Utilization by the Isolated Perfused Rat Liver of *N*-Acetyl-D-[1-¹⁴C]galactosamine and *N*-[³H]Acetyl-D-galactosamine for the Biosynthesis of Glycoproteins

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The isolated perfused rat liver system has been used to monitor the utilization of *N*-[³H]acetyl-D-galactosamine and *N*-acetyl-D-[1-¹⁴C]galactosamine for the biosynthesis of radiolabelled glycoproteins, which are subsequently secreted into the plasma. Both radiolabels appear in a number of different glycoproteins, predominantly as sialic acid and *N*-acetylglucosamine. The ratio of labelled sialic acid to labelled *N*-acetylglucosamine varies for different glycoproteins, but the bulk of *N*-acetyl-D-galactosamine is incorporated without deacetylation.

It has been established (Wood *et al.*, 1973), that ³⁵S-labelled chondroitin sulphate is degraded in the liver of rats. This degradation would presumably result in the release of hexosamine residues, and some information is available on the fate of such molecules *in vivo*. For example, a study (Maley *et al.*, 1968) on the fate of *N*-acetyl-D-[1-¹⁴C]galactosamine after intraportal administration to rats revealed that radio-labelled UDP-*N*-acetylglucosamine accounted for the major proportion of radioactivity present as nucleotide *N*-acetyl-hexosamines in the HClO₄-soluble fraction of the liver, whereas radiolabelled galactosamine 1-phosphate and sialic acid were relatively minor components. Other studies, in which the metabolism of *N*-acetyl-D-[1-¹⁴C]galactosamine was investigated in the isolated perfused rat liver system, demonstrated utilization of the hexosamine for glycoprotein biosynthesis (MacNicoll *et al.*, 1977). Radiolabelled glycoprotein secreted by the liver accounted for approximately half of the radioactivity in the perfusate after 6h, and ¹⁴C-labelled sialic acid and glucosamine were the major radioactive components of the glycoprotein(s). However, it is not known whether *N*-acetyl-galactosamine itself is a suitable substrate for the transformations or whether hexosamine utilization follows an obligatory deacetylation. The present work was undertaken to study this possibility by measuring the distribution of radioactivity in glycoproteins synthesized by the liver in the presence of *N*-[³H]acetyl-D-galactosamine and *N*-acetyl-D-[1-¹⁴C]galactosamine.

Materials and Methods

Radiolabelled chemicals

D-[6-³H]Glucosamine hydrochloride (14–25 mCi/μmol), *N*-acetyl-D-[1-¹⁴C]galactosamine (53 mCi/mmol), *N*-[³H]acetyl-D-[1-¹⁴C]galactosamine (500 mCi/mmol), *n*-[³H]hexadecane (5.04 × 10⁶ d.p.m./g) and *n*-[¹⁴C]hexadecane (1.12 × 10⁶ d.p.m./g) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Experimental animals

Adult male and female MRC Hooded rats, maintained on a standard diet, were used throughout.

Isolated-liver perfusion

The method used was that of Curtis *et al.* (1970), and perfusion was carried out at a flow rate of approx. 40 ml/min for 6h with homologous heparinized blood containing *N*-[³H]acetyl-D-galactosamine (32.6 μCi; 66.5 nmol) and *N*-acetyl-D-[1-¹⁴C]galactosamine (5.9 μCi; 182.5 nmol). The packed cell volume was monitored at hourly intervals and ranged between 31 and 33.5% cells. Bile was collected hourly, and samples were analysed for radioactive contents. At the end of the perfusion the perfusate was collected and the plasma separated by centrifuging (3000g) for 30 min at 4°C. The liver was infused with 0.15 M-NaCl (10 ml) via the hepatic portal vein cannula, and was chopped and homogenized in ice-cold 0.15 M-NaCl (final volume 15 ml) with a Potter–Elvehjem homogenizer (0.23 nm clearance). HClO₄ (40 ml; 6%, w/v) was added to 15 ml of the liver homogenate and the

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acid-insoluble material was separated by centrifuging (3000g for 30min at 4°C). The precipitate was resuspended in a further 30ml of HClO₄ and centrifuged. The combined supernatants (60ml) were adjusted to pH 7.4 with 10M-KOH and the precipitated material was separated by centrifuging (3000g for 30min at 4°C). The neutralized HClO₄ extracts were stored frozen until required.

