myeloma protein*

Tumour cells were suspended in Eagle's medium containing isotopically labelled precursor and 50 mM-sodium pyruvate, the cell concentration being adjusted to approx. 10^6 cells/ml. The suspension was divided into equal portions, which were separately incubated with slow swirling at $37°C$, with O_2+CO_2 (95:5) as the gas phase. At each time-interval one portion of cells was withdrawn from the water bath, and the cells and medium were separated. Total radioactivity was determined on a portion of each sample, and the remainder was analysed for radioactivity precipitable with α-chain antibody. In the experiments with [^{14}C]glucosamine, the trichloroacetic acid-insoluble material and the immunoprecipitates were hydrolysed, and the fraction containing sialic acid was separated from the fraction containing glucosamine by column chromatography as indicated in the text. The radioactivities of these fractions were counted separately. Isotope concentrations in the incubation medium were 1 μCi/ml for ^{14}C and 5 μCi/ml for ^{3}H. The specific radioactivities of the isotopically labelled compounds were: L-[U-^{14}C]lysine, 312 mCi/mmol; D-[1-^{14}C]glucosamine, 55 mCi/mmol; L-[1-^{3}H]fucose, 920 mCi/mmol. The volumes of the samples incubated were identical for each experiment, but varied slightly (5–6 ml) between experiments. The results are expressed as the total radioactivities (d.p.m.) for each sample incubated. ●, Total intracellular protein; ■, α-chain-precipitable intracellular protein; ▼, α-chain-precipitable protein in the medium. (*a*) Incorporation of [^{14}C]lysine; (*b*) incorporation of [^{14}C]glucosamine; (*c*) incorporation of [^{14}C]sialic acid; (*d*) incorporation of [^{3}H]fucose.

amino sugar fractions were measured. Paper-chromatographic analysis confirmed that sialic acid and glucosamine each accounted for 95% of the radioactivity of the respective eluates.

In experiments with [^{3}H]fucose, chromatographic analysis showed that fucose accounted for 70–75% of the incorporated radioactivity; the remainder co-chromatographed with galactose.

The incubations employed in these experiments differed from those of Melchers (1970, 1971) and Choi et al. (1971) in that pyruvate replaced glucose as an energy source and in that $O_2 + CO_2$ was used to replace air $+ CO_2$. The presence of glucose in the incubation medium depressed the cellular utilization of [^{14}C]glucosamine, and thus [^{14}C]sialic acid incorporation was low. Under the conditions used here O_2 uptake and lysine incorporation were linear with time during the experiments. The cell membranes were judged to have remained undamaged, since there was no detectable leakage of lactate dehydrogenase, of [^{14}C]glucosamine phosphates or of nucleotide-bound [^{14}C]glucosamine from the cells during incubation.

The incorporation of [^{14}C]lysine, [^{14}C]glucosamine, [^{14}C]sialic acid and [^{3}H]fucose into total and immunoprecipitable protein is shown in Fig. 1. For lysine, glucosamine and sialic acid the radioactivity incorporated into intracellular myeloma protein increased throughout the experiments, whereas the radioactivity of fucose-labelled myeloma protein within the cells began to level off after 120 min. Negligible amounts of lysine-, glucosamine- or sialic acid-labelled myeloma protein were secreted by the cells, whereas appreciable quantities of fucose-labelled myeloma protein were found in the medium. In experiments conducted for longer time-intervals, [^{14}C]lysine-labelled myeloma protein accumulated in the medium after approx. 180 min, indicating that the cells had not lost the ability to secrete protein. This conclusion was supported by studies of lysine and glucosamine incorporation in vivo, which showed that newly synthesized myeloma protein was secreted by tumours and not retained within the cells. However, no detailed incorporation studies were conducted in vivo for periods longer than 150 min, since O_2 uptake by cells began to decline after this time.

In experiments where the incubation medium contained puromycin (5 μM), incorporation of lysine was inhibited by 95%, but incorporation of the sugars was decreased by only 10–20% after 140 min.

The theory of sequential addition of monosaccharides to glycoproteins requires that the cells should contain pools of glycoproteins at various stages of completion. Assuming a steady state of synthesis and secretion, incubation with isotopically labelled precursor will result in the radioactivity of the intracellular myeloma protein increasing to a maximum with time as the intermediate pools reach isotopic equilibrium in sequence. Thereafter labelled protein will accumulate outside the cells at a constant rate. The time taken for the intracellular radioactivity to reach a maximum and for the secretion of radioactive protein to become linear with time will depend on the labelled compound used. This time will be longer for compounds incorporated early in the sequence, e.g. amino acids, and shorter for those compounds incorporated at the end of the sequence, e.g. the terminal sugars, sialic acid and fucose.

It has already been shown that plasmacytoma cells contain incompleted myeloma protein and that these pools may take 3–4 h to reach isotopic equilibrium (Melchers, 1970, 1971; Choi et al., 1971). That large intracellular pools of myeloma protein occurred in MPC-1 cells was indicated by the finding that puromycin had little effect on the incorporation of sugars into myeloma protein. The continued intracellular accumulation of myeloma protein labelled with lysine, glucosamine or sialic acid, together with the secretion of only small amounts of radioactive protein, shows that these intermediate pools had not reached isotopic equilibrium by the end of the incubation period. In contrast, the results with fucose indicate that isotopic equilibrium was being attained towards the end of the incubation period. It may therefore be concluded that the addition of fucose to the myeloma IgA occurs later in the assembly sequence than the addition of sialic acid.

The difference in time of attachment of the two terminal sugars suggests a morphological separation of the two processes. Studies with rat liver suggest that both sugars are transferred to glycoprotein in the Golgi membranes (Schachter et al., 1970; Wagner & Cynkin, 1971). Thus these membranes may be topologically organized with respect to glycoprotein synthesis.

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