Glycosylation of Ribonuclease A Catalysed by Rabbit Liver Extracts

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Crude extracts of rabbit liver catalyse *in vitro* the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to bovine pancreatic ribonuclease A. The enzymic activity is contained in rough endoplasmic reticulum. It has an absolute requirement for a bivalent metal ion: $\text{Co}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+}$. Mg^{2+} is ineffective. There is enzymic activity in the absence of detergent, but increased activity is observed in the presence of Triton X-100. The site of glycosylation of ribonuclease A is asparagine-34, and glycosylation occurs only at this point. These findings agree with the hypothesis that the sequence Asn-X-Thr(Ser) (where X may be one of a number of types of amino acid) is a necessary, but not sufficient, condition for *N*-acetylglucosamylation of a protein-bound asparagine residue.

For several years it has been thought that in glycoproteins formation of the carbohydrate-peptide linkages of the form 4-*N*-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine results from the glycosylation of an asparagine, rather than an aspartic acid, residue. Examination of the distribution of radioactivity in the serum glycoproteins of rats that had been injected with L-[^{14}C]asparagine or L-[^{14}C]aspartic acid confirmed that an L-asparagine residue was at least the major, if not the only, site of glycosylation in which this type of carbohydrate-peptide linkage is formed (Kohno & Yamashina, 1973).

There is a marked specificity as to which L-asparagine residue(s) in a polypeptide chain can undergo glycosylation. If such a residue is followed in a position next-but-one on its C-terminal side by an L-serine or an L-threonine residue, the amide moiety of the L-asparagine residue might undergo substitution more readily (Marshall, 1967). These and other considerations led to the proposal that the sequence in a protein $\cdots\text{Asn-X-Thr(Ser)}\cdots$ (where X may be one of a number of types of amino acid) is a necessary but not sufficient condition for glycosylation to occur (Neuberger & Marshall, 1968). The studies of Melchers (1969), Catley *et al.* (1969) and later workers (Hunt & Dayhoff, 1970; Jackson & Hirs, 1970; see also Marshall & Neuberger, 1970; Marshall, 1972) support this belief, although direct evidence is lacking.

To test this hypothesis we have used ribonuclease A as a substrate because it contains a requisite sequence for glycosylation to occur (Marshall, 1967; Neuberger & Marshall, 1968). Rabbit liver contains a UDP-*N*-acetyl-D-glucosamine transferase which catalyses the formation of the carbohydrate-peptide linkage involving asparagine. It will be referred to as GlcNAc-Asn synthetase. The activity appears to be present in the rough endoplasmic reticulum and requires, at

least under the assay conditions that have been used, relatively high concentrations of Co^{2+} or Mn^{2+} ions.

Experimental

Materials

Bovine pancreatic ribonuclease A (chromatographically pure, cat. no. 15410) and yeast ribonuclease (cat. no. 15332) were bought from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. UDP-*N*-Acetyl-D-glucosamine (grade 1; Na^+ salt; cat. no. U-4375) and UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine ($>200\text{mCi/mmol}$; NH_4^+ salt) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and The Radiochemical Centre, Amersham, Bucks., U.K., respectively. Each of these materials was chromatographed on Whatman no. 1 (descending) in ethanol-1M-ammonium acetate-acetic acid (50:20:33, by vol.). They ran with identical R_F values (0.28) and there was no sign of any other radioactive component in the ^{14}C -labelled preparation. The unlabelled substance was detected under a u.v. lamp.

Rabbit liver was frozen solid immediately after removal from the animal (Honee Bun Farm Products Ltd., Little Venn, Wollsey, Bideford, Devon, U.K., or Buxted Rabbit Co., Great Totiase Farm, Buxted, Sussex, U.K.).

CM-cellulose (Whatman CM 70; 0.6 mequiv./g; fibrous), Sephadex G-25 (fine grade), sulphopropyl-Sephadex C-25 (2.3 ± 0.3 mequiv./g; particle size 40–120 μm) and Amberlite CG-120 (200–400 mesh; BDH Chemicals Ltd., Poole, Dorset, U.K.) were used in the chromatographic procedures.