Analysis of radiolabelled amino sugars in the HClO₄-soluble extracts of rat liver

The neutralized extract (10ml) was subjected to ion-exchange chromatography on Dowex 1 (X2; formate form, 200–400 mesh) and Dowex 50 (X8; H⁺ form, 200–400 mesh) (from Sigma Chemical Co., St. Louis, MO, U.S.A.). The amino sugars and metabolites were separated by the method of Akamatsu *et al.* (1976) and the radioactive content of portions (0.5ml) of each fraction was determined by liquid-scintillation counting.

Measurement of radiolabelled macromolecules secreted by the perfused liver

The proportion of radioactivity (³H and ¹⁴C) present as polymeric material in the 6h plasma was determined by molecular-exclusion chromatography on Sephadex G-200 and by dialysis.

Samples (250μl) of the plasma were applied to a column (580mm×9mm) of Sephadex G-200 equilibrated with 0.2M-NaCl at 4°C. The column was eluted with 0.2M-NaCl at a flow rate of 1ml/h. The effluent was collected as 1ml fractions and portions (500μl) were assayed for total ³H and ¹⁴C by liquid-scintillation counting. A bulk preparation of the ³H- and ¹⁴C-labelled polymeric materials in plasma was obtained by dialysis of 50ml of plasma against 2×2 litres of 0.15M-NaCl for 16h at 4°C. After dialysis, portions (250μl) of the non-diffusible material were oxidized by combustion and assayed for total ³H and ¹⁴C by liquid-scintillation counting. Portions (1ml) of the diffusible radioactivity were assayed for the total ³H and ¹⁴C by liquid-scintillation counting without combustion.

Ethanol fractionation of ³H and ¹⁴C-labelled macromolecules secreted by the liver

Samples (10ml) of the non-diffusible material described above were subjected to further dialysis against 2×2 litres of 0.15M-NaCl/0.75M-trisodium citrate/0.035M-citric acid, pH 7.1. This material was then fractionated with ethanol as described by Cohn *et al.* (1950). Portions (0.05–0.5 ml) of each fraction were assayed for total ³H and ¹⁴C content by liquid-scintillation counting. The ethanol was removed from Cohn fractions II, I-III-3, V and VI under

reduced pressure and the remaining aqueous solutions were freeze-dried. Each fraction was dissolved in 4–8ml of 0.15M-NaCl/5.4mM-trisodium citrate.

Neuraminidase digestion of ³H and ¹⁴C-labelled macromolecules

The radiolabelled polymers obtained after dialysis of the 6h plasma and the Cohn fractions II, I-III-3, V and VI were made 0.05M with respect to sodium acetate and 1mM with respect to CaCl₂. The pH of each fraction was adjusted to 5.5 and neuraminidase (type V from *Clostridium perfringens*, EC 3.2.1.18; Sigma) was added to a final concentration of 1mg/50mg of protein. The mixtures were incubated for 24h at 37°C. The distribution of ³H and ¹⁴C before and after neuraminidase treatment was determined by molecular-exclusion chromatography on Sephacryl S-200. Samples (250μl) were applied to a column (580mm×9mm) of Sephacryl S-200 [Pharmacia (G.B.) Ltd., London W.5, U.K.] equilibrated with 0.15M-NaCl in 8M-urea. The column was eluted with the same buffer and portions (0.5ml) of the 1ml fractions were assayed for total ³H and ¹⁴C content by liquid-scintillation counting.

Subtilisin digestion of ³H and ¹⁴C-labelled macromolecules

A sample (0.5ml) of the non-diffusible radioactive material obtained after dialysis of the 6h plasma was reduced and carboxymethylated by the method of Crestfield *et al.* (1963). After reaction the mixture was dialysed against water (2×2 litres for 16h at 4°C), freeze-dried and dissolved in 0.5ml of 0.5% (w/v) NH₄HCO₃, pH 8.5. Subtilisin (Type VII from *Bacillus amyloliquefaciens*, EC 3.2.21.14; Sigma) was added to a final concentration of approx. 1mg/50mg of protein and the mixture was incubated for 2h at 37°C. The distribution of ³H and ¹⁴C-labelled material before and after subtilisin digestion was determined by molecular exclusion on Sephacryl S-200 as described above.

Measurement of radiolabelled hexosamines

The fractions corresponding to the broad peak of radioactivity eluted from Sephadex S-200 after digestion with subtilisin were pooled, dialysed against water (2×2 litres for 16h at 4°C) and freeze-dried. After hydrolysis in 5ml of 4M-HCl (in sealed tubes under N₂ at 100°C for 8h) in the presence and absence of marker *N*-acetyl-D-[1-¹⁴C]galactosamine, the hydrolysates were evaporated to dryness in a Rotary Evapomixer (Buchler Instruments Ltd., Fort Lee, NJ, U.S.A.) and redissolved in 0.5ml of a citrate buffer containing salt. This buffer was prepared by mixing 220.7g of trisodium citrate, 2H₂O, 150ml of aq. (1:1, v/v) Brij 35 (BDH Chemicals Ltd., Poole,

Dorset, U.K.), 375 ml of 2M-NaOH, 30 ml of octanoic acid (BDH) and made up to 15 litres with water. To 7.48 litres of this stock solution were added 280.6 g of NaCl and 60 ml of 10M-HCl. The volume was made up to 8 litres with water, and the final pH of this salt/citrate buffer was 5.0.

