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UDP-*N*-ACETYL-D-GLUCOSAMINE–ASPARAGINE SEQUON *N*-ACETYL-β-D-GLUCOSAMINYL-TRANSFERASE-ACTIVITY IN HUMAN SERUM*†

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ABSTRACT

Serum contains a sugar transferase which is able to catalyse the glycosylation *in vitro* of the asparagine residue present in the sequence Asn.Leu.Thr in bovine pancreatic ribonuclease. UDP-2-Acetamido-2-deoxy-D-glucose (UDP-*N*-acetyl-D-glucosamine) acts as the donor, although the mechanism of the transfer is unexplored. Spermidine and Mn^{2+}, as well as CDP-choline, can act as activators for the reaction. Monoglycosylated ribonuclease (ribonuclease-GlcNAc) has been separated (23% yield) from unreacted ribonuclease A by affinity chromatography on a column of wheat-germ agglutinin bound to Sepharose, and characterised. A possible reason for the presence of the enzyme in serum is suggested.

INTRODUCTION

A number of proteins which are produced by the known processes of transcription and translation subsequently undergo an intracellular glycosylation with the formation of one or more linkages along the polypeptide chain of the type 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn)[1]. For those glycoproteins formed in liver, the mechanism of formation of linkages of this nature is known to involve glycosylation of an asparaginyl, rather than an aspartyl, residue[2,3]. This mechanism is likely to be general throughout nature. A necessary, but not sufficient, condition for glycosylation of an asparaginyl residue appears to be its occurrence in an amino acid sequence of the form ...Asn.X.Thr... or ...Asn.X.Ser..., where X may be one of a large number of types of amino acid residue[4,5]. It has been suggested that this type of sequence might be described as an asparagine sequon for glycosylation[6]. The frequency with which a threonine residue occurred adjacent to or within one residue of a glycosylated asparagine residue was commented upon earlier[7],

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although the threonine moieties to which attention was drawn occurred in a variety of positions on either the *N*- or *C*-terminal sides of the asparagine residue in question[6].

Preparations of rough endoplasmic reticulum from rabbit liver are able to catalyse the formation *in vitro* of the GlcNAc–Asn linkage at an appropriate asparagine sequon. The enzyme preparation was reported to have a requirement for relatively high concentrations of Mn^{2+}, Co^{2+}, or Ni^{2+} ions for maximum activity[3].

Attempts to examine the mechanism of action of the hepatic enzyme were frustrated by the rapid losses in activity which attended solubilisation. A more stable form of the enzyme is now reported to be circulating in human serum. In the experiments to be described, either whole serum or a fraction precipitated with ammonium sulphate was used as a source of this enzyme. The acceptor substrate used was bovine pancreatic ribonuclease A, which is a convenient substrate because it contains the requisite asparagine sequon for *N*-acetyl-*β*-D-glucosaminylation[4,5]. The enzyme preparation may be activated *in vitro* by naturally occurring polyamines, and spermidine is more effective than Mn^{2+} ions in this respect. Uridine diphosphate inhibits the enzyme, as does Triton X-100 at high concentrations.

EXPERIMENTAL

Materials. — Bovine pancreatic ribonuclease A (chromatographically pure, Cat. No. 15410) and yeast ribonucleic acid (Cat. No. 15332) were bought from Boehringer Corp. (London) Ltd. UDP-*N*-Acetyl-D-glucosamine (grade 1; Na^+ salt; Cat. No. U-4375) and UPD-*N*-acetyl-D-glucosamine-*U*-^{14}C (>200 mCi/mmol; NH_4^+ salt) were obtained from Sigma (London) Chemical Co. Ltd., and The Radiochemical Centre (Amersham, Bucks., U.K.), respectively. They were chromatographically pure (Whatman No. 1 paper; ethanol–M ammonium acetate–acetic acid, 50:20:33).

CM-Cellulose (Whatman CM 23; 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) were used in the various chromatographic procedures.

Crystalline trypsin (Boehringer Corp., Cat. No. 15330) was purified before use, to remove extraneous proteinases[8]. Wheat-germ agglutinin bound to Sepharose was either given by Dr. M. Monsigny or bought from Miles Laboratories Ltd. (Stoke Poges, Bucks.).

Serum samples. — In experiments involving whole serum, fresh blood (A, Rh negative) was taken from a subject (ZK) immediately before use. The sample was allowed to clot and the serum removed by centrifugation. For experiments in which partially purified enzyme was used, outdated samples of blood (AB, Rh positive), which had been kept for 20 days at 4° in the presence of citrate–phosphate anticoagulant from the time of taking, were obtained from the Department of Haematology, St. Mary's Hospital.

Radioactivity counting. — This was effected in 10 ml of scintillator[9] (a mixture of

2 parts of toluene, containing 0.5% of 2,5-diphenyloxazole and 0.02% of 1,4-bis-(5-phenyloxazol-2-yl)benzene, and 1 part of Triton X-100). A Nuclear–Chicago scintillation counter was used (efficiency 85%) and corrections were applied where necessary for quenching by protein.

Fractionation of serum by ammonium sulphate. — Serum was dialysed against 2 × 40 volumes of Tris-phosphate buffer (0.04M in Tris, pH 8.6). Ammonium sulphate (5.7 g) was dissolved in 50 ml of the dialysed serum, and the preparation was kept in a cold room overnight. The precipitate (*A*) was collected by centrifugation (8,000 *g*, 10 min, 4°) and dissolved in 10 ml of 0.04M Tris-phosphate buffer (pH 8.6). The solution was dialysed against the same buffer (2 × 2 l) overnight at 4° and freeze-dried.

Further fractions (*B* to *G*) were obtained from the several supernatants by successive additions of 1.45, 1.50, 1.50, 1.55, 1.60, and 1.65 g of ammonium sulphate, respectively, with removal of each precipitate as described above. These weights of ammonium sulphate are those needed to give the degrees of saturation at 25° shown in Table I[10], wherein are also shown the recoveries of protein, assessed as total absorbance units at 280 nm.

Incubation conditions. — The various conditions used are described in Table II.

Enzyme assays. — The reactions in the incubated mixtures in some cases were stopped by the addition of 2 ml of a cold solution containing 1% of phosphotungstic acid and 5% of trichloracetic acid. The precipitates formed were collected on DAWP 02500 Millipore filters, carefully washed with the precipitating acid (10 × 5 ml), and air-dried. The incorporation of *N*-acetyl-D-glucosamine into the precipitates was determined by radioactive counting. Corrections for quenching by the added protein

TABLE I

RECOVERIES OF TOTAL PROTEIN (ABSORBANCES AT 280 nm) AND OF ENZYME ACTIVITIES (TRANSFER OF *N*-ACETYL-D-GLUCOSAMINE INTO RIBONUCLEASE A) IN FRACTIONS PRECIPITATED BY VARIOUS CONCENTRATIONS OF AMMONIUM SULPHATE FROM 50 ml OF DIALYSED HUMAN SERUM[a]

Fraction No.	*Degree of saturation[10] by ammonium sulphate* (%)	*Total[b] E_{280}*	*Enzyme activity*	
			Specific[c]	*Total[c] × 10^{-3}*
Serum	—	2600	72	187
A	20	386	135	52
B	25	276	237	65
C	30	478	419	200
D	35	285	460	131
E	40	257	361	93
F	45	147	129	19
G	50	201	99	20

[a]Details of these experiments are given in the text. [b]The ratio $E_{280}/_{260}$ was close to 1.41 for all fractions. [c]The units of specific activity are defined as pmoles of *N*-acetyl-D-glucosamine incorporated into ribonuclease A per h under the assay conditions described in Table II (column 1). The specific activity is expressed per unit of E_{280} also.