Crystalline trypsin (Boehringer Corp., cat. no. 15330) was purified before use, to remove extraneous

proteinases (Desnuelle *et al.*, 1962). Trypsin (17 mg) was dissolved in 1.5 ml of sodium citrate buffer (0.05 M in citrate) containing 0.013 M-CaCl₂, pH 6.0. The solution was placed at 4°C on a column (9.0 cm × 0.9 cm) of CM-cellulose, which had been pre-equilibrated with the same buffer. The column was eluted with 14 ml of this buffer, followed by 30 ml of 0.1 M-sodium citrate buffer, pH 6.0, containing 0.013 M-CaCl₂. Flow rate was 5 ml/h and the eluate was collected in 2 ml volumes. Two u.v.-absorbing peaks (280 nm) appeared. The second one (fractions 13–25) contained trypsin. The solution was desalted by eluting in 0.2 M-acetic acid from Sephadex G-25 in the cold room, and the eluate was freeze-dried. Approximately one-third of the crystalline trypsin, as purchased, consisted of proteins other than trypsin. These extraneous proteins chromatographed in the position of the proteinases identified by Desnuelle *et al.* (1962).

Methods

Radioactivity counting. This was effected in 10 ml of scintillator [a mixture of 2 parts of toluene containing 0.4% of 2,5-diphenyloxazole and 0.1% of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 1 part of Triton X-100 (Tait, 1970)]. A Nuclear-Chicago scintillation counter was used (efficiency 85%) and corrections were applied where necessary for quenching by protein.

Preparation of liver extract. Frozen rabbit liver (2 g) was cut up finely and homogenized in the cold in a Potter-Elvehjem homogenizer with 4–5 ml of buffer (0.05 M-Tris-HCl, pH 7.4 at 20°C).

Incubation conditions. These differed from one experiment to another, and pertinent data are discussed (Table 1) in relation to the various types of experiment in the Results and Discussion section.

Preparation of rough endoplasmic reticulum. The procedure of Dallner (1963) as described by Lawford & Schachter (1966) was used. Amounts of about 10 g of rabbit liver were used.

Glucosamine. This was determined after acid hydrolysis (4 M-HCl; 3–6 h; 100°C) by an Elson-Morgan reaction (Kraan & Muir, 1957) or by chromatography on the amino acid analyser (Locarte Mini Analyser). Two programmes were used for the analyser: programme I was used to separate most of the amino acids, pH 3.25 (0.2 M-sodium citrate), 55 min; pH 4.25 (0.2 M-sodium citrate), 110 min; pH 6.65 (1.0 M-sodium citrate), 140 min. Programme II allowed the separation of leucine and glucosamine, pH 4.25 (0.2 M-sodium citrate), 100 min; pH 6.65 (1.0 M-sodium citrate), 140 min. Leucine and glucosamine are eluted by and separated in the first of these buffers.

Chromatography of ribonuclease. The size of CM-cellulose column used for chromatography was re-

lated to the amount of ribonuclease present in the incubation mixture. A column 9 cm × 0.9 cm was used for up to 20 mg of ribonuclease, but one of 18 cm × 0.9 cm was used for 41 mg of ribonuclease. The samples, either ribonuclease solution or an incubation mixture after centrifugation (2000 g; 10 min; 4°C), was applied to the column previously equilibrated with 0.01 M-Tris-HCl, pH 7.0 at 4°C. Elution was effected with a linear gradient of NaCl in the Tris buffer. In those experiments where crude liver homogenate was used, the gradient was prepared from 2 × 200 ml volumes, one containing buffer only and the other 0.1 M-NaCl in the starting buffer. The gradient was prepared from 2 × 100 ml volumes when rough endoplasmic reticulum was used as the enzyme.

The position of ribonuclease in the eluate was determined by the method of Kalnitsky *et al.* (1959).

Isolation of glycopeptides from glycosylated ribonuclease. Glycosylated ribonuclease (1–18 mg) was cyanoethylated (Plummer & Hirs, 1964). A solution of cyanoethylated, glycosylated ribonuclease (1%, w/v) was digested in the presence of purified trypsin (0.02%) at 25°C for 4 h in water adjusted to pH 8.0. Adjustment to pH 8.0 was made at frequent intervals with 0.02 M-NaOH. The mixture was then left for 12 h at 4°C, when the pH was about 4.5–5.0. The pH was adjusted to 3.3 with acetic acid and the mixture was centrifuged (10000 g; 15 min; 4°C). The pellet was taken up in 0.2 M-acetic acid and the mixture was re-centrifuged under the same conditions. The supernatant was added to the previous (pH 3.3) supernatant and chromatographed. The precise conditions are described in the Results and Discussion section. The precipitate contained very little radioactivity.