The hydrolysates were applied to a column (1400 mm $\times$ 5.2 mm) of Chromobeads (type A) cation-exchange resin (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.) equilibrated in the same buffer. The eluate was collected in 3 min fractions and assayed for radiolabelled hexosamines by liquid-scintillation counting.

Measurement of the radiolabelled hexosamines in the diffusible fraction of the plasma obtained after 6 h perfusion was carried out as follows. Plasma (10 ml) was dialysed against water (50 ml). The dialysate was freeze-dried, redissolved in water (6 ml) and portions (2 ml) were each made up to 5 ml of 4.0M-HCl in the presence and absence of marker *N*-acetyl-D-[1- 14 C]-galactosamine. Hydrolysis and hexosamine analysis was carried out as previously described.

Measurement of radioactivity

Portions (up to 0.5 ml) of plasma, bile or liver homogenate containing both ^3H - and ^{14}C -labelled material were oxidized (Oxymat liquid-scintillation sample oxidizer model in 4101; Intertechnique, Plaisir, France). The scintillant used for the recovery of ^3H was prepared by dissolving 20 g of naphthalene and 7 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole in 700 ml of 1,4-dioxan (scintillation grade) and 300 ml of toluene. The scintillant used for the recovery of ^{14}C was prepared by dissolving 37.5 g of 2,5-diphenyloxazole and 2.7 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.) in 600 ml of ethanolamine, 1800 ml of methanol and 1600 ml of toluene. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer model 3375 (Packard Instrument Co. Inc., Downers Grove, IL, U.S.A.) by using preset ^3H and ^{14}C windows. Absolute amounts of ^3H and ^{14}C in each sample were determined by treating known amounts of each isotope in an identical manner. Less than 2% of the total ^{14}C was recovered in the ^3H channel and less than 1% of the total ^3H was recovered in the ^{14}C channel.

Alternatively, samples (up to 0.5 ml) containing both ^3H and ^{14}C were assayed for radioactivity in 5 ml of PCS scintillation 'cocktail' (Amersham/Searle Corporation, Arlington Heights, IL, U.S.A.) in vials that had previously been measured for background radioactivity. At an amplification of 100% and a channel-discriminator setting of 0-500, the efficiencies of counting unquenched samples of ^3H and ^{14}C were 22.02 and 5.23% respectively. At an

amplification of 6.5% and a channel-discriminator setting of 100-1000, the efficiencies of counting for ^3H and ^{14}C were 0.20 and 65.04% respectively. The radioactive content of each sample was expressed as d.p.m. by reference to quench curves prepared by the addition of water (0-0.5 ml) to 5 ml of PCS scintillation 'cocktail' containing known amounts of *n*-[^3H]-hexadecane or *n*-[^{14}C]-hexadecane.

Results and Discussion

Analysis of the ^3H and ^{14}C content of the plasma obtained after 6 h perfusion showed that 32.5% of the total ^3H in plasma and 39.5% of the total ^{14}C in plasma was associated with polymeric material (Fig. 1). Alternatively, after dialysis of the 6 h plasma, 36.4% of the total ^3H in plasma and 44.8% of the total ^{14}C in plasma was retained inside the dialysis tubing, presumably as non-diffusible radioisotope. This small but consistent difference between the recoveries of ^3H and ^{14}C in the polymeric material secreted by the liver suggests that some deacetylation of *N*-[^3H]acetyl-D-galactosamine (or its metabolites) is occurring in the liver. The higher ^{14}C content of the polymers can then be explained by assuming that 20% of the ^{14}C is derived from labelled precursor(s) that have undergone deacetylation. However, for the incorporation of the remaining 80% of the ^{14}C label, it is not necessary to propose a deacetylation step.

