INVESTIGATIONS OF THE MECHANISM OF THE BIOSYNTHESIS OF GLYCOPROTEINS.

A Thesis submitted by

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ABSTRACT

The biosynthesis of the carbohydrate portion of glycoproteins is a post-translational event which involves the enzymic assembly of polysaccharide moieties and the formation of glycopeptide bonds. In animals the carbohydrate is attached either through an O-glycosidic linkage to serine, threonine or hydroxylysine residues or by the way of a glycosylamine bond to an asparagine moiety.

The formation of carbohydrate-asparagine linkages occurs provided the asparagine residue is located in an appropriate amino acid sequence of the type Asn-X-Thr(Ser).

Glycosylation of appropriate asparagine residues of serum glycoproteins occurs, at least partly, at the polyribosomal level and an enzyme has been shown to be present in preparations of rough endoplasmic reticulum of rabbit liver which can catalyse the incorporation of N-acetyl-glucosamine into asparagine 34 of bovine pancreatic ribonuclease A.

The presence of the enzyme could not be demonstrated in preparations of rat liver, although when the animal was subjected to surgical injuries or in regenerating liver enzymic activity was apparent. A soluble form of the enzyme was found in human serum.

The enzyme, irrespective of its source, be it serum or liver, had a requirement for metal ions for the expression of its full activity. It was shown for the serum enzyme that it is also activated by naturally occurring polyamines, spermidine being the most effective in this respect. The enzyme is precipitated from serum at 25-35% saturation with ammonium sulphate.

Interestingly enough, a similar enzymic activity could be demonstrated in plants; yeast cell membrane preparations were shown to be able to catalyse the incorporation of N-acetyl-glucosamine into ribonuclease A.

The product of the enzymic reaction 4-N-(2-acetamide-2-deoxy-β-D-glucopyranosyl)-^{34}L-asparagine ribonuclease has been isolated and purified from ribonuclease A and its properties have been elucidated.

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CHAPTER 1

CONSIDERATIONS OF THE STRUCTURE AND SYNTHESIS OF GLYCOPROTEINS

The synthesis of proteins occurs by a mechanism which involves transcription of the sequence of bases in deoxyribonucleic acid, and thus of the information contained in the latter. That information is concerned, as far as we know, only with the incorporation of 20 L-amino acids into a polypeptide chain together with initiation and termination of the chain. But many proteins have substituents which are added by post-translational mechanisms, and among these substituents are phosphate, acetyl, methyl and hydroxyl groups (of proline and lysine residues in collagen), as well as carbohydrate moieties in a very large number of proteins. There is no final agreement on the definition of a glycoprotein (see Gottschalk, 1972), but it seems to be most reasonable to describe those proteins which have undergone glycosylation *in vivo* as glycoproteins (Neuberger & Marshall, 1968; Spiro, 1973; Sharon, 1975; Kornfeld & Kornfeld, 1976).*

Glycoproteins are one of the essential constituents of the vertebrates, invertebrates and higher plants (Table 1). Their presence has also been shown in fungi and bacteria. They may occur *inter alia* as soluble secreted materials or as components

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* In this and subsequent Chapters a few abbreviations will be used, but only those which are generally accepted (see "Instructions to Authors", The Biochemical Journal, 1976, 153, 1-21). Additionally, the term "Carb" will be used to denote a carbohydrate moiety, and ribonuclease-GlcNAc is used to indicate the monoglycosylated form of ribonuclease A.

Glycoprotein	Source	M.W.	Man	Gal	Fuc	GlcNAc	GalNAc	NeuNAc	Glc	Ara	Xyl	References
ENZYMES												
Animals												
Deoxyribonuclease B	Bovine pancreas	31,000	6	-	-	2	-	-	-	-	-	Catley *et al.* (1969)
Sulphatase A	Cattle liver	107,000	14	8	Trace		-	8	-	-	-	Graham & Roy (1973)
Hyaluronidase	Testes	61,000	19	-	-	7	-	-	-	-	-	Borders & Rafferv (1968)
Proteinase b	Snake venom	95,000	43[a]			34	-	10	-	-	-	Dahima *et al.* (1968)
Amine oxidase	Bovine plasma		5	-	-	2	-	-	-	-	-	Watanabe & Yasunobu (1970)
Plants												
Bromelain	Pineapple stem	33,000	3	-	1	2	-	-	-	-	1	Yasuda *et al.* (1970)
Taka amylase	Aspergillus oryzea	51,000	6	-	-	2	-	-	-	-	-	Yamaguchi *et al.* (1969)
Invertase	Yeast	270,000	750	-	-	38	-	-	-	-	-	Neumann & Lampen (1967)
Peroxidase see also lectins	Horseradish[b]	40,000	34		10	47			4.0	2.0	9.0	Clarke & Shannon (1975)
Microbial												
β-D-glucosidase	Aspergillus fumigatus	41,000	17	-	-	2	-	-	-	-	-	Rudick & Elkein (1973)
HORMONES												
Thyroglobulin	Human	670,000	930	50	21	114	13	23	-	-	-	Arima *et al.* (1972)
Chorionic gonadotrophin	Pregnant human	27,000	8.0	8.0	1	11	3	8	-	-	-	Bahl (1969)
Thyrotropin	Bovine	25,000	6.9	0.3	0.9	7.8	3.3	-	-	-	-	Liao *et al.* (1969)
Luteinizing hormone	Sheep pituitary gland	28,000	7.0	2	1.0	8.0	3.0	0 or 1	-	-	-	Walborg & Ward (1963)
Thyroid stimulating hormone (β-subunit)	Bovine	13,600	3	-	0.9	3.0	2.0	-				Liao & Pierce (1970)
Follicle stimulating hormone	Ovine	32,000	6	6	1	6	3	6	-	-	-	Cahill *et al.* (1968)
IMMUNOGLOBULINS												
IgG (γ-chain)	Human serum	140,000	5	3	2	9	-	1.0	-	-	-	Rosevear & Smith (1961)
IgA (α-chain)	Human	170,000	14	12	2	12	6	5	-	-	-	Dawson & Clamp (1968)
IgM (μ-chain)	Human[c]	(180,000)5	35	11	6	27	-	9	-	-	-	Johnson & Clamp (1971)
Bence Jones (MOPC 46)	Mouse urine	23,000	2	3	Trace	3.6	0.7	-	-	-	-	Coleman *et al.* (1967)
IgG	Rabbit	160,000	5	1	1	4	2	2				Fanger & Smyth (1972) Nolan & Smith (1962a)
CARRIER												
Transferrin	Human serum	90,000	8	4	-	8	-	4	-	-	-	Jamieson *et al.* (1971)
Caeruloplasmin	Human serum	160,000	22	14	2	12	-	9	-	-	-	Jamieson (1965)
Haptoglobin (β-chain)	Human	14,000	8	16	1-3	16		8				Cheftel *et al.* (1965)
Gastroferrin	Human gastric juice	263,000	-	461	250			30				Multani *et al.* (1970)
Intrinsic factor[d]	Hog stomach	53,000	3.5	11.8	4.0	9.1	3.6	3.7				Highley *et al.* (1967)
MUCINS												
Submaxillary mucin	Sheep[e]	1,000,000		3	5	-	112	105				Tettamanti & Pigman (1968)
Cervical mucin oestrus	Cattle[e]	4 x 1,000,000		153	31	86	64	45				Gibbons (1959)
Ovarian cyst fluid A*	Human[e]	- [f]		94	110	98	109	7				Aminoff *et al.* (1959), Watkins (1972)
Gastric mucosa F IIb	Pig[e][h]	- [g]		250	76	121	51	4				Slomiany & Meyer (1972)