TABLE II

CONDITIONS OF INCUBATION USED IN THE ENZYMIC *N*-ACETYL-β-D-GLUCOSAMINYLATION OF BOVINE, PANCREATIC RIBONUCLEASE A. THE CONCENTRATIONS EXPRESSED ARE THOSE PRESENT IN THE INCUBATION MIXTURE. ALL INCUBATIONS WERE AT 37° FOR 1 HOUR, UNLESS INDICATED OTHERWISE

Assay conditions	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Tris-HCl buffer (mM)[a]	45	45	25	33	45	42	50
$MnCl_2$ (mM)	50	50	50	50	50	[h]	50
CDP-choline (mM)	0	0	3.40	2.26	0	0	0–4.7[l]
UDP-GlcNAc (mM)	0.086	0.75	4.0	2.6	12	0.24	0.57
(c.p.m./nmole)	258	310	50	40	75	5050	420
Ribonuclease Conc. (μM)	20.4	178	428	285	2850	159	143
Amount (mg)	0.1	0.25	3	3	20	0.2	0.2
Enzyme vol. (μl)	50[b]	25[d]	250[e]	250[e]	150[f]	25[k]	30[m]
Total volume (μl)	350	100	500	750	500	90	100
Ribonuclease glycosylated (%)	12[c]	5	23	14	9.5[g]		

[a]pH 7.2; concentrations refer to the Tris component. [b]The enzyme source used in this series of experiments was either pooled, outdated serum or one of the various ammonium sulphate-precipitated fractions (see Table I). The amount of total protein corresponded to 2.60 E_{280} units in the case of serum and to 0.75 unit for the other fractions. [c]See Fig. 1. The amounts of *N*-acetyl-D-glucosamine incorporated into the ribonuclease by serum and by fractions A–G, which had been freshly prepared, were 186, 101, 178, 314, 345, 271, 97, and 74 pmoles, respectively. [d]Containing 0.5 mg of a fraction precipitated from serum between 35–45% ammonium sulphate saturation. [e]Fresh, human serum (250 μl). [f]Containing 1.5 mg of a fraction precipitated between 30–40% saturation with ammonium sulphate. [g]This is the mean of seven results (9.9, 10.9, 9.2, 8.6, 9.1, 9.6, 9.0%). The same incorporation was found if incubation was continued for 2 h. The first of the preparations mentioned was analysed for glucosamine by a colorimetric procedure[11] (5 mg yielded 6.2 μg of glucosamine ≡0.097 residue of sugar/mol.). [h]Various concentrations of Mn^{2+}, spermine, or spermidine were used, as described in Fig. 9. The solutions of the polyamines were adjusted with dilute HCl solution to pH 7.2 before use. [k]Containing 100 μg of a fraction precipitated between 30–40% saturation with ammonium sulphate. [l]See the results described in Fig. 10. [m]Containing 300 μg of 30–40% ammonium sulphate-fractionated material.

were applied. In all cases, control experiments were performed in which ribonuclease A was not added to the incubation medium, because there was uptake of *N*-acetyl-D-glucosamine by endogenous glycoproteins in the enzyme preparations used and it was necessary to apply corrections for these reactions.

In those experiments in which it was desired to isolate and characterise glycosylated ribonuclease, the reaction was terminated by cooling the incubation mixture to 4° rather than by addition of acid.

Amino acid and hexosamine analyses. — The amino acid composition of acid hydrolysates of glycosylated ribonuclease was determined with a Locarte Analyser (The Locarte Co., Emperor's Gate, London, S.W.7). Acid hydrolysates (4M HCl; 100°; 4 h) were analysed by one of two procedures. The first was used to separate the amino acids; 2-amino-2-deoxyglucose (glucosamine) does not separate from leucine adequately. This programme was pH 3.25 (0.2M sodium citrate), 50 min; pH 4.25

(0.2M sodium citrate), 80 min; pH 6.65 (M sodium citrate), 140 min. The second programme, which allowed the separation of leucine and glucosamine, was pH 4.25 (0.2M sodium citrate), 100 min; pH 5.28 (0.35M sodium citrate), 140 min. Leucine and glucosamine are eluted by, and separated in, the first of these buffers.

A modification[11] of the Elson–Morgan procedure was also used to determine glucosamine in acid hydrolysates (4M HCl; 6 h; 100°) of various preparations of glycosylated ribonuclease.

Chromatography on CM-cellulose. — A sample, either a solution of ribonuclease A or an incubation mixture after centrifugation (2000 *g*; 10 min; 4°), was applied to a column (9 × 0.9 cm) of CM-cellulose previously equilibrated with 0.01M Tris-HCl (0.01M in Tris, pH 7.0) at 4°. Elution was effected with a linear gradient of NaCl in the Tris buffer[12]. The gradient was prepared from buffer (100 ml) and 0.1M NaCl in starting buffer (100 ml). The activities of the ribonuclease in the various fractions were assessed[13].

Chromatography on wheat-germ agglutinin–Sepharose. — A column (2 × 0.3 cm) was prepared at 4° in 0.01M Tris-HCl buffer (pH 7.0). Application of a mixture of ribonuclease A and [^{14}C]-glycosylated ribonuclease (~20 mg of total protein) was followed by elution with Tris-HCl buffer and then with a linear gradient of *N*-acetyl-D-glucosamine in the buffer (2 × 25-ml volumes, the first containing starting buffer, and the second 0.5mM *N*-acetyl-D-glucosamine in starting buffer). Volumes of 1 ml were collected. Protein in the effluent prior to application of the *N*-acetyl-D-glucosamine gradient was assessed from absorbance measurements at 220 nm, and the gradient was not applied until the value had fallen to zero (~20 fractions). The concentrations of *N*-acetyl-D-glucosamine in the various fractions were determined by a modification[14] of the Morgan–Elson reaction. The position of the [^{14}C]-glycosylated ribonuclease was found by counting measured portions of the various eluted fractions. Those fractions containing glycosylated ribonuclease were combined, and dialysed (18/32 Visking dialysis-tubing) exhaustively against many changes of 200 volumes of 0.05M Tris-HCl buffer (pH 7.2) until no free *N*-acetyl-D-glucosamine remained[14]. The residual solution of glycosylated ribonuclease, hereinafter referred to as ribonuclease-GlcNAc, was freeze-dried.