Results and Discussion

Incorporation, catalysed by liver homogenates, of N-acetyl-D-glucosamine into a fraction precipitable by phosphotungstic acid

Ribonuclease A was incubated with homogenates of rabbit liver under the conditions described in Table 1 (column 2), and the radioactivity incorporated into material precipitable by 1% (w/v) phosphotungstic acid was determined. There was incorporation of label into endogenous protein, but the amounts of label incorporated in those experiments in which ribonuclease A was used were greater (Fig. 1).

Incorporation of N-acetyl-D-glucosamine into ribonuclease A

The ribonuclease A used was shown to contain little or no glucosamine: the sugar could not be detected when Elson-Morgan assays (Kraan & Muir, 1957) were carried out on hydrolysates (4 M-HCl; 3 h;

Table 1. Conditions used in studies on the extent of incorporation of *N*-acetyl-D-glucosamine into bovine pancreatic ribonuclease A

The concentrations shown are those present in the actual incubation mixture. UDP-GlcNAc, UDP-*N*-acetyl-D-glucosamine.

Assay conditions	2	3	4	5	6	7
Tris-HCl buffer (mM)	143	200	200	200	200	200
MnCl ₂ (mM)	10	59	20	26	†	58
UDP-GlcNAc (mM)	0.042	2.17	3.83	2.81	0.025	0
(c.p.m./nmol)	2240	0	77.3	69.6	11.6	—
Ribonuclease (μM)	6.8	555	992	629	35.7	1410
(mg)	0.1	7.77	41.7	20	0.2	16.8
Triton X-100 (%)	0	0	0.3	0.3	†	0.1
Total vol. (ml)	1.05	0.85	3.0	2.27	0.4	0.85
Liver homogenate (mg)	20–200	100	—	—	25	50
Rough endoplasmic reticulum*	—	—	300	300	—	—
Temperature (°C)	30	37	37	37	37	37
Incubation time (h)	0.5	6	6	6	6	6

* The amount stated is the equivalent amount of liver in mg from which the preparation was derived.
† See the text.

Fig. 1. Relationship between the amount of *N*-acetylglucosamine transferred from UDP-*N*-acetylglucosamine to bovine pancreatic ribonuclease A in the presence of increasing amounts of rabbit liver homogenate

Conditions of incubation are described in column 2, Table 1. Experiments were carried out in the presence (●) and the absence (○) of added pancreatic ribonuclease. The difference curve is represented by ■.

Fig. 2. Chromatography on a CM-cellulose column (9.0 cm × 0.9 cm) of an incubation mixture of ribonuclease A, UDP-*N*-acetyl-D-glucosamine, MnCl₂ and liver homogenate as described in column 3, Table 2 (●, E₂₂₀)

The chromatographic conditions are given in the text. In one control experiment, no added ribonuclease at all was used (■, E₂₂₀) and in another 2.6 mg of ribonuclease A was added after incubation (○, E₂₂₀). The salt gradient (—) is given on the right-hand ordinate. For details of the glucosamine analysis of fraction D see the text.

100°C; *in vacuo*) of ribonuclease (6 mg). It chromatographed on CM-cellulose as a single peak and was eluted at about 0.07 M-NaCl when a linear gradient of NaCl in 0.01 M-Tris-HCl buffer, pH 7.0, was used. This finding confirms that reported by Taborsky (1959) for the chromatography of ribonuclease A. The forms of ribonuclease that contain several sugar residues, such as the B form, are eluted from the column at lower concentrations of NaCl.

Ribonuclease A was incubated with unlabelled UDP-*N*-acetyl-D-glucosamine (for conditions see Table 1, column 3). The supernatant was chromatographed on a CM-cellulose column, under the conditions described in the legend to Fig. 2, after centrifugation of the mixture for 5 min at 2000g at 4°C. The pancreatic ribonuclease was contained in the last

peak of the protein profile. This was concluded from the following observations. First, the peak of this absorption occurred at a region of the chromatogram where the NaCl concentration was 0.074 M. The peak did not occur in a chromatogram derived from a control experiment in which ribonuclease A had been omitted from the incubation mixture (Fig. 2). In another control experiment a mixture containing all the components listed (Table 2, column 3), but without the addition of pancreatic ribonuclease A, was incubated. To one-half of this mixture was then added 2.6 mg of ribonuclease A and, after centrifugation, chromatography was effected on CM-cellulose. The ribonuclease fraction gave rise to absorption at the NaCl concentration expected (Fig. 2).