Glycoprotein	Source	M.W.	Man	Gal	Fuc	GlcNAc	GalNAc	NeuNAc	Glc	Ara	Xyl	References
CONNECTIVE TISSUES												
Glomerular basement membrane	Human[e]		3.4	14	1.0	5.9	0.8	-	13	-	-	Beisswenger & Spiro (1970)
Aorta glycoprotein	Ox	60,000			1.8							
Carp swim bladder	Citrate soluble[e]		0.3	1.5	-		0.9[i]		1.2			Spiro (1969b)
Achilles tendon[e][j]			0.9	2.5	0.2		2.5[i]	0.2	1.3	-	-	Spiro (1969b)
PLASMA AND BODY FLUIDS												
Fetuin	Foetal calf serum	45,000	8	12	-	15	2	13	-	-	-	Spiro (1960, 1962)
α_1-acid glycoprotein	Human plasma	40,000	12	17	2	24	-	14	-	-	-	
Lung lavage	Human	36,000	4.4	4.0	1.0	6.0		3.0				Bhattacharyya et al. (1976)
Fibrinogene	Human plasma	330,000	22	19	-	19	-	6	-	-	-	Mester & Szabados (1968)
Thyroid hormone-binding protein	Human plasma	17,300	2	2	-	2	-	-	-	-	-	Nilsson & Peterson (1975)
LECTINS												
Wax bean		130,000	3	4	1	2	-	-	2	5	-	Takahashi & Liener (1968)
Lima bean agglutinin[k]			6-7	-	1	2	-	-	-			Galbraith & Goldstein (1972)
Limulin[l]	Limulus polyphemus	115,000										Roche & Mousigny (1974)
Phaseolus vulgaris		12,800	20	-	3	15	-	-	4	8	1	Mialonier et al. (1973)
Solarium tuberosum		47,000	41.6[m]									Allen & Neuberger (1973); Delmotte et al. (1975)
EGG WHITE												
Ovalbumin		45,000	3	-	-	3	-	-	-	-	-	Johansen et al. (1961)
Ovomucoid		28,000	7	2		23		1				Chatterjee & Montgomery (1962)
Avidin (subunit)			3			4						Huang & De Lange (1971)
Ovotransferrin		80,000	4	-	-	8	-	-	-	-	-	Williams (1968)
Phosvitin		40,000	3	3	-	5	-	2	-	-	-	Shainkin & Perlmann (1971b)
URINE												
Tamm and Horsfall	Hamster	82,000	11.0	12.4	8.4	51.8	28.9	0-1.4	-	-		Dunstan et al. (1974)

(a) Determined as total hexose (mannose and galactose): (b) The carbohydrate composition given is from isoenzyme c before tryptic digestion:
(c) The sugar content is given as residues per 180,000 molecular weight: (d) The sugar composition is given as g/100 g of protein:
(e) The sugar content is expressed as mole/10^5 g dry weight: (f) The molecular weight not reported: (g) Trypsin digestion was used for isolation:
(h) This mucin contains 36 moles sulphate/10^5 g dry weight: (i) This value refers to the total hexosamines: (j) This collagen contains 0.4 moles hexuronic acid/10^5 g dry weight: (k) The agglutinating activity of the lima bean resides in two molecular species (isolectins) each with the same amino acid and carbohydrate composition but with different molecular weights, component II, 195,000-269,000 and component III, 110,000-138,000 (Galbraith & Goldstein, 1970). The carbohydrate values are given as mole/31,000 g protein: (l) The total carbohydrate of this lectin is low, 3.5% (1.8% neutral sugars and 1.8% glucosamines).
(m) This value refers to total sugars and is given as percentage.

TABLE 1. Examples of some glycoproteins showing their functions and carbohydrate content as residues per mole.

of cellular membranes. They may be enzymes, hormones or immunoglobulins. Most serum, egg, milk and urine proteins contain covalently-bound carbohydrate. Glycoproteins are also present in blood vessels, skin and epithelial secretions. Certain Bence Jones proteins contain carbohydrates. The role of glycoproteins as part of the body defence mechanism (immunoglobulins), lubricants (respiratory or intestinal mucus), carriers (transcobalamine) and metabolic effectors (hormones and enzymes) make their vital importance quite obvious. A point of considerable interest is the way in which the carbohydrate moiety is linked to the polypeptide chain.

1. General aspects of carbohydrate-peptide linkages in glycoproteins

With the use of relatively recent techniques, more information has been obtained regarding the type of linkages between the carbohydrate and amino acid in a glycoprotein. The glycopeptide containing a few amino acids (Montgomery & Wu, 1963) or a complex heterosaccharide attached to only one amino acid (Marks et al., 1962a) could be obtained by proteolytic cleavage of the glycoprotein, followed by conventional separation techniques. In some cases the peptide can be shortened by the action of carboxypeptidase A after blocking the terminal α-amino group with an appropriate moiety (Bogdanov et al., 1961; Marks et al., 1962b 1963) or by leucine aminopeptidase or by Edman degradation (Edman, 1950).

It has been shown that of those amino acid residues which possess side chains which might conceivably undergo glycosylation, only a few actually become involved in carbohydrate-peptide linkages. Those commonly involved (see Gottschalk, 1972) are