Fractionation of the labelled polymers in the 6 h plasma either by molecular-exclusion chromatography (Fig. 2a) or ethanol fractionation (Table 1) also shows that the ratio of ^3H to ^{14}C in the polymeric substances is virtually constant, regardless of the size or nature of the polymer. The only exception is observed in Cohn fraction II, which has approx. 30% less ^3H than ^{14}C . However, the recoveries of ^3H

Fig. 1. Molecular-exclusion chromatography of plasma obtained after 6 h liver perfusion with *N*-[^3H]acetyl-D-galactosamine and *N*-acetyl-D-[1- ^{14}C]galactosamine. Elution profile on Sephadex G-200 showing the distribution of radioactivity ($\bullet$, ^3H ; $\circ$, ^{14}C) in untreated plasma. The column (580 mm $\times$ 9 mm) was eluted with 0.2M-NaCl.

Table 1. *Ethanol fractionation of the non-diffusible fraction of plasma obtained after 6h liver perfusion with N-[³H]-acetyl-D-galactosamine and N-acetyl-D-[1-¹⁴C]galactosamine*

For details see the text.

Cohn fraction no.	Major components	Radioactivity (expressed as % of total in all fractions)	
		³ H	¹⁴ C
II	γ-Globulins	7.6	10.5
III-0	β-Globulins	2.5	3.0
III-1,2	Prothrombin	0.2	0.2
	isoagglutinins		
I, III-3	Fibrinogen	33.2	30.4
	cryoglobulins		
VI	Acid glycoproteins	31.8	32.0
V	Albumins	23.2	21.3
IV-6, 7	α-Globulins	1.6	1.7
IV-1	Glycolipids	2.4	1.9

and ¹⁴C in this fraction are relatively small. Consequently, the difference in ³H and ¹⁴C content in this fraction alone is not sufficient to account for the lower recovery of ³H in the heterogeneous mixture of labelled polymers secreted by the liver. Digestion of the non-diffusible material in the 6h plasma with subtilisin followed by gel filtration on Sephacryl S-200, showed an identical shift in the elution profiles of both isotopes (Fig. 2*b*). After digestion, both isotopes were retarded on the column, which suggests that all the radioactively labelled polymeric material was susceptible to proteolytic digestion.

The effect of neuraminidase treatment on the distribution of ³H and ¹⁴C in the non-diffusible material obtained from the plasma is shown in Fig. 2*c*). Analysis by gel-filtration on Sephacryl S-200 showed that, after neuraminidase treatment, an additional radioactive peak of low molecular weight could be separated. This retarded peak accounted for 29.7% of the ³H and 28.3% of the ¹⁴C content of the radiolabelled polymeric material in the plasma, presumably as [³H]sialic acid and [¹⁴C]sialic acid. However, the radiolabelled sialic acid is not restricted to one polymer. Neuraminidase digestion of Cohn fractions II, III-3, VI and V, followed by gel-filtration on Sephacryl S-200, showed that each fraction was susceptible to neuraminidase digestion. After digestion, an additional peak of radioactivity could be separated that was not present in the undigested fractions (Figs. 3 and 4). The proportion of this peak assumed to be radiolabelled sialic acid varied in each fraction, ranging between 24.6 and 47.0% of the total ³H and ¹⁴C in each fraction. When the non-diffusible radioactivity in 6h plasma was subjected to hexosamine analysis after subtilisin digestion and

Fig. 2. *Chromatography on Sephacryl S-200 of the non-diffusible fraction of plasma obtained after 6h liver perfusion with N-[³H]acetyl-D-galactosamine and N-acetyl-D-[1-¹⁴C]galactosamine*

Elution profiles on Sephacryl S-200 showing the distribution of radioactivity (●, ³H; ○, ¹⁴C) in (a) the non-diffusible fraction of plasma, (b) after digestion with subtilisin, and (c) after digestion with neuraminidase. Columns (580 mm × 9 mm) were eluted with 0.15 M-NaCl, 8 M-urea and the void volume is indicated by V₀.

acid hydrolysis, approx. 68% of the total ¹⁴C-label was recovered as hexosamines. By contrast, the ³H label could not be detected in the hexosamine peak, presumably owing to deacetylation by acid hydrolysis. Of the total [¹⁴C]hexosamine recovered, 95% corresponded to [¹⁴C]glucosamine and 5% to galactosamine. Hexosamine analysis of the diffusible radioisotope from the 6h plasma showed that 95% of the total ¹⁴C was present as hexosamine. Of this material, 98% co-chromatographed with [¹⁴C]galactosamine and only 2% with [¹⁴C]glucosamine. The redistribution of radioactivity in Cohn fraction V after neuraminidase treatment (cf. Figs. 3*d* and 4*d*) and the lack of significant labelling of amino acids in the hexosamine analysis suggests that the extensive labelling of the albumin fraction is due to contaminating glycoproteins.