Ribonuclease which had been incubated with the liver homogenate now contained glucosamine. Pooled fractions 272–290 (fraction D, Fig. 2) were combined, dialysed exhaustively against water and evaporated in a rotary evaporator to dryness. The residue was taken up in water and by a modification of the Elson–Morgan reaction was shown to contain 0.14 mol of glucosamine/mol of the ribonuclease contained in the later fractions of the peak (Fig. 2). If it is assumed that the glucosaminylated ribonuclease co-chromatographs identically with ribonuclease A, this is equivalent to 0.23 mol of glucosamine/mol. However, as demonstrated (see below), the glucosaminylated ribonuclease tends to occur in the earlier part of the peak of ribonuclease activity, so that the value obtained is likely to be an underestimate. The control experiments were carried out by making an Elson–Morgan assay on equivalent numbers of fractions derived from the experiment in which ribonuclease

had been omitted from the incubation mixture, and also in that experiment where the enzyme was added to the mixture after incubation had occurred; in neither case was glucosamine present.

The results of these experiments demonstrate that *N*-acetyl-D-glucosamine is incorporated into ribonuclease A by transfer of the sugar moiety from UDP-*N*-acetyl-D-glucosamine, under the influence of an enzyme in rabbit liver. The glycosylated ribonuclease is not readily separable from ribonuclease A under the chromatographic conditions used, which readily separated bovine pancreatic ribonucleases A and B (Taborsky, 1959).

Presence of enzyme in the rough endoplasmic reticulum

The enzyme that transfers *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine to ribonuclease A is present in the rough endoplasmic reticulum of rabbit liver, as has been predicted (Redman & Cherian, 1972). Ribonuclease A (41.7 mg) was incubated with a portion of the rough endoplasmic reticulum in the presence of UDP-*N*-acetyl-D-[U-¹⁴C]-glucosamine (for conditions see Table 1, column 4). Chromatography on CM-cellulose (Fig. 3a) led to the separation of ribonuclease. Clearly the radioactivity associated with *N*-acetyl-D-glucosamine is eluted with the earlier part of the peak of ribonuclease. The total incorporation of radioactivity was equivalent to 0.08 mol of glucosamine/mol of ribonuclease A, assuming that there is a quantitative recovery of the latter enzyme from the CM-cellulose column. The extent of conversion of ribonuclease A into a glycosylated form is less in this experiment than in that in

Fig. 3. Partial separation of the ¹⁴C-labelled monoglycosylated ribonuclease from ribonuclease A by chromatography on CM-cellulose

For conditions of chromatography see the text. (a) CM-cellulose chromatography of the mixture gave a largely non-glycosylated fraction (E) which was a potential acceptor for *N*-acetylglucosamine. (b) Fraction E was reincubated with UDP-*N*-acetyl-D-[¹⁴C]glucosamine which led to the production of further ¹⁴C-labelled monoglycosylated ribonuclease, which was separated on CM-cellulose by using a shallower NaCl gradient than in (a). ●, E_{280} ; ○, radioactivity; —, NaCl gradient.

which the whole liver homogenate was used, in spite of the fact that Triton X-100 was used in the experiments involving rough endoplasmic reticulum. This detergent, as will be shown below, leads to greater activity of the transferring enzyme.

The ribonuclease A present in the latter part of the peak (Fig. 3a) could be converted in part into a glycosylated form. Combined fractions 66–82 (fraction E) were dialysed and evaporated on the rotary evaporator. This combined fraction was again glycosylated with radioactively labelled UDP-*N*-acetyl-D-[14 C]-glucosamine (Table 1, column 5) and the mixture chromatographed with a slightly shallower gradient, in the hope that there would be separation of the glycosylated and non-glycosylated enzymes. The results indicated that a further 0.05 mol of *N*-acetyl-D-glucosamine had been incorporated into 1 mol of ribonuclease. Again the peak of radioactivity was displaced towards the early part of the ribonuclease peak (Fig. 3b).

Thus the monoglycosylated form of ribonuclease, which we propose to call ribonuclease-GlcNAc and which is produced under the conditions described, is slightly less tightly bound to CM-cellulose than is ribonuclease A, but the chromatographic conditions used do not lead to a complete separation of the two forms of enzyme. Since this work was completed it has been reported that chromatography on Amberlite IRC-50 appears to be more effective (Tarentino *et al.*, 1974). Further proof that the radioactive *N*-acetylglucosamine was covalently linked to ribonuclease was provided by carrying out electrophoresis on those fractions apparently containing radioactive ribonuclease (Fig. 3a) in polyacrylamide gels containing sodium dodecyl sulphate (Marshall & Zamecnik, 1969). The gels were stained for protein and the ribonuclease band was seen. The stained gels were cut into thin slices, and after drying were triturated in scintillator and counted for radioactivity. Label appeared only in the region of the ribonuclease.