	Glycoprotein	Attached sugar	Amino acid	Sequence around the linkage region	References
1.	Ox submaxillary glycoprotein	N-acetyl-D-galactosamine	THR, SER	Ser-THR-Gly-Ser	Ozeki & Yosizawa (1971)
2.	Chondroitin-4-sulphate	N-acetyl-D-galactosamine	SER	Gly-Gly-SER-Gly	Katsura & Davidson (1966)
3.	α-Amylase	N-acetyl-D-galactosamine	SER	SER-Glu-Asp-Gly-(Ala-Thr)	Isugita & Akabori (1959)
4.	Human chorionic gonadotrophin	N-aretyl-D-galactosamine	SER	Pro-Pro-Pro-Ser-Leu-Pro-SER-Pro-SER-Arg-Leu-Pro	Carlsen et al. (1973)
5.	Human γ-chain (IgG_3-Zuc)	N-acetyl-D-galactosamine	SER	Pro-Gly-Gly-SER-Ser-Glu-Pro-Lys	Frangione & Milstein (1969)
6.	Ox encephalitogen	N-acetyl-D-galactosamine	THR	Val-Thr-Pro-Arg-THR-Pro-Pro-Pro-Ser-Glu-Gly-Lys	Hagopian et al. (1971)
7.	Rabbit γ-chain	N-acetyl-D-galactosamine	THR	Ser-Lys-Pro-THR-Cys-Pro-Pro-Pro	Smyth & Utsumi (1967)
8.	Antifreeze glycoprotein	N-acetyl-D-galactosamine	THR	Ala-THR-Ala-Ala-THR-Ala-Ala-THR-Ala	De Vries et al. (1971)
9.	Human α-chain (IgA_1-Pat,Oso)	N-acetyl-D-galactosamine	THR, SER	Pro-Ser-THR-Pro-Pro-THR-Pro-SER-Pro-SER-THR-Pro-Pro-THR-Pro	Frangione & Wolfenstein-Todel (1972)
10.	Keratan sulphate (skeletal)	N-acetyl-D-galactosamine	THR	Gly-Pro-THR	Hopwood & Robinson (1974)
11.	Human skin collagen	β-D-galactose	HYL	Gly-Phe-HYL-Gly-Ile-Arg	Morgan et al. (1970)
12.	Guinea-pig skin collagen	β-D-galactose	HYL	Gly-Met-HYL-Gly-His-Arg	Morgan et al. (1970)
13.	Carp swim bladder collagen	β-D-galactose	HYL	Gly-Met-HYL-Gly-His-Arg	Morgan et al. (1970)
14.	Cuttlefish skin collagen	β-D-galactose	HYL	Gly-Glu-HYL-Gly-Ala-Arg, Gly-Pro-HYL-Gly-Ala-Arg	Isemura et al. (1973a)
15.	Sea cucumber collagen	β-D-galactose	HYL	Gly-Pro-HYL-Gly-Asp	Isemura et al. (1973b)
16.	Chlamydomonas (green alga)	β-D-galactose	HYP	$(Gal)_2$	Miller et al. (1972)
17.	Urinary glycopeptide	β-D-galactose	Cysteine	CYS-Glu-His-Ser-His-Asp-Gly-Ala	Lote & Weiss (1971)
18.	Bovine nasal cartilage	O-β-D-xylose	SER	Gly-Ala-SER-Gly, Leu-SER-Gly	Johnson & Baker (1973)
19.	Bovine intervertebral disc chondroitin sulphate	O-β-D-xylose	SER	Glu-Ala-SER-Gly-Pro-THR(GalNAc)	Hopwood & Robinson (1974)
20.	Glycopeptide from normal human urine	Fucose	THR	$(Glc)_3$	Hallgren et al. (1973)
21.	Glycopeptide from human erythrocyte membrane	Glucose	Cysteine	Cys-Glu-Gly-His-Ser-His-Asp-His-Gly-Ala	Weiss et al. (1971)
22.	Aspergillus glucamylase	D-mannose	SER		Pazur & Aronson (1972)
23.	Potato lectin	L-arabinose	HYP		Allen & Neuberger (1973)
24.	Various glycoproteins listed in Table 3	β-D-GlcNAc	ASN		

TABLE 2. Amino acid sequences of glycopeptides containing glycosylated Ser, Thr, Hyl, Hyp and cysteine residues

asparagine, threonine and serine. Animal collagens contain glycosylated hydroxylysine residues (Spiro, 1967; Butler & Cunningham, 1966), but so far it would appear hydroxyproline residues occur in a glycosylated form only in plants (Fincher et al., 1973, 1974; Lamport, 1967, 1969; Miller et al., 1972; Heath & Northcote, 1971). A linkage involving glutamine or glutamic acid has also been reported in bovine nasal (Hascall & Riolo, 1972) and tracheal keratan sulphate (Heinegård, 1972), but confirmation of this is perhaps needed.

The sugar moieties involved in the linkage are also limited to a certain number of types, i.e. N-acetyl-β-D-glucosamine, N-acetyl-α-D-galactosamine, β-D-xylose, D-mannose, β-D-galactose, L-arabinose and L-fucose (Table 2). L-Arabinose has also been reported to be glycosidically linked to L-serine or L-threonine (Wardi et al., 1969) in hyaluronic acid. The structures of the sugars, that is whether D or L, will not be stated usually in the following text, but it is implied that they are as above.

2. The asparagine sequon for glycosylation

Isolation of glycopeptides followed by sequencing the amino acids in the vicinity of the carbohydrate-peptide linkage has revealed that the amino acid residue bound to asparagine on either carboxyl or amino side differs from one type of protein to the other, and even in a given glycoprotein at different sites of attachment (some examples are given in Table 3). As can be seen, the amino acid may be basic, neutral or acidic, polar or non-polar. Beeley (1974) reported the presence, in ovomucoid, of a cystine residue linked to the carboxyl group of the asparagine residue. Proline has also been reported in a