Analysis of ³H- and ¹⁴C-labelled amino sugars in liver

Of the total ³H and ¹⁴C added to the perfusate, 7.8% of the ³H and 8.1% of the ¹⁴C label was measurable in the washed liver after homogenization.

Fig. 3. Chromatography on Sephacryl S-200 of plasma fractions obtained after 6h liver perfusion with *N*-acetyl-D-[1- 14 C]galactosamine

The non-diffusible fraction of plasma was first subjected to ethanol fractionation. Elution profiles show the distribution of radioactivity ($\bullet$, ^3H ; $\circ$, ^{14}C) in (a) Cohn fraction II, (b) Cohn fractions I, III-3, (c) Cohn fraction VI and (d) Cohn fraction V. Columns (580mm $\times$ 9mm) were eluted with 0.15M-NaCl, 8M-urea, and the void volume is indicated by V_0 .

Approximately one-half (3.6% of the ^3H label and 3.8% of the ^{14}C label) of each isotope was soluble in HClO_4 . Fractionation of the HClO_4 -soluble material by cation-exchange chromatography showed that approx. 50% of both isotopes (57.0% of the ^3H and 53.5% of the ^{14}C label) appeared in the fraction containing free *N*-acetylhexosamines. A small amount of radioisotope only (2.7% of ^3H and 2.2%

Fig. 4. Chromatography on Sephacryl S-200 of neuraminidase digested plasma fractions obtained after 6h liver perfusion with *N*-[^3H]acetyl-D-galactosamine and *N*-acetyl-D-[1- 14 C]galactosamine

The non-diffusible fraction of plasma was subjected to fractionation with ethanol. Fractions were digested with neuraminidase, and the elution profiles ($\bullet$, ^3H ; $\circ$, ^{14}C) show the distribution of radioactivity in (a) Cohn fraction II, (b) Cohn fractions I, III-3, (c) Cohn fraction VI and (d) Cohn fraction V. Low-molecular-weight material accounted for (a) 39.3% of the ^3H , 43.7% of the ^{14}C ; (b) 38.5% of the ^3H , 24.6% of the ^{14}C ; (c) 47.3% of the ^3H , 47.0% of the ^{14}C and (d) 37.0% of the ^3H , 36.4% of the ^{14}C , expressed as a percentage of the total of each isotope eluted from each column. Columns (580mm $\times$ 9mm) were eluted with 0.15M-NaCl, 8M-urea, and the void volume is indicated by V_0 .

of ^{14}C) was associated with non-acetylated hexosamines. The remainder of both isotopes in the HClO_4 -soluble material was distributed between three fractions: the nucleotide *N*-acetylhexosamines (18.5% ^3H and 19.4% ^{14}C), *N*-acetylhexosamine phosphates (10.3% ^3H and 12.4% ^{14}C) and sialic acid (10.8% ^3H and 11.3% ^{14}C).

Collectively the results demonstrate that both *N*-[^3H]acetyl-D-galactosamine and *N*-acetyl-D-[1- ^{14}C]galactosamine are utilized by the liver for the biosynthesis of glycoproteins, which are secreted

into the plasma. The ^3H and ^{14}C labels are present in a number of glycoproteins predominantly as sialic acid and *N*-acetylglucosamine, showing that the epimerization step is a major event in the utilization of *N*-acetylgalactosamine by the isolated perfused liver. Although the ratio of labelled sialic acid to labelled *N*-acetylglucosamine varies from one Cohn fraction to another, the ratio of ^3H to ^{14}C in each fraction remains virtually constant and it is apparent that the bulk of the *N*-acetyl-D-galactosamine is eventually incorporated into glycoproteins without deacetylation.

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References

- Akamatsu, N., Nakajima, H. & Miyata, S. (1976) *Biochem. J.* **158**, 589–592
- Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Deronaux, G., Gillespie, J. M., Kahnt, F. W., Lever, F. W., Liu, C. H., Mittleman, D., Mouton, R. F., Schmid, K. & Uroma, E. (1950) *J. Am. Chem. Soc.* **72**, 465–474
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Curtis, C. G., Powell, G. M. & Stone, S. L. (1970) *J. Physiol. (London)* **213**, 14P–15P
- MacNicoll, A. D., Wusteman, F. S., Powell, G. M. & Curtis, C. G. (1977) *Biochem. Soc. Trans.* **5**, 442–443
- Maley, F., Tarentino, A. L., McGarrah, J. F. & Del Giacco, R. (1968) *Biochem. J.* **107**, 637–644
- Wood, K. M., Wusteman, F. S. & Curtis, C. G. (1973) *Biochem. J.* **134**, 1009–1013