The finding of enzymic activity in the rough endoplasmic reticulum is not wholly unexpected. Other workers showed that puromycin will release 14 C-labelled glycoprotein from liver polyribosomes of rats that had previously been injected with D-[14 C]glucosamine (Lawford & Schachter, 1966; Molnar & Sy, 1967; Robinson, 1969). These earlier results suggested that at least some of the glucosamine present in serum glycoproteins is incorporated into the protein at the rough endoplasmic reticulum.

Effects of metal ions on the extent of glycosylation of ribonuclease A

Incubation mixtures were set up in the absence of Triton X-100 containing the components described in column 6 (Table 1), but with various concentrations of the following metal ions as chlorides: Co^{2+} , Mn^{2+} ,

Fig. 4. *Effect of metal ions on the incorporation of N-acetyl-D-[14 C]glucosamine into ribonuclease A*

(i) Co^{2+} : $\circ$, pancreatic ribonuclease added; $\bullet$, no ribonuclease added. (ii) Mn^{2+} : $\square$, ribonuclease added; $\blacksquare$, no ribonuclease added. (iii) Ni^{2+} : $\triangle$, ribonuclease added; $\blacktriangle$, no ribonuclease added.

Ni^{2+} or Mg^{2+} . Some mixtures were prepared in the absence of ribonuclease A. After incubation as described (Table 1), the radioactivity present in the material precipitable by 1% phosphotungstic acid in 0.5M-HCl was measured. The results (Fig. 4) show that Co^{2+} and Mn^{2+} ions are effective in facilitating the incorporation of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A as well as into endogenous acceptors. Ni^{2+} ions are less effective and Mg^{2+} was shown to have no effect at all.

Effects of Triton X-100 on the extent of glycosylation of ribonuclease A

In these experiments a constant concentration of 50mM- Mn^{2+} was used, but the other conditions were identical with those shown in column 6, Table 1. The concentration of Triton X-100 was varied from one experiment to another. The amounts of *N*-acetylglucosamine incorporated into phosphotungstic acid-precipitable material increased from that found in absence of Triton X-100 by a factor of 1.70 in the presence of 0.04% detergent, by a factor of 1.68 (0.08%), 1.56 (0.1%), 1.67 (0.2%), 1.56 (0.3%), 1.52 (0.5%) and 1.40 (1.0%). In control experiments where the incubation medium lacked ribonuclease, the radioactivity incorporated was largely unaltered regardless of the concentration of Triton X-100.

These experiments demonstrate that Triton X-100 leads to an increase in the amount of *N*-acetylglucosamine incorporated into ribonuclease A under the catalytic influence of the liver enzyme.

Characterization of the position at which glycosylation of ribonuclease A occurs

Ribonuclease which had been partially glycosylated with *N*-acetyl-D-[14 C]glucosamine under the activity of rough endoplasmic reticulum was *S*-cyanoethylated and the product digested with trypsin. The radioactive glycopeptides were isolated and their origins in ribonuclease were determined.

[14 C]Glycosylated ribonuclease (fractions 68–102; Fig. 3b) was dialysed against water and the contents of the dialysis sac were evaporated to dryness on a rotary evaporator. A portion of the residue (7 mg) was *S*-cyanoethylated and digested with trypsin (see under 'Methods' for details). A portion of the digest was subjected to high-voltage electrophoresis (Atfield & Morris, 1961) on paper [Whatman 3MM; 120V/cm; 40 min; in acetic acid–formic acid–water buffer (78 g of acetic acid + 25 g of formic acid made up to 1 litre with water), pH 1.8] and two radioactive regions were found by monitoring with a Panax gas-flow strip-scanner. They had moved distances of 14.1 and 20.2 cm respectively towards the cathode. The faster-moving peak had about 55% of the total radioactivity.