Isolation of glycopeptides from ribonuclease-GlcNAc. — Ribonuclease-GlcNAc (4.5 mg) was cyanoethylated[15] and the product was digested with purified trypsin. The general methods used for the isolation of the glycopeptides were described earlier[3].

Disc gel-electrophoresis of ribonuclease-GlcNAc. — This was carried out by the procedure of Marshall and Zamecnik[16] in 9.4% polyacrylamide gel. The running buffer contained 0.1% of sodium dodecyl sulphate, as the original[16] experiments. The need for detergent in this buffer should be obvious, although this was not made clear in the description of the original procedure[16]. The gel was stained with Coomassie Blue and cut into 2-mm sections, each of which was dissolved in 30% hydrogen peroxide solution (2 ml) in scintillation vials[17]. The solutions were evaporated to dryness and the residues were counted for radioactivity.

RESULTS

Portions of the whole serum and of the fractions *A–G* obtained by precipitation with ammonium sulphate were assayed for enzyme activity under the conditions of incubation shown in Table II (column 1). From the results obtained, the specific and total activities of the serum and of the fractions were calculated (Table I). There were 187 units of activity in the original 50 ml of dialysed serum, but there was a total recovery of 579 units (309%). It seems likely that an inhibitor present in whole serum has been removed upon fractionation with ammonium sulphate. The most active fractions (*C* and *D*), which are precipitated between 25–30% and 30–35% of ammonium sulphate, are about six times as active as the whole serum.

Incorporation of *N*-acetyl-D-glucosamine into ribonuclease is not directly proportional to the amount of partially purified enzyme-fractions under the conditions of assay used (Fig. 1). Amounts of ribonuclease up to 12% of that originally present in the reaction mixtures had undergone glycosylation in this series of experiments. The amounts glycosylated were calculated from the incorporation of radioactivity in general, and the values obtained agreed with those obtained by colorimetric estimation of glucosamine, as discussed later.

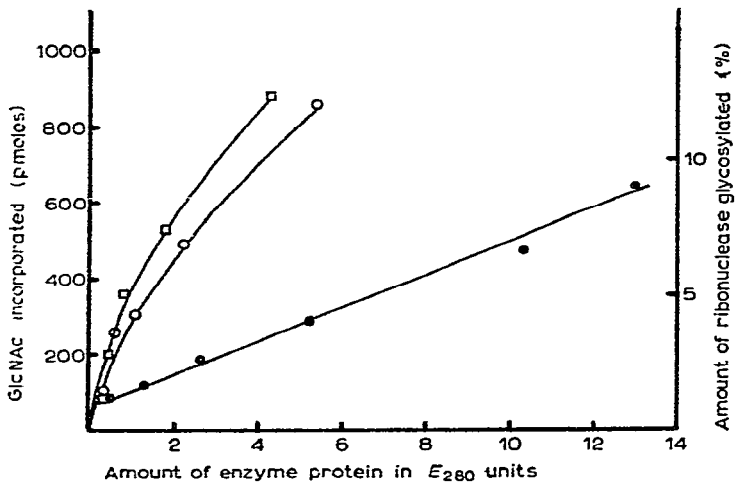

Fig. 1. Relationship between the amount of enzyme protein used and the extent of enzymic incorporation of *N*-acetyl-D-glucosamine into ribonuclease A. The conditions of the experiment are described in the text, and the incubation conditions in Table II (column 1): —●—●—, dialysed whole serum; —○—○—, fraction *C*, precipitated between 25–30% ammonium sulphate saturation; —△—△—, fraction *D*, precipitated between 30–35% ammonium sulphate saturation. Fractions *C* and *D* had been stored at −20° for several months.

In all the experiments so far described, incubation periods of 1 h were used, and it was desirable to study the extent of incorporation after different periods of time. For this experiment, the incubation conditions are described in Table II (column 2).

The results are shown in Fig. 2, which includes the differences in the extent of incorporation of *N*-acetyl-D-glucosamine into acid-precipitable protein both in the presence and absence of added ribonuclease A. The incorporation into ribonuclease A was found to be largely complete after ~2 h, with ~5% of the ribonuclease glycosylated. This value, which was obtained with 500 μg of an ammonium sulphate-fractionated enzyme preparation, is similar to that found when the same amount of partially purified enzyme was used in the earlier experiments (Fig. 1).

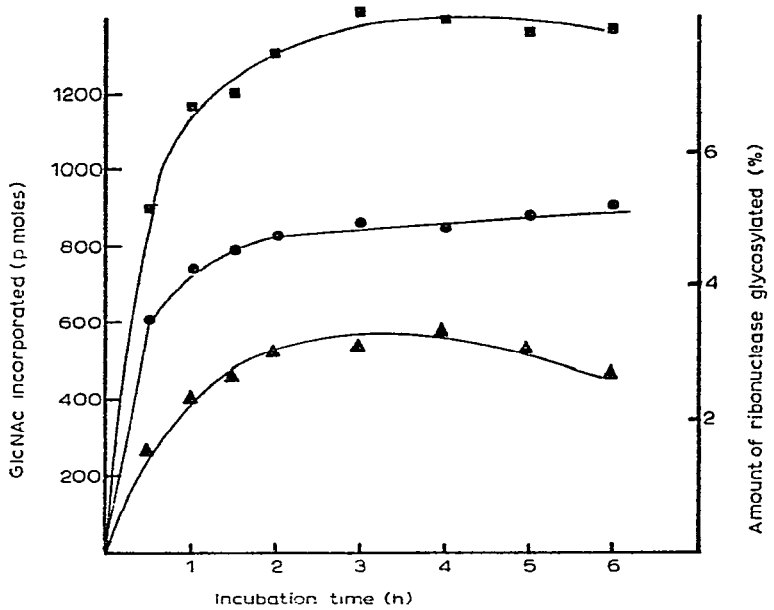

Fig. 2. Rate of incorporation of *N*-acetyl-D-glucosamine into ribonuclease A. Incorporation into protein precipitable by phosphotungstic acid/trichloracetic acid mixture (see Methods) when ribonuclease A was added to the incubation mixtures under the conditions of incubation described in Table II (column 2), —■—■—; incorporation when no ribonuclease A was added to the incubation mixtures (control), —▲—▲—. The difference curve, which represents incorporation into ribonuclease A, is shows as —●—●—.

It was also found, under these conditions of incubation, that UDP acted as an inhibitor. At concentrations of UDP of 0.02, 0.10, and 0.20mM, the glycosylation of ribonuclease A was inhibited to the extent of 2, 15, and 27%, respectively.

Triton X-100 at 0.1% and 0.2% concentrations had no effect. At a concentration of 1%, there was a 47% inhibition of enzyme activity.