	Glycoprotein	Sequences of the amino acids around glycosylated asparagine residues	References
1.	Ovalbumin	Glu-Lys-Tyr-ASN-Leu-Thr-Ser	Cunningham et al. (1963); Lee & Montgomery (1962); Johansen et al. (1961)
2.	Ovotransferrin	Leu-Ile-His-ASN-Arg-Thr-Gly	Williams (1968); Ellman & Williams (1970)
3.	Human transferrin	Ala-Glu-Asn-Tyr-ASN-Lys-Ser, Ser-Tyr-ASN-Lys-Ser	Spik & Montreuil (1968); Charet et al. (1969)
4.	Avidin (hen)	Leu-Gly-Ser-ASN-Met-Thr-Ile	De Lange (1970)
5.	Fibrinogen (human)	Gln-Val-Glu-ASN-Lys-Thr-Ser	Mester (1969)
6.	Deoxyribonuclease (bovine)	Ser-ASN-Ala-Thr	Catley et al. (1969)
7.	Thyroglobulin (human)	Ala-Leu-Glu-ASN-Ala-Thr-Arg	Rawitch et al. (1968)
8.	Horseradish peroxidase	ASN-Phe-Ser-Asp-Gly-Ile, Asp-ASN-Leu-Thr, Asn-Val-Gly-Leu-ASN-Arg	Clarke & Shannon (1976)
9.	Bence Jones k (mouse) MOPC 46	Ala-Ser-Gln-ASN-Ile-Ser-Asn-Asn-Leu	Melchers (1969)
10.	Bence Jones λ chain Eul, human	Cys-Ser-Gly-ASN-Ser-Ser	Melchers (1969)
11.	Pancreatic ribonuclease (guinea-pig)	Arg-Arg-ASN-Met-Thr-Gln, Ser-Pro-Ser-ASN-Ser-Ser-Asn	Van Den Berg & Beintema (1975)
12.	α-Lactalbumin	ASN-Gln-Ser	Hopper & McKenzie (1973)
13.	Pineapple stem bromelain	Ala-Arg-Val-Pro-Arg-Asn-ASN-Glu-Ser-Ser-Met	Yasuda et al. (1970)
14.	Ovomucoid	Arg-Phe-Pro-ASN-Ala-Thr, Val-Pro-Met-ASN-Cys-Ser, Ser-Tyr-Ala-ASN-Thr-Thr-Ser	Beeley (1972); Murthy et al. (1973)
15.	Hen egg yolk phosvitin	Ser-ASN-Ser-Gly-PSer	Shainkin & Perlmann (1971)
16.	Ox aorta glycoprotein	Ala-Phe-Gly-ASN-Gly	Radhakrishnamurthy & Berenson (1966)
17.	Egg white vitellin (hen)	ASN-Thr-Ser-(Ala-Gly-Val)-Ile	Augustyniak & Martin (1968)
18.	α_1-acid glycoprotein (human)	Ile-Thr-ASN-Ala-Thr-Leu, Arg-Glu-ASN-Gly-Thr-Ile Phe-Thr-Pro-ASN-Lys-Thr-Glu, Glu-Glu-Tyr-ASN-Lys-Ser-Val Ile-Tyr-ASN-Thr-Thr-Tyr	Schmid et al. (1971)
19.	Human γG globulin	Asp-Tyr-ASN-Ser-Thr	Rosevear & Smith (1961)
20.	Rabbit γG globulin	Gln-Phe-ASN-Ser-Thr-Ile	Nolan & Smith (1962a,b); Hill et al. (1966)
21.	Ox γG globulin	Glx-Phe-ASN-Ser-Thr-Tyr	Duquesne et al. (1965); Howell et al. (1967)
22.	Ovine luteinizing hormone	Pro-Ile-ASN-Ala-Thr-Leu	Liu et al. (1972)
23.	Bovine visual pigment 500	Met ASN-Gly-Thr	Heller & Lawrence (1970)
24.	Taka amylase A	Val-Ser-ASN-Tyr-Ser-Ile-Asp	Isemura & Ikenaka (1973)

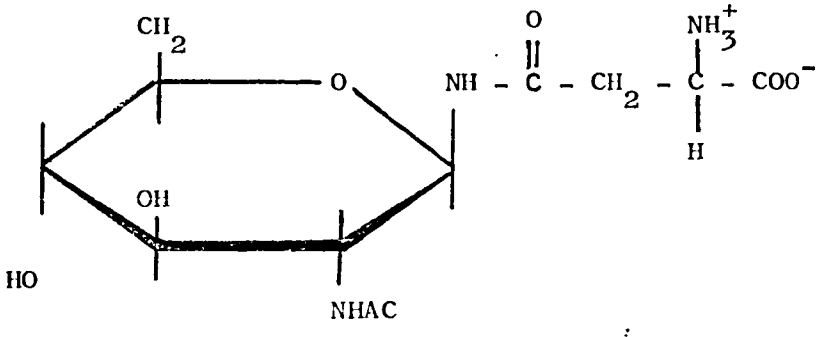
The glycopeptide from hen egg yoke phosvitin (item 15) is reported with an exceptional amino acid sequence.

TABLE 1. Sequences of glycopeptides with the structure ASN-(Carb)-X-Ser and ASN-(Carb)-X-Thr

similar position (Beintema et al., 1973). Eylar (1965) first commented on the frequency of the occurrence of a threonine residue in the neighbourhood of the glycosylated asparagine, suggesting that it might occupy any of the positions A, B, C or D in the sequence ...A.B.Asn(Carb).C.D... This hypothesis was not wholly correct. Further consideration of the problem led to the conclusion that an asparagine residue must be followed in a position next-but-one on its C-terminal side by a serine or threonine residue in order to undergo glycosylation. There is no clear evidence as to why there is this need for a serine or threonine residue but, as suggested, it could act as a signal for the sugar transferase, or the hydroxyl group of the amino acid might be involved in hydrogen bonding (Neuberger & Marshall, 1968). According to Neuberger & Marshall (1968), (see also Marshall, 1967; Marshall & Neuberger, 1972a) occurrence of asparagine in a sequence of the type mentioned is a necessary but not sufficient condition for glycosylation to occur. This type of sequence, namely ...Asn.X.Thr... or ...Asn.X.Ser... has been termed an asparagine sequon (Marshall, 1974). Not all sequences of this type are glycosylated; examples are rat ribonuclease (Beintema & Gruber, 1967) and guinea-pig immunoglobulin K chain (Lamm & Lisowska-Bernstein, 1968). There is only one recent report of glycosylation at an asparagine residue which does not occur in the appropriate sequon; that in hen egg phosvitin Asn.Ser.Gly (Shainkin & Perlmann, 1971a) and, if correct, is of considerable interest.

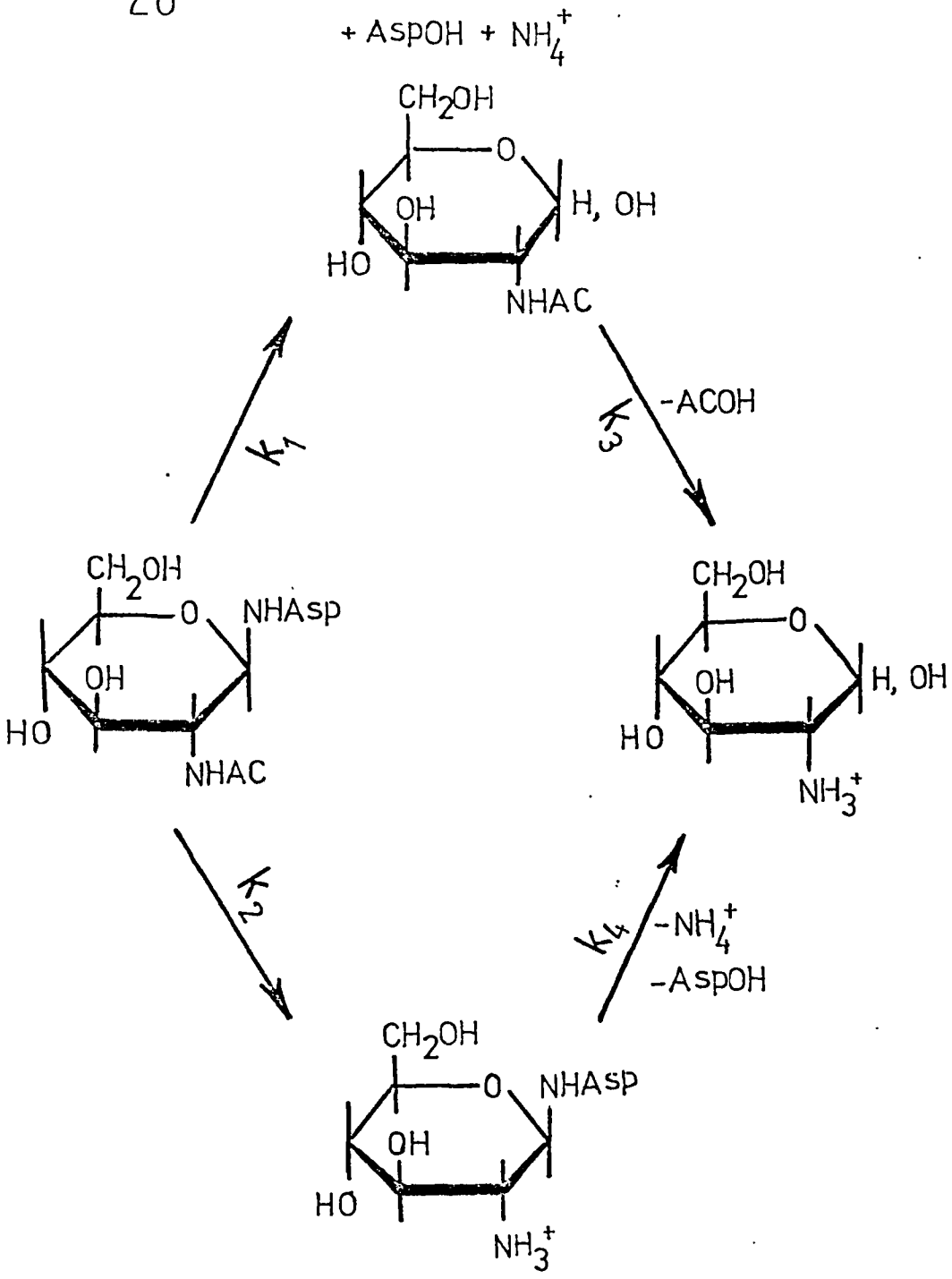
3. The occurrence of 4-N-2-acetamido-2-deoxy-β-glucopyranosyl-asparagine in glycoproteins