The remainder of the tryptic digest was subjected to gel filtration in 0.2M-acetic acid on a column (150 cm $\times$ 0.9 cm) of Sephadex G-25, when a partial separation into two radioactive peaks occurred. The radioactive fractions were combined, freeze-dried and the residue was re-chromatographed on a column (200 cm $\times$ 0.6 cm) of Sephadex G-25. There was a clear separation into two radioactive glycopeptides, which overlap with other non-radioactive peptides (Fig. 5). Combined fractions 72–84 were chromatographed on the cation-exchange resin Amberlite CG-120 (Fig. 6),

Fig. 5. *Rechromatography in 0.2M-acetic acid on Sephadex G-25 (200 cm $\times$ 0.6 cm) of part of the effluent from Sephadex G-25 of a tryptic hydrolysate of monoglycosylated ribonuclease A*

For details see the text. ○, E_{226} ; ●, radioactive glycosylated residues.

when a separation into two radioactive peaks occurred. The one eluted first (peak I) had the composition described in Table 2. The amino acid composition indicates that the material could have been derived only from residues 34–37 in the original ribonuclease A, which has the sequence $\cdots$ Met-Met-Lys-Ser-Arg-Asn-Leu-Thr-Lys-Asp-Arg-Cys-Lys $\cdots$ in positions 29–41 (Smyth *et al.*, 1963). The asparagine residue is partially substituted by *N*-acetyl-D-glucosamine as is deduced by the low amounts of glucosamine (Table 2); and it seems likely that peak (I) consists of a mixture of the peptide Asn-Leu-Thr-Lys and the *N*-acetyl- β -D-glucosaminylated form of this peptide. From other similar experiments in which the peaks from the Sephadex G-25 column (Fig. 5) have been chromatographed separately on an Amberlite CG-120 column, it was demonstrated that fraction (I) corresponds to the second of the radioactive fractions on the Sephadex G-25 column.

The second radioactive peak (Fig. 6) from the Amberlite CG-120 column (fraction II) corresponds to the first radioactive peak on the Sephadex G-25 column (Fig. 5). The value of V_e/V_0 for this fraction was 3.0 and for angiotensin II (octapeptide) was 2.93, whereas the value for fraction (I) was 3.40. Fraction (II) therefore appears to be of higher molecular weight than fraction (I) and might be expected to be of approximately the same size as an octapeptide.

Amino acid analysis of peak (II) from the Amberlite CG-120 column (Fig. 6) revealed the presence of Asp (1.0), Thr (0.83), Ser (1.2), Glu (1.0), Gly (1.3), Ala (0.9), Leu (0.36), Lys (1.15), His (0.76), Ile (0.19), and small amounts of arginine and glucosamine. These values seemed surprising if peak (II) were indeed homogeneous with regard to amino acid sequence, and carried radioactive glucosamine. The amino acid that chromatographed in the position of glutamic acid may have been, at least in part, *S*-carboxyethylcysteine (Plummer & Hirs, 1964). Sometimes peak (II) appeared in a form which indicated that there were overlapping substances present. Further purification of peak (II) material on a sulphopropyl-Sephadex C-25 column (Fig. 7) revealed the presence of three ninhydrin-positive peaks (II-1, II-2 and II-3), the second of which was also radioactive. The amino acid analysis for the material of peak (II-2) is described in Table 2. That for peak (II-3) was Lys (1.9), Glu (1.0), Thr (1.1) and Ala (2.7). This peptide was almost certainly initially present in the ribonuclease at positions 1–7 (Lys-Thr-Glu-Ala-Ala-Lys $\cdots$).

Fraction (II-2) (Table 2) is probably a mixture of the glycosylated peptide from amino acid residues 32–37 together with non-glycosylated material. But the reasons for the low value obtained for arginine were not wholly clear, and some control experiments were performed. In one of these, ribonuclease A was treated with liver under conditions described in

Fig. 6. Chromatography on Amberlite CG-120 of combined fractions 72-84 from the Sephadex G-25 column (Fig. 5)

The fractions were combined and freeze-dried. The residue was dissolved in 1.0 ml of pyridine-formate buffer (0.2 M pyridine, pH 3.26) and applied to the column (7 cm $\times$ 0.9 cm), which had been equilibrated with this buffer at room temperature (24°C). Elution was effected first with 50 ml of the same buffer and then with a gradient made with two 75 ml vessels: in the first was the starting buffer, in the second was pyridine-formate buffer (0.4 M pyridine, pH 6.5). Fractions (2 ml) were collected and assayed with ninhydrin after alkaline hydrolysis (Plummer & Hirs, 1964; Hirs, 1967) (●, E_{570}). Radioactivity was also assessed (○). —, pH gradient. For details of fractions (I) and (II) see the text.

Table 2. Composition of the radioactively labelled glycopeptides obtained from ribonuclease that had been glycosylated with the use of rough endoplasmic reticulum

The results are expressed in residues relative to aspartic acid.