It has been assumed in the aforementioned results that the differences obtained in incorporation of *N*-acetyl-D-glucosamine into acid-precipitable protein in the presence and absence of added ribonuclease A could be interpreted as an indication that the latter was in fact the acceptor of the sugar. This reasonable assumption was confirmed by isolation and identification of the glycosylated ribonuclease, and the point in the polypeptide chain at which glycosylation had occurred was determined.

Enzymic formation and isolation of glycosylated ribonuclease

In the first of this series of experiments, UDP-*N*-acetyl-D-glucosamine-*U*-^{14}C was allowed to react with bovine, pancreatic ribonuclease A in the presence of Mn^{2+} with fresh human serum, which was taken from a subject (ZK) immediately before the experiment. The conditions of incubation are described in Table II (column 3) and appropriate control experiments were performed (see below).

The incubation mixture, which originally contained ribonuclease A, was chromatographed on CM-cellulose[12]. Four radioactive peaks were found (Fig. 3*a*). The fourth of these (Fractions 47–51), which was later shown to be glycosylated ribonuclease, occurred in a position slightly ahead of, but overlapping, that of ribonuclease A, which is eluted at 0.07M NaCl. Control experiments yielded results which supported the conclusion that some of the ribonuclease A had undergone *N*-acetylglucosaminylation: Firstly, a mixture prepared as in the above experiment, apart from having no ribonuclease A, was incubated as before and then divided into two equal parts. One part was chromatographed directly on CM-cellulose, and the results showed (Fig. 3*b*) that there was no peak of radioactivity at the position occupied by glycosylated ribonuclease (seen as peak number 4 in the results of the previous experiment). Moreover, there was no protein peak, although there was absorption, at the point where the NaCl concentration of the gradient was 0.07M. The other control experiment was performed by adding 2.5 mg of ribonuclease A to the second half of the incubation mixture just described, followed by chromatography on a CM-cellulose column. The results (Fig. 3*c*) showed the presence of a protein eluting at 0.07M NaCl, which was due to the added ribonuclease A, but there was now no radioactivity in the vicinity of the ribonuclease peak.

The results of this series of experiments show that, under the appropriate conditions, incubation of ribonuclease A with human serum and UDP-GlcNAc-^{14}C leads to incorporation of radioactivity into ribonuclease A. There were 49.2 nmoles of *N*-acetylglucosamine incorporated per 3 mg of ribonuclease A, which is equivalent to 0.23 residue of sugar per mole of protein. Under slightly different conditions of incubation (column 4, Table II) in which the ribonuclease concentration was two-thirds of that in the experiment described above, there were 30.2 nmoles of *N*-acetyl-glucosamine incorporated per 3 mg of ribonuclease A (0.14 residue per mole of ribonuclease A).

Separation of glycosylated ribonuclease from ribonuclease A

The results described in Fig. 3 indicated the likelihood that the glycosylated ribonuclease and ribonuclease A have only slightly different chromatographic properties on CM-cellulose. A method of separation by affinity chromatography was therefore devised using immobilised wheat-germ agglutinin.

For this purpose, a mixture of the glycosylated ribonuclease and ribonuclease A was prepared by incubating ribonuclease A under the conditions described (column 5, Table II). In these experiments, an enzyme fraction obtained by ammonium sulphate fractionation of serum was used. Measurements of radioactivity of the product

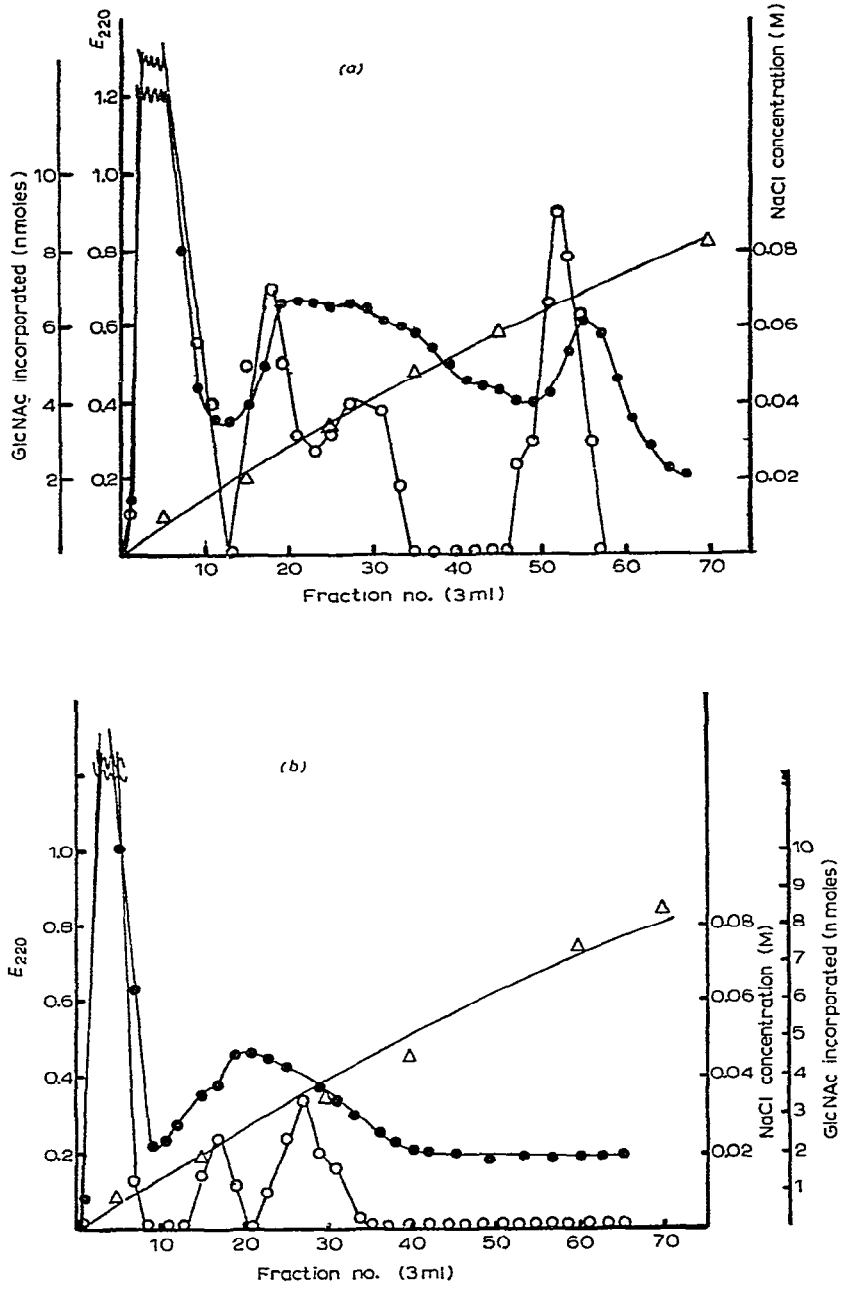

Fig. 3. Chromatography on a CM-cellulose[12] column (9 × 0.9 cm) of a mixture incubated as described in Table II (column 3). The chromatographic conditions are described in the text. E_{220} is represented as —●—●—; *N*-acetyl-D-glucosamine incorporated as —○—○—, and NaCl gradient as —△—△—. Results obtained (*a*) when ribonuclease A was added prior to incubation, (*b*) when ribonuclease A was omitted from the mixture, (c) when ribonuclease was added to the mixture before chromatography but after incubation.