This type of linkage (GlcNAc-Asn) occurs in a variety of glycoproteins, including serum proteins such as transferrin, fetuin, fibrinogen and caeruloplasmin (Table 3). Ribonucleases B, C and D from cattle pancreas, and that from pig pancreas also, contain this type of linkage (see Table 6) as do also the egg white proteins such as albumin, ovomucoid and avidin (Table 3). The structure assigned to this particular linkage is:



This type of linkage was first investigated in hen egg albumin by Cunningham et al. (1957), Johansen et al. (1958) and Jevons (1958). These authors showed that aspartic acid was the site of attachment to the carbohydrate moiety, but later studies showed that the amide nitrogen of the asparagine residue is linked to the sugar molecule (Johansen et al., 1961; Nuenke & Cunningham, 1961). The question as to whether the amino acid residue which had undergone glycosylation in vivo was asparagine or aspartic acid was not resolved for a number of years. Most workers had assumed that it was an asparagine residue, and the results of interesting studies by Yamashina and his colleagues in Kyoto confirmed this.

When [^{14}C]-aspartic acid or [^{14}C]-asparagine was injected into rats, the distribution of the radioactivity in the serum glycoproteins showed that asparagine is the major, if not the



$k_1 = 20 \times 10^{-3}\ min^{-1}$

$k_2 = 22 \times 10^{-3}\ min^{-1}$

$k_3 = 21 \times 10^{-3}\ min^{-1}$

$k_4 = 7 \times 10^{-3}\ min^{-1}$

Fig. 1. The major pathways by which GlcNAc-Asn can undergo hydrolysis in 2M HCl, 100°C (from Marshall, 1969)

only, site of attachment to the sugar (Kohno & Yamashina, 1973).

The linkage compound, GlcNAc-Asn, was first isolated from digests of hen's egg albumin by hydrolysis of glycopeptide with 2N HCl or H_2SO_4 at 100°C for a period of 12-30 min (Bogdanov et al., 1962, 1964; Marks et al., 1962a, 1963; Yamashina et al., 1963, 1965; Marshall & Neuberger, 1964; Tsukamoto et al., 1964; Yamamoto & Tsukamoto, 1965). The compound was purified by conventional chromatographic and electrophoretic techniques. GlcNAc-Asn has also been obtained by periodate oxidation of glycopeptides at 5°C with buffered 0.2M sodium periodate in the dark (Spiro, 1964; Makino & Yamashina, 1966; Hughes & Jeanloz, 1966). The compound has also been prepared by techniques of classical organic chemistry, through condensation of a derivative of 2-acetamido-2-deoxy-β-glucopyranosylamine with 1-benzyl-N-benzyloxycarbonyl-aspartate in the presence of dicyclohexyl carbodiimide. The sugar derivative most often used is 2-acetamido 3,4,6-tri-O-acetyl-2-deoxy-β-glucopyranosylamine (Marks et al., 1962a, 1963; Bolton & Jeanloz, 1963; Tsukamoto et al., 1964; Marshall & Neuberger, 1964). The properties of the linkage compound have been studied extensively. It has been shown clearly that a β-glycosidic linkage (Delbaere, 1974; Austen & Marshall,1974a) connects the nitrogen of the asparagine to the anomeric carbon atom of the sugar.

This type of linkage is relatively stable to acidic conditions. The model compound is split mainly by two pathways in 2N HCl at 100°C (Marshall, 1969; Cowley et al., 1971) (p. 26). The corresponding rate of release of aspartic acid from hen's egg albumin glycopeptide which contained aspartic acid as the

only amino acid was found to be 13 x 10^{-3} min^{-1} (Marshall, 1969). The rate constant for the destruction of the linkage compound under these conditions is thus 42 x 10^{-3} min^{-1} ($t_{\frac{1}{2}} \sim 16$ min).

In less concentrated HCl (1$\underline{N}$) the linkage is more stable (Marks et al., 1962a, 1963; Yoshimura & Hashimoto, 1967). In alkaline solutions the linkage seems to be relatively stable. The destruction of GlcNAc-Asn linkage in 0.2$\underline{M}$ NaOH at 100°C is a first-order reaction. The overall yield of aspartic acid produced upon alkaline hydrolysis is related to the concentration of the alkali used (Austen & Marshall, 1974b).

The GlcNAc-Asn has two titrating groups within easily accessible ranges of pH; the α-carboxyl group with an apparent pK_a value of 2.05, the α-amino group with a pK_a of 8.6 (Marshall & Neuberger, 1964).

4. Other types of carbohydrate linkage

There are other types of carbohydrate-peptide linkages in glycoproteins as indicated above, but the general purpose of the experimental work described in this thesis is not concerned with the other types. They are listed in Table 2, but their chemical properties are not described. The relevance of them to the work herein discussed lies in the general biosynthetic pattern concerning the addition of sugar to a polypeptide chain. For this reason some other aspects of these other types of carbohydrate-peptide linkages will be mentioned later.