Component	Fraction (I) (Fig. 6)	Fraction (II-2) (Fig. 7)†
Serine	0	0.86
Arginine	0	0.37
Aspartic acid	1.0	1.0
Leucine	0.87	0.93
Threonine	0.88	0.87
Lysine	1.13	0.94
Glucosamine	0.12*	0.18‡

* Determined on acid hydrolysates with the use of the amino acid analyser (programme 2).

† Glycine (0.12) and alanine (0.06) were also present.

‡ Determined by an Elson-Morgan procedure (Kraan & Muir, 1957).

Fig. 7. Rechromatography of fraction (II) (Fig. 6) on a column (12 cm $\times$ 1.6 cm) of sulphopropyl-Sephadex C-25

Elution was effected with a pyridine-formate gradient (100 ml of pyridine-formate buffer, pH 3.75, 0.2 M in pyridine; 100 ml of pyridine-formate, pH 5.25, 0.4 M in pyridine). ○, E_{570} [ninhydrin assays on small fractions were performed after removal of the buffer followed by hydrolysis in NaOH (Hirs, 1967)]; —, pH.

column 7 (Table 1), i.e. in the absence of UDP-*N*-acetyl-D-glucosamine. The ribonuclease A was re-separated on CM-cellulose and digested with trypsin

Fig. 8. Chromatography on sulphopropyl-Sephadex C-25 (under the conditions described in the legend of Fig. 7) of a peptide from ribonuclease A corresponding in behaviour to the glycopeptide (II) obtained from monoglycosylated ribonuclease

○, E_{570} ; —, pH.

Table 3. Analysis of peptides arising from tryptic digests of ribonuclease A, and chromatographing in the same place as fraction (II-2), which was derived from glycosylated ribonuclease

In one experiment the ribonuclease A was first incubated with liver (column 7, Table 1) in the absence of UDP-*N*-acetyl-D-glucosamine and peptide (II'-2') is derived from this. Peptide (II'-2'') is derived from ribonuclease A that had not been incubated with liver.

Component	Fraction (II'-2') (Fig. 8)	Fraction (II'-2'')
Serine	1.2	1.1
Arginine	0.60	0.67
Aspartic acid	1.0	1.0
Leucine	1.1	0.98
Threonine	1.2	1.0
Lysine	1.0	1.3

under conditions identical with those used for digestion of glucosaminylated ribonuclease. The tryptic hydrolysate was chromatographed on Sephadex G-25 under conditions described in Fig. 5. The region corresponding to fraction (II) was further fractionated on Amberlite CG-120 followed by chromatography on sulphopropyl-Sephadex (Fig. 8) and the material in the peak corresponding in position to that of peak (II-2) is described as fraction (II'-2'). Analysis of peak (II'-2') gave rise to the results described in Table 3. A similar control experiment was carried out in which ribonuclease A, not previously treated with a liver homogenate, was digested with trypsin and a corre-

sponding peptide (fraction II'-2'') was analysed (Table 3). The results of these experiments show that there is incomplete recovery of arginine in this peptide, although the loss appears to be less than that observed in the experiments with glycosylated ribonuclease. The reasons for the losses have not been examined further apart from carrying out controls with a mixture of the free amino acids of the type occurring in the peptide. They were dissolved in the pyridine-formate buffer, with or without 8M-urea, the buffer was removed in the same way as in the main experiments, and acid hydrolysis was effected. Little or no loss of arginine occurred.

General conclusions

There is present in the rough endoplasmic reticulum of rabbit liver an enzyme that has the ability to catalyse the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to asparagine-34 of bovine pancreatic ribonuclease. As predicted, no other of the total of 10 asparagine residues in ribonuclease appears to undergo glycosylation. Greater activity is found in the presence of Triton X-100, which supports the contention that the enzyme is membrane bound. The enzyme requires Mn^{2+} or Co^{2+} at relatively high concentrations for full activity.

It was suggested that the UDP-*N*-acetylglucosaminyl transferase found in rat liver microsomal fractions might be the enzyme that transfers *N*-acetylglucosamine to unidentified nascent ribosomal peptides (Mookerjea, 1972). The relationship, if any, between that enzyme and the one described in our studies is obscure. Evidence was provided that pancreatic ribonuclease may stimulate incorporation of *N*-acetylglucosamine into endogenous proteins (Letts & Schachter, 1973) in the absence of Mn^{2+} . It is clear from our experiments that the presence of Mn^{2+} potentiates the enzyme involved in *N*-acetyl- β -D-glucosaminylation of ribonuclease A.