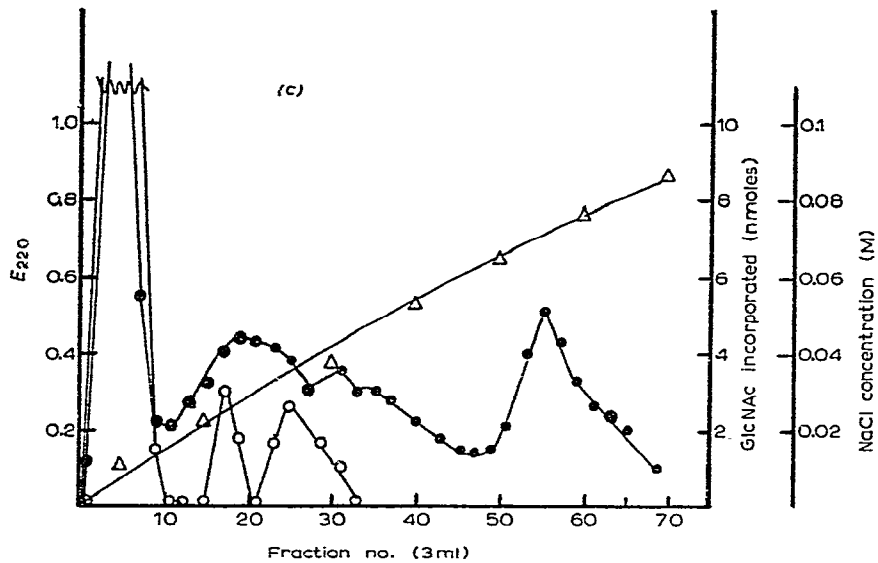

Fig. 3. (*Legend on p. 00 below*).

showed that ~10% of the ribonuclease became glycosylated. Specific hexosamine analysis (see footnote *g*, Table II) indicated that the preparation contained 9.7% of glycosylated ribonuclease. The latter result shows that the glucosamine-incorporation value deduced from radioactive measurements is a reliable estimate.

Application of partially glycosylated ribonuclease (~20 mg; 10,860 c.p.m. = 144 nmoles of GlcNAc) to a column (2.0 × 0.3 cm) of agarose–wheat-germ agglutinin was followed firstly by elution with 0.01M Tris-HCl buffer (pH 7.0) and then by a gradient of *N*-acetylglucosamine in the same buffer (Fig. 4). The substance emerging from the column at a concentration of the eluting sugar of 0.175mM contained 9100 c.p.m. (≡ 123 nmoles of GlcNAc; 85% recovery), and was shown to be ribonuclease-GlcNAc by the procedures discussed below. There was ~1.7 mg of ribonuclease-GlcNAc. Recovery of radioactivity in parallel experiments varied from 77% to 88%.

Some properties of ribonuclease-GlcNAc

Ribonuclease-GlcNAc was found to be eluted from CM-cellulose slightly more readily than is ribonuclease A. Ribonuclease-GlcNAc (1.5 mg) and ribonuclease A (1.0 mg) were separately chromatographed on a column (9 × 0.9 cm) of CM-cellulose in 0.01M Tris-HCl buffer (pH 7.0). Elution was effected with a linear gradient of NaCl (Fig. 5). The results of this experiment indicate clearly why chromatography on CM-cellulose was ineffective, in the earlier experiments, in separating ribonuclease-GlcNAc from ribonuclease A (see Fig. 3).

Ribonuclease-GlcNAc is also eluted more readily from Amberlite CG-50 than is ribonuclease A. Ribonuclease-GlcNAc (0.5 mg) and ribonuclease A (0.8 mg) were

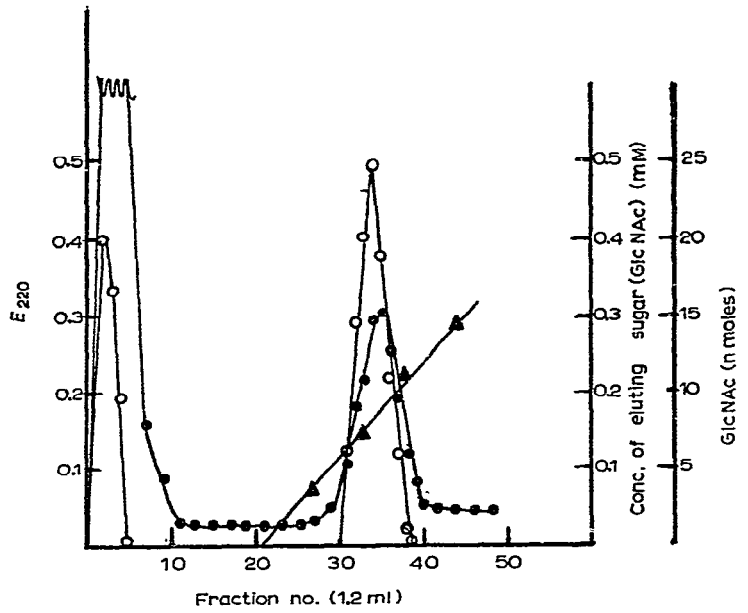

Fig. 4. Separation by chromatography on a column (20×0.3 cm) of wheat-germ agglutinin–Sepharose of ribonuclease A and ribonuclease GlcNAc: —●—●—, E_{220}; —○—○—, *N*-acetyl-D-glucosamine-^{14}C; —▲—▲—, *N*-acetyl-D-glucosamine assayed by a Morgan–Elson procedure[14].