5. Functions of carbohydrate moieties of glycoproteins

The contribution of the carbohydrate moieties of glycoproteins to the physical properties and function of

Specificity	Structure	References
A	α-GalNAc-(1→3)-β-Gal-(1→3 or 4)-GlcNAc ↑1,2 α-Fuc	Painter et al. (1965) Lloyd et al. (1966)
B	α-Gal-(1→3)-β-Gal-(1→3 or 4)-GlcNAc ↑1,2 α-Fuc	Painter et al. (1965) Lloyd et al. (1966)
H	β-Gal-(1→3 or 4)-GlcNAc ↑1,2 α-Fuc	Rege et al. (1964a) Lloyd et al. (1966)
Le^a	β-Gal-(1→3)-GlcNAc ↑1,4 α-Fuc	Rege et al. (1964b) Lloyd et al. (1968)
Le^b	β-Gal-(1→3)-GlcNAc ↑1,2 ↑1,4 α-Fuc α-Fuc	Marr et al. (1967) Rovis et al. (1973)

TABLE 4. Structure assigned to the blood group specific determinants.

these types of macromolecule has often been speculated upon. Eylar (1965) suggested a hypothesis that the carbohydrate units act as an essential prosthetic group to promote the exit of the protein from the cell, but there are many extracellular proteins, e.g., human serum albumin, which are not glycosylated.

Studies so far have revealed many activities for the carbohydrate units of the glycoproteins. The A, B, H and Lewis blood group specific glycoproteins isolated from ovarian cyst fluids are composed of 80-90% carbohydrate and 10-20% amino acids. Despite the similarities in their overall compositions, structural differences exist between them (Table 4). The indirect methods of serological inhibition by simple sugars of both the agglutination of the erythrocytes by blood group specific lectins (Morgan & Watkins, 1953) and also the inhibition of specific precipitation by human antisera (Kabat & Leskowitz, 1955) gave the first clues that these differences reside on the carbohydrate part of the molecule. N-Acetyl-α-galactosamine was found to be the "immunodominant" sugar in the A determinant, α-galactose in the B determinant, α-fucose in the H and Le^a determinants. Later studies on the serologically active fragments isolated from acid, base and enzymic hydrolysates of the glycoproteins resulted in our present knowledge of the structures of the carbohydrate moieties (Ginsburg et al., 1971; Morgan, 1970; Kabat, 1970; Watkins, 1972).

The hormonal activity of some glycoproteins was at one time attributed to their sugar moieties. Human chorionic gonadotrophin and erythropoietin contain relatively small percentages of sugar. When sialic acid was removed by treatment

with neuraminidase (Mori, 1969; Lowy *et al.*, 1960), the molecule was reported to be inactive. However, tests of activity were of necessity done *in vivo* and the question of metabolic turnover must also be considered (Marshall & Neuberger, 1968). It had been suggested earlier (Walborg & Ward, 1963) that removal of sialic acid could also influence the transport of the macromolecule into the receptive target cells of the ovary and bone marrow respectively. The effects of sialic acid residues on the functions of a number of glycoproteins were first illuminated by the detailed studies of Morell at the Einstein Medical School, New York, and Ashwell at the National Institutes of Health in Bethesda, Maryland. The principle derived from these studies may be considered from the results of the experiments they and their colleagues did with human caeruloplasmin.

Crystalline caeruloplasmin was substantially freed from sialic acid residues by the action of *Vibrio cholerae* neuraminidase with the formation of asialo-caeruloplasmin. Treatment of the latter with galactose oxidase converted the non-reducing terminal galactose residues to the 6-aldehydo derivatives and subsequent reduction of these with tritiated sodium borohydride resulted in the conversion of the 6-aldehydo entities to galactose residues labelled with tritium (Morell *et al.*, 1966). The half life for the *in vivo* turnover of caeruloplasmin is $\sim$ 2-3 days. When the labelled asialo-caeruloplasmin was injected into rabbits, 90% of it was removed from the serum within 15 min and was found in the parenchymal cells, but not the Kupffer cells, of the liver (Morell *et al.*, 1968). Rapid clearance of asialo-caeruloplasmin occurs because intact

galactose residues are exposed by removal of sialic acid; treatment of asialo-caeruloplasmin with β-galactosidase or oxidation of exposed galactose residues with galactose oxidase from Polyporus circinatus yields products with much larger survival times. When sialic acid is reintroduced into the acceptor sites for this sugar of asialo-caeruloplasmin (Hickman et al., 1970), the product exhibits its normal half life.

Certain glycoproteins in the blood of some antarctic fish have been shown to contribute to the lowering of the freezing point of the blood (DeVries et al., 1970). When the protein is subjected to proteolytic digestion, its ability to lower the freezing point is lost. The activity is also found to be due to the Cis-3,4-hydroxyls of the D-galactose residues. An anti-freeze glycoprotein has also been found in the serum of winter flounder (Duman & DeVries, 1973). The curious amino acid sequence in these anti-freeze glycoproteins $(Ala\text{-}Ala\text{-}Thr)_x$ where x = 15, 25 or 32 might suggest a mechanism of synthesis different from that generally in operation for proteins.

There are also many examples known where no specific activity can be, at least at present, ascribed to the carbohydrate moiety of a glycoprotein. Tarentino et al. (1974) presented evidence that when bovine pancreatic ribonuclease B or deoxyribonuclease A, which have few sugar residues, are subjected to the action of endo-β-N-acetyl-glucosaminidase which releases all the oligosaccharide side chains apart from the N-acetyl-glucosamine residue linked directly to the polypeptide chain, no detectable differences in circular dichroism or

moreover enzymic activities between the native and glycosidase-treated enzymes could be discerned, although their electrophoretic and chromatographic mobilities were changed.

Likewise when immunoglobulin IgM is subjected to the same treatment with endo-β-N-acetyl-glucosaminidase the immunological properties of the glycoprotein as determined by complement fixation and immunodiffusion tests (Tarentino et al., 1974) are unchanged.

In cases such as ribonuclease A and B which differ structurally only in that the latter is glycosylated at asparagine-34, no difference in enzymic properties between the two forms are apparent (Plummer & Hirs, 1964).