It was proposed that formation of a carbohydrate moiety at an appropriate asparagine receptor in a glycoprotein is mediated through a dolichol pyrophosphate oligosaccharide (Behrens *et al.*, 1973). The results of the present studies do not permit any clear-cut decision as to whether a lipid such as dolichol monophosphate *N*-acetylglucosamine (Leloir, 1971; Richards & Hemming, 1972) or dolichol pyrophosphate *N*-acetylglucosamine (Behrens *et al.*, 1973) is involved as an intermediate or whether there is direct transfer of the sugar from UDP-*N*-acetylglucosamine to ribonuclease A. This is an important aspect because the results of experiments by Tetas *et al.* (1970) and by Mookerjea *et al.* (1972) may also be interpreted as involving a lipid intermediate. Further, the possibility exists that the mechanism involved *in vitro* may differ from that *in vivo* (Marshall, 1974).

References

- Atfield, G. N. & Morris, C. J. O. R. (1961) *Biochem. J.* **81**, 606-614
- Behrens, N. H., Carminatti, H., Staneloni, R. J., Leloir, L. F. & Cantarella, A. I. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 3390-3394
- Catley, B. J., Moore, S. & Stein, W. H. (1969) *J. Biol. Chem.* **244**, 933-936
- Dallner, G. (1963) *Acta Pathol. Microbiol. Scand. Suppl.* **166**, 1-94
- Desnuelle, P., Rovey, M. & Marous, S. (1962) *Biochim. Biophys. Acta* **56**, 202-204
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 325-329
- Hunt, L. & Dayhoff, M. O. (1970) *Biochem. Biophys. Res. Commun.* **39**, 757-765
- Jackson, R. L. & Hirs, C. H. W. (1970) *J. Biol. Chem.* **245**, 624-636
- Kalnitsky, G., Hummel, J. P. & Dierks, C. (1959) *J. Biol. Chem.* **234**, 1512-1516
- Kohn, M. & Yamashina, I. (1973) *J. Biochem. (Tokyo)* **73**, 1089-1094
- Kraan, J. G. & Muir, H. (1957) *Biochem. J.* **66**, 55 P
- Lawford, G. R. & Schachter, H. (1966) *J. Biol. Chem.* **241**, 5408-5418
- Leloir, L. F. (1971) *Science* **172**, 1299-1303
- Letts, P. J. & Schachter, H. (1973) *Can. J. Biochem.* **51**, 101-105
- Marshall, R. D. (1967) *Abstr. Int. Congr. Biochem. 7th Colloquium XIV-4*
- Marshall, R. D. (1972) *Annu. Rev. Biochem.* **41**, 673-702
- Marshall, R. D. (1974) *Biochem. Soc. Symp.* **40**, 17-26
- Marshall, R. D. & Zamecnik, P. C. (1969) *Biochim. Biophys. Acta* **181**, 454-464
- Marshall, R. D. & Neuberger, A. (1970) *Advan. Carbohydr. Chem. Biochem.* **25**, 407-478
- Melchers, F. (1969) *Biochemistry* **8**, 938-947
- Molnar, J. & Sy, D. (1967) *Biochemistry* **6**, 1941-1947
- Mookerjee, S. S. (1972) *Can. J. Biochem.* **50**, 1082-1093
- Mookerjee, S. S., Cole, D. E. C., Chow, A. & Letts, P. (1972) *Can. J. Biochem.* **50**, 1094-1108
- Neuberger, A. & Marshall, R. D. (1968) *Fifth Symposium on Foods: Carbohydrates and their Roles* pp. 115-132, Avi Publishing Co., Westport, Conn.
- Plummer, T. H. & Hirs, C. H. W. (1964) *J. Biol. Chem.* **239**, 2530-2538
- Redman, C. M. & Cherian, M. G. (1972) *J. Cell Biol.* **52**, 231-245
- Richards, J. B. & Hemming, F. W. (1972) *Biochem. J.* **130**, 77-93
- Robinson, G. B. (1969) *Biochem. J.* **115**, 1077-1078
- Smyth, D. G., Stein, W. H. & Moore, S. (1963) *J. Biol. Chem.* **238**, 227-234
- Taborsky, G. (1959) *J. Biol. Chem.* **234**, 2652-2656
- Tait, G. H. (1970) *Biochem. J.* **118**, 819-830
- Tarentino, A. L., Plummer, T. H. & Maley, F. (1974) *J. Biol. Chem.* **249**, 818-824
- Tetas, M., Chao, H. & Molnar, J. (1970) *Arch. Biochem. Biophys.* **138**, 135-146