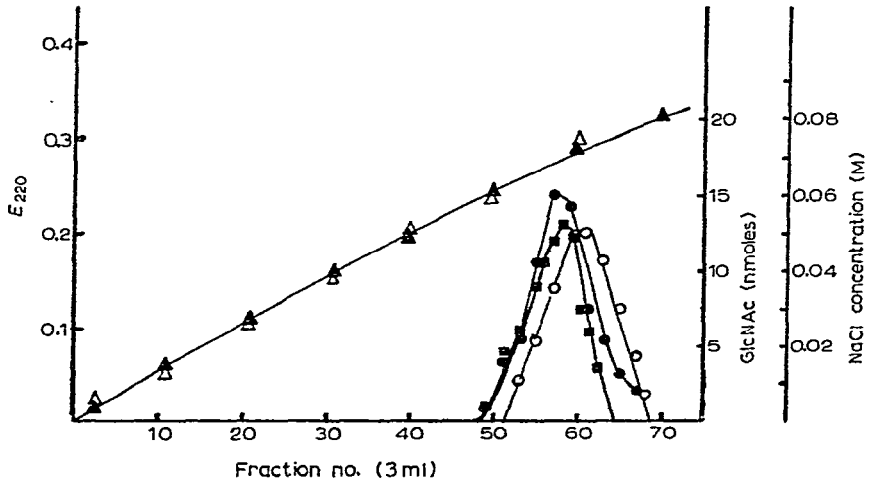

Fig. 5. Chromatography on a CM-cellulose column[12] (9×0.9 cm) of either 1.5 mg of ribonuclease-GlcNAc (—●—●—, E_{220}; —■—■—, amount of *N*-acetyl-D-glucosamine covalently bound to ribonuclease; —▲—▲—, NaCl gradient) or 1.0 mg of ribonuclease A (—○—○—, E_{220}; —△—△—, NaCl gradient). The conditions of chromatography are described in the text.

chromatographed in separate experiments (Fig. 6) on a column (45 × 0.9 cm) of Amberlite CG-50 in 0.2M sodium phosphate buffer (pH 6.02)[18]. The relative mobility of ribonuclease A to that of ribonuclease-GlcNAc is 1.22, close to the value found by others[18]. The ribonuclease-GlcNAc was able to catalyse the digestion of ribonucleic acid[13] (Fig. 6).

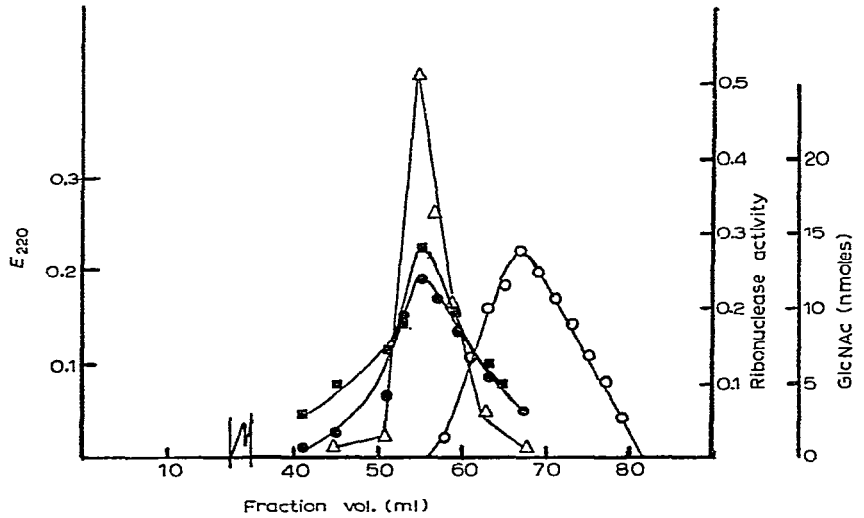

Fig. 6. Chromatography on an Amberlite CG-50 column[18] (45 × 0.9 cm) of either 0.5 mg of ribonuclease-GlcNAc (—●—●—, E_{220}; —■—■—, amount of *N*-acetyl-D-glucosamine covalently bound in ribonuclease-GlcNAc; —△—△—, enzyme activity of ribonuclease-GlcNAc), or of 0.8 mg of ribonuclease A (—○—○—, E_{220}). The chromatographic details are given in the text.

Ribonuclease-GlcNAc exhibits a mobility of 0.73 relative to Bromophenol Blue on electrophoresis in 9.4% polyacrylamide gel containing sodium dodecyl sulphate[16] (detection with Coomassie Blue or by measurement of radioactivity in the [^{14}C]-labelled glycosylated ribonuclease. This value is similar to that reported earlier[16] for ribonuclease A). The results of this experiment demonstrate also that the glucosamine incorporated into ribonuclease A cannot be released in a dissociating medium.

Amino acid and amino sugar analyses performed on the putative ribonuclease-GlcNAc yielded the information described in Table III. Ribonuclease-GlcNAc appears to differ from ribonuclease A only by the presence of approximately one residue of glucosamine. These results leave no doubt that the product is mono-glycosylated ribonuclease, which can also catalyse hydrolysis of ribonucleic acid (Fig. 6). By isolation of glycopeptides from tryptic digests of carboxyethyl-ribonuclease-GlcNAc, it was confirmed that the asparagine sequon at residues 34–37 of ribonuclease A was the one which had undergone substitution by *N*-acetyl-D-glucosamine.

TABLE III

AMINO ACID AND HEXOSAMINE CONTENT OF RIBONUCLEASE-GlcNAc

Component	*Residues per mole in*	
	RNAase-GlcNAc	*RNAase A*
Aspartic acid	15.6	15
Threonine	9.9	10
Serine	15.3	15
Glutamic acid	12.2	12
Proline	4.0	4
Glycine	3.0	3
Alanine	11.8	12
Cystine/2	6.7	8
Valine	8.6	9
Methionine	2.9	4
Isoleucine	2.7	3
Leucine	1.9	2
Tyrosine	5.2	6
Phenylalanine	2.9	3
Histidine	3.7	4
Lysine	10	10
Arginine	3.8	4
Glucosamine	0.78[a]	0[b]

[a]Determined by an Elson–Morgan procedure[11] after hydrolysis in 4M HCl at 100° for 4 h. The value is not corrected for the water and ash content of freeze-dried ribonuclease-GlcNAc. [b]No glucosamine could be detected in the preparation of ribonuclease A used, which had been purified by chromatography on CM-cellulose.

Identification of the glycosylated sequon in ribonuclease-GlcNAc

Ribonuclease-GlcNAc (4.5 mg ≡ 321 nmoles) was converted into its carboxyethyl derivative[15], which was digested with highly purified trypsin[8]. The digest was fractionated first on Sephadex G-25 (Fig. 7), when a single, but asymmetric, radioactive peak (Fractions 139–146) containing a total of 304 nmoles of glucosamine (95% recovery; based on radioactive measurements) emerged.

A portion of the combined fraction (≡ 200 nmoles of D-glucosamine-^{14}C) was chromatographed on Amberlite CG-120. The first of the radioactive fractions (Peak I, Fig. 8) contained 104 nmoles of D-glucosamine-^{14}C. Measured aliquots of this were hydrolysed (4M HCl; 4 h; 100°) and subjected to analysis on an amino acid analyser. The composition of this glycopeptide (Table IV) showed that it must have arisen from residues 34–37 of the original ribonuclease A, where the sequence is known to be ...Asn.Leu.Thr.Lys....