6. Some aspects of the structures of the carbohydrate moieties of glycoproteins

There are a number of techniques available for elucidation of the structure of the carbohydrate moieties of glycoproteins. These will not be discussed in any detail here and the reader is referred to some comprehensive reviews (Jeanloz, 1966; Marshall & Neuberger, 1972b; Hughes, 1973; Spiro, 1973; Kornfeld & Kornfeld, 1976). There are also numerous articles which deal with the sizes and structure of the carbohydrate moieties of glycoproteins (Roden, 1970; Schmid, 1972; Spiro, 1973; Marshall & Neuberger, 1972). Usually they are of a molecular size varying from two, or even one in some cases, to about 15 sugar moieties. As an example of the complexity of the structure one may consider the α_1-glycoprotein, orosomucoid. It has a molecular weight of $\sim$ 40,000 of which 41.4% is carbohydrate. The latter is distributed as five separate moieties linked at asparagine residues

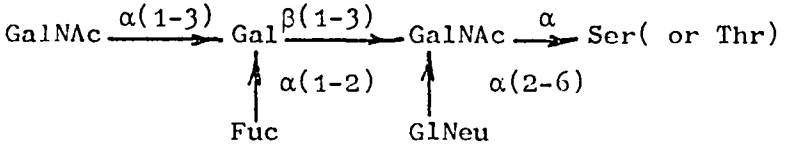
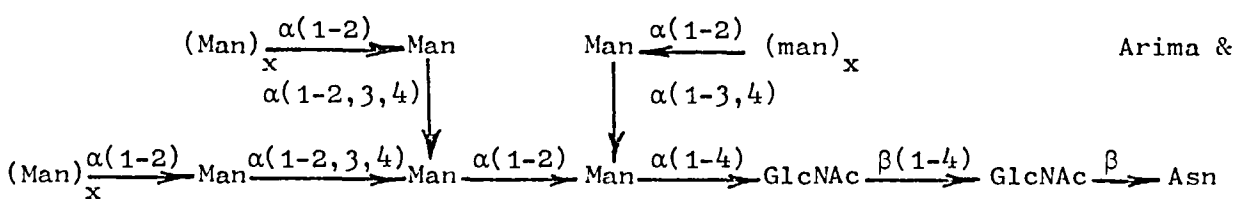
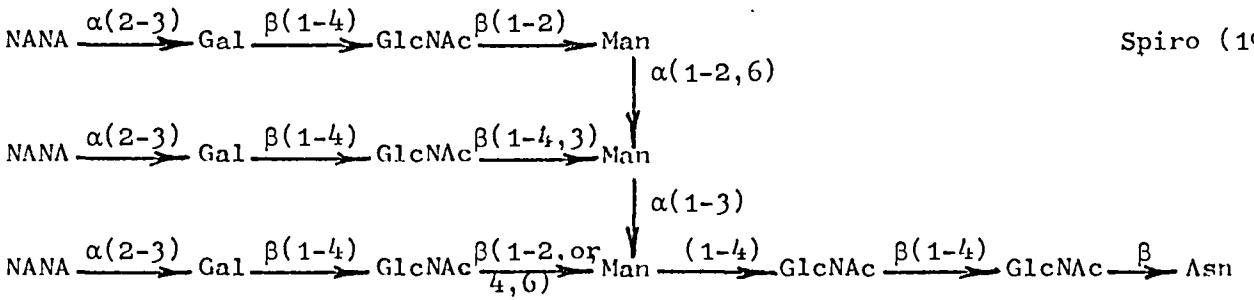
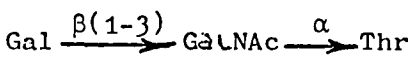
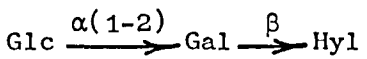
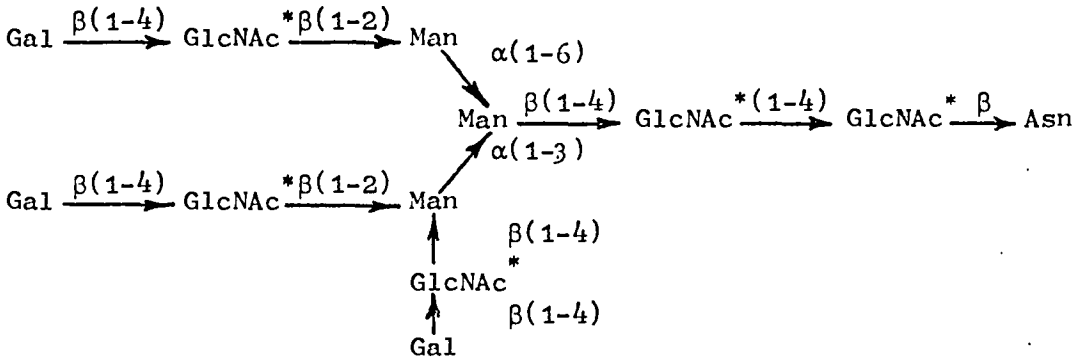
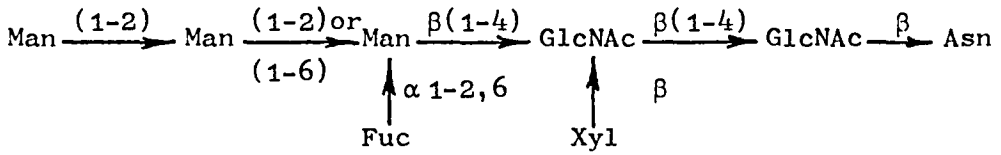
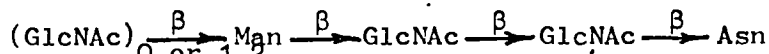
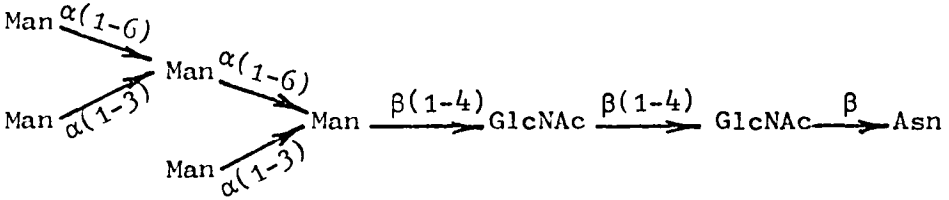
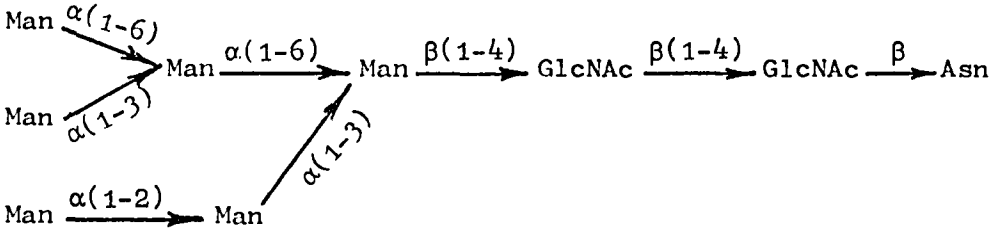
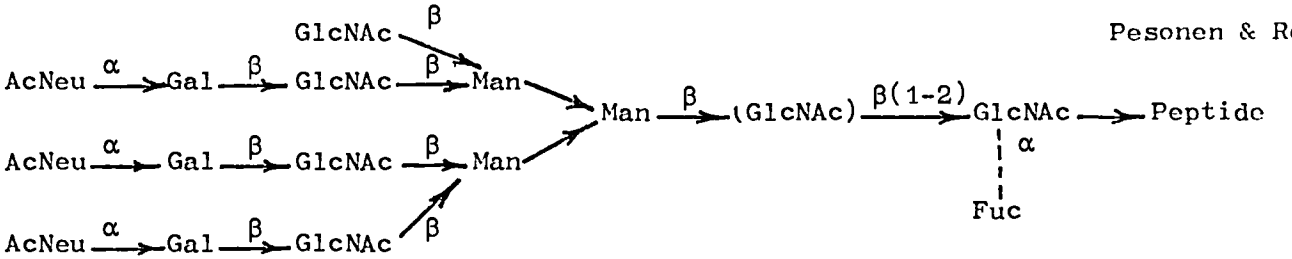
Glycoprotein	Structure	References
1. Pig submaxillary glycoprtein		Carlson (1968)
2. Thyroglobulin Unit A		Arima & Spiro (1972)
3. Fetuin		Spiro (1962, 1964, 1973)
4. Antifreeze glycoprotein		DeVries et al. (1971) Shier et al. (1975)
5. Basement membrane, collagen		Spiro (1967)