The second peak (II, Fig. 8) contained radioactivity equivalent to 39 nmoles of glucosamine, and it was purified further by chromatography on a column of sulphopropyl-Sephadex C-25 under conditions closely similar to those described earlier[3]. The radioactive peak (II^1; 26 nmoles based on D-glucosamine-^{14}C assays) was hydrolysed by acid in the usual way, and the hydrolysate contained only Ser, Arg, Asp, Thr, Leu, Lys in addition to glucosamine (Table IV). It is suggested that

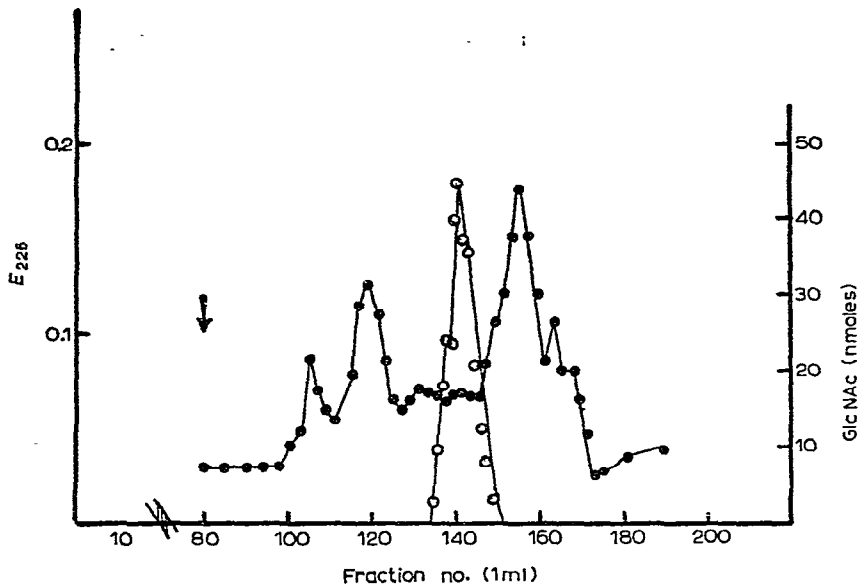

Fig. 7. Fractionation at room temperature of tryptic digest of carboxyethyl derivative of ribonuclease-GlcNAc-^{14}C (4.5 mg) on Sephadex G-25 medium [two connected, parallel columns (each 125 × 1.0 cm)]. The eluting buffer was 0.1M acetic acid: —●—●—, E_{226}; —○—○—, *N*-acetyl-D-glucosamine incorporated. The exclusion volume (V_0; blue dextran) is indicated by the arrow.

TABLE IV

AMINO ACID AND GLUCOSAMINE CONTENTS OF GLYCOPEPTIDES I AND II[1] ISOLATED FROM TRYPTIC DIGESTS OF CARBOXYETHYL-RIBONUCLEASE-GlcNAc-^{14}C

Substance	*Glycopeptide I*			*Glycopeptide II*[1]		
	Amount	*(nmoles)*	*Residues/mole*	*Amount*	*(nmoles)*	*Residues/mole*
Glycopeptide[a]	7.3	14.6	1.00	26		1.00
Serine	0	0		46		1.76
Arginine	0	0		26		0.50
Aspartic acid	7.0	14.3	0.97	24		0.92
Threonine	7.4	16.3	1.07	36		1.38
Leucine	6.7	16.4	1.02	25		1.04
Lysine	8.3	13.1	1.02	37		0.77
Glucosamine			1.01[b]			

[a]The amount of glycopeptide was calculated from the specific activity of UDP-GlcNAc-^{14}C used and the number of counts in the radioactive glycopeptide; it was assumed there was one residue of glucosamine per glycopeptide. This was used as the basis of comparison. [b]Assessed from a ratio of leucine to glucosamine in a separate experiment.

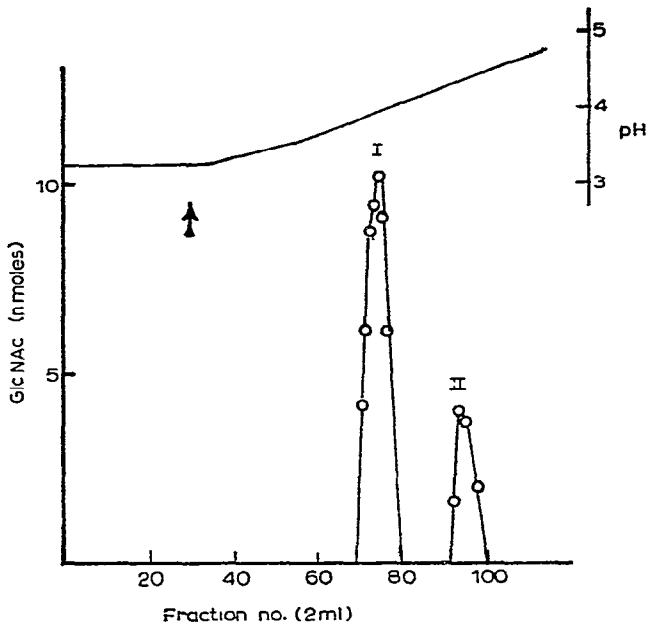

Fig. 8. Chromatography on an Amberlite CG-120 column (7×0.9 cm) of a measured aliquot (≡200 nmoles of D-glucosamine-^{14}C) of combined Fractions 139–146 from the Sephadex G-25 column (Fig. 7): —O—O—, *N*-acetyl-D-glucosamine incorporated. Elution was effected with a linear pH gradient (————) of pyridine–formate [50 ml of pyridine–formate buffer (0.2M pyridine, pH 3.25) followed by 2×100-ml interconnected vessels, one containing starting buffer, the second containing pyridine–formate (0.4M pyridine, pH 5.25)]. The arrow indicates the point at which the gradient was begun.

glycopeptide II[1] had arisen from residues 32–37 in the orignal ribonuclease A. Thus, at least 72% of the glucosamine incorporated into ribonuclease A to form ribonuclease-GlcNAc is almost certainly accounted for by substitution at asparagine residue 34 of the protein chain.

Replacement of Mn^{2+} ions by polyamines as an activator for the transfer reaction

The effects of Mn^{2+} ions, spermidine, and spermine on the extent of glycosylation of ribonuclease A were assessed. Incubation mixtures were prepared with concentrations of components as described in Table II (column 6). The final concentrations of neutralised spermine and spermidine ranged from 0–84mM. Each of the substances caused an increase in the extent of incorporation of glucosamine, both into endogeneous protein and into added ribonuclease A. Spermidine at concentrations of ~10–15mM was most effective in accelerating glycosylation of ribonuclease A, although Mn^{2+} ions at concentrations of ~50mM were ~70% as effective (Fig. 9).

Effect of CDP-choline on incorporation of glucosamine into ribonuclease

The effects of adding CDP-choline on the extent of glycosylation of ribonuclease

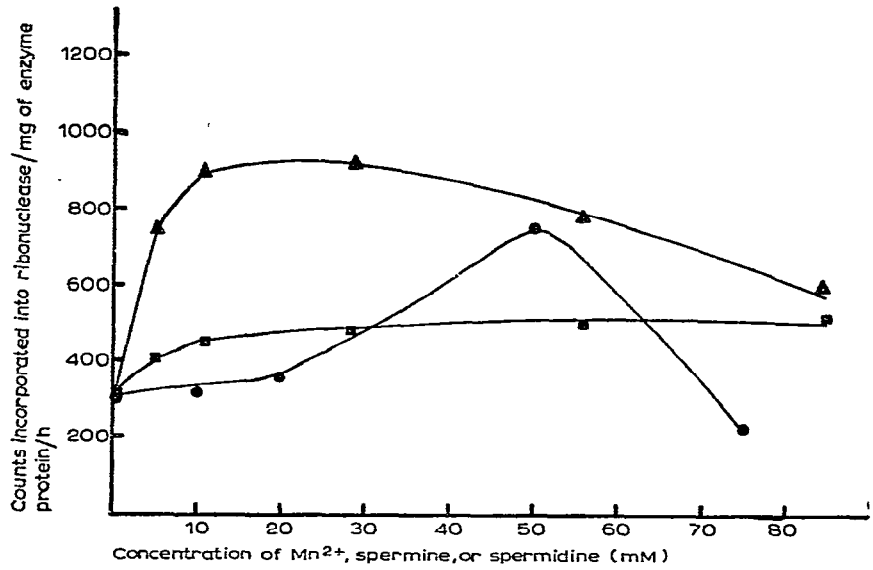

Fig. 9. Effects of Mn^{2+} (—●—●—), spermine (—■—■—), and spermidine (—▲—▲—) on the transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A. The conditions of incubation are described in Table II (column 6).

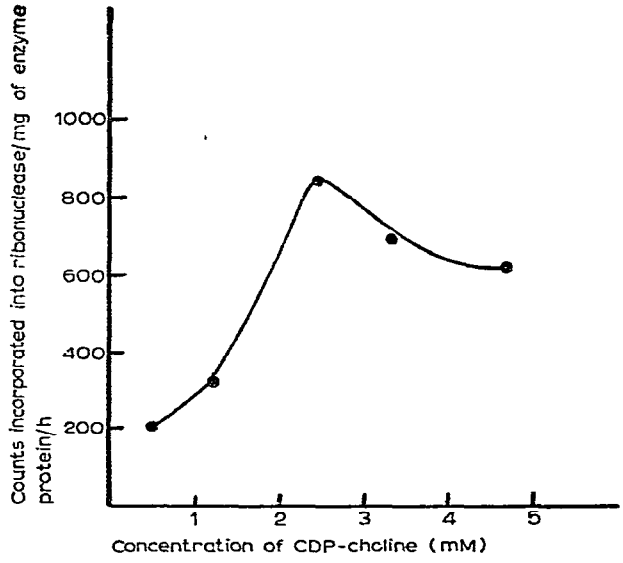

Fig. 10. Effect of CDP-choline on the incorporation of *N*-acetyl-D-glucosamine into ribonuclease A, with UDP-*N*-acetyl-D-glucosamine as the donor. The incubation conditions are described in Table II (column 7).

A were assessed. In this series of experiments, Mn^{2+} ions were also present in the medium at a concentration of 50mM and the general conditions of incubation used are described in Table II (column 7). The results (Fig. 10) show that the nucleotide enhances the ability of partially purified enzyme to catalyse the transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into added ribonuclease A. There is also enhanced incorporation into endogenous proteins or glycoproteins present in the enzyme preparation. The most effective concentration of CDP-choline is ~2.4mM.

DISCUSSION

An enzyme which is able to catalyse the glycosylation *in vitro* of asparagine residue 34 of bovine pancreatic ribonuclease, with UDP-*N*-acetyl-D-glucosamine as the sugar donor, is present in human serum. Monoglycosylated ribonuclease (ribonuclease-GlcNAc) is produced, and may be separated from unreacted ribonuclease A by affinity chromatography on wheat-germ agglutinin linked to Sepharose. Ribonuclease-GlcNAc is bound to the lectin–affinity column relatively weakly (see Fig. 4). This weak interaction may be due, in part, to the fact that ribonuclease-GlcNAc contains only a single *N*-acetyl-D-glucosamine residue, which is a relatively poor acceptor for the lectin compared with larger oligomers[19–23]. A second factor which may contribute to the relatively poor interaction is that the column chromatography was performed at a pH (7.0) where both ribonuclease-GlcNAc and wheat-germ agglutinin are probably positively charged overall. The isoelectric point of ribonuclease-GlcNAc is likely to be similar to that of ribonuclease A (pI = 7.8) and that of wheat-germ agglutinin is also likely to be quite high because the lectin does not bind to DEAE-cellulose at either pH 7 (Ref. 24) or pH 8.6 (Ref. 20).

The mechanism of transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine to the polypeptide chain is unknown. A lipid intermediate could be involved, but we have no evidence about this. The relationship of the results presently obtained to the possibility that addition of whole oligosaccharide chains to a polypeptide chain is the usual pathway for synthesis of at least some glycoproteins is unknown[25–27].

The transferase may be largely separated from albumin because of the ability of the former to precipitate from serum at 25–35% saturation with ammonium sulphate. Perhaps not surprisingly, because it is in soluble form, the enzyme is not stimulated by the detergent Triton X-100, which tends to inhibit its activity at higher concentrations. The enzymic activity of the preparation is increased in the presence of Mn^{2+}, but a more effective activator is spermidine. There is also increased activity in the presence of CDP-choline, but whether this has any significance with respect to the probable requirement of choline for glycoprotein formation by the whole animal[28–29] is presently unknown.

In some experiments, as much as 23% conversion of ribonuclease A into ribonuclease-GlcNAc occurred. The reaction was probably complete at that stage, and the most likely explanation why the reaction is not quantitative is that the

products are inhibitory. In particular, uridine diphosphate acts as an inhibitor for the enzyme.

The presence in serum of UDP-*N*-acetyl-D-glucosamine–asparagine sequon *N*-acetyl-β-D-glucosaminyl-transferase raises a number of important questions, one of which is the source of the enzyme. It is generally accepted that *N*-acetyl-β-D-glucosaminylation of the nascent apoprotein occurs, at least largely, whilst the latter is associated with the polysomes[30–34]; hence, the presence of the enzyme in rough endoplasmic reticulum[3] is perhaps expected.

There may be also an enzyme having similar specificity on the outer surface of the plasma membrane of a number of types of cells, where several other sorts of glycosyltransferases probably occur[35,36]. Its function could be recognition of proteins that contain an unsubstituted asparagine sequon by a mechanism similar to that suggested[37] whereby a cell surface might recognise a glycoprotein. Natural turnover of the plasma membranes might lead to appearance of the enzyme in serum, where a variety of other glycosyl transferases occur[38].

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