TABLE 5. Continued

Glycoprotein	Structure	References
6. Orosomucoid^{+} (desialised, human)		Hatcher & Jeanloz (1974)
7. Pineapple stem bromelain		Yasuda et al. (1970) Sukeno et al. (1971)
8. Proteoglycan ("internal"region)		Rodén (1968)
9. Hen egg albumin		Huang et al. (1970) Lee (1971) Sugahara et al. (1972)

The structure Gal $\xrightarrow{\beta(1-4)}$ GlcNAc $\xrightarrow{\beta(1-3)}$ could be attached to each GlcNAc* residue.

TABLE 5. Continued

Glycoprotein	Structure	References
10. Hen egg albumin		Tai et al. (1975)
	Glycopeptide V	
		
	Glycopeptide VI	
11. Semliki virus glycoprotein		Pesonen & Renkonen (1976)

+ The term α_1-acid glycoprtein and orosomucoid are synonymous

TABLE 5. Examples of the structure of the carbohydrate moieties of some glycoproteins

numbers 15, 38, 54, 75 and 85 (Ikenaka et al., 1972). The structure of the carbohydrate moiety is represented in Table 5, item 6, but there may well be some degree of heterogeneity within a given carbohydrate moiety and this aspect will be dealt with in the following section. The purpose of the experimental work described in this thesis was not directly concerned with the structures of the whole carbohydrate moieties of glycoproteins, but there are several relevant principles which may be considered because they have a bearing on the present studies. It is considered useful, in order to facilitate the discussion, to list a number of structures of carbohydrate moieties in various glycoproteins (Table 5), but it is also important to keep in mind the problems of heterogeneity.

7. Heterogeneity of carbohydrate moieties of glycoproteins

Recent developments in physical and chemical techniques provide the possibility of a thorough analysis of a glycoprotein. It has been found that even a highly purified preparation of a given glycoprotein contains molecular species which differ in their structures. Heterogeneity of glycoproteins could arise from the presence of "families" of molecules which differ only in their carbohydrate structures. It is important to stress that heterogeneity residing in the polypeptide chain, such as the variants of orosomucoid with either a glutamine or an arginine residue at position 20 (Nimberg et al., 1971), will not be considered generally.

Heterogeneity could arise from the presence of a carbohydrate prosthetic group on one molecular species and its absence in the others. Differences observed in a given glycoprotein found

in different species is of considerable interest too. It is not necessarily limited to differences in carbohydrate chains; it may also occur with regard to glycosylation of the polypeptide chain of the glycoprotein molecule. Some examples of differences of this nature will be discussed.

Early studies on ovalbumin glycopeptides (Kaverzneva & De Fan, 1961; Nuenke & Cunningham, 1961) indicated that the single oligosaccharide moiety (Neuberger, 1938) was not idential in all ovalbumin molecules. Cunningham (1968) clearly demonstrated that ovalbumin is heterogeneous with respect to the oligosaccharide moiety. The author in an elegant work showed that ovalbumin upon digestion with pronase, pepsin or a mixture of "trypsin and chymotrypsin followed by carboxypeptidase" yielded five glycopeptides. Thorough analyses of these glycopeptides revealed that although they all contained glucosamine and mannose, they differed in the composition of the carbohydrate prosthetic group. The heterogeneity observed was demonstrated not to be the result of the use of pooled ovalbumin from a number of individual hens, since glycopeptides from ovalbumin from single hens showed similar heterogeneity. Huang et al. (1970) characterized five homogeneous glycopeptides isolated from pronase digests of ovalbumin and subjected them to periodate oxidation. They showed that the glycopeptides differed only in carbohydrate composition. The carbohydrate-peptide linkage is, as discussed above, of the Asn-GlcNAc type. The overall structure is represented in Table 5, item 9. A somewhat different structure was proposed recently by Tai et al. (1975; Table 5, item 10). Specific α-D-mannosidase and N-acetyl-β-D-glucosaminidase were used by

Huang et al. (1970) in elucidating the structure.

A preparation of bovine pancreatic juice contains several types of ribonuclease (Plummer & Hirs, 1963, 1964; Reinhold et al., 1968). They showed different chromatographic behaviour but had similar enzymic properties. Amino acid compositions of the four different types A, B. C and D were identical. Ribonuclease A, the most abundant form, was devoid of any sugar, as mentioned above, while forms B, C and D of the enzyme were all glycosylated at asparagine 34 of the polypeptide chain. Ribonuclease B contained six residues of mannose and two of glucosamine. Ribonucleases C and D, in addition to mannose and glucosamine, contained fucose, sialic acid and galactose. The sugar compositions of these forms are:

Sugar	RNase A	RNase B	RNase C	RNase D
Glucosamine	-	2	4	4
Mannose	-	6	5	5
Galactose *	-	-	2.6	3.6
Sialic acid	-	-	2	4
Fucose *	-	-	1.4	1.7

*The values for galactose and fucose in RNase C and D were calculated from the ratios of $\frac{\text{Man}}{\text{gal}}$ and $\frac{\text{Fuc}}{\text{gal}}$ (Plummer, 1968).

Another interesting and similar example is provided by bovine α-lactalbumin which exists in two forms, A and B. Both the variants consist of four components, a major one (M) and three minor forms, F, S_1 and S_2; only the last two appear

to be glycoproteins. Component S_1 has 6-7 hexosamine, 6 hexose, one 6-deoxyhexose and one sialic acid residues. The component S_2 has similar sugar residues, except it does not contain sialic acid (Hopper & McKenzie, 1973). The site of attachment of the sugar to the protein is suggested to be through asparagine 45 in the molecule, because this is the only place where an asparagine sequon, Asn-Gln-Ser, occurs in the non-glycosylated form, (Hopper & McKenzie, 1973).

Worth mentioning is the rare occurrence of β-lactoglobulin Droughtmaster (Dr) in the milk of Droughtmaster cattle. This variant of β-lactoglobulin is a glycoprotein and it would appear that it carries carbohydrate at some point in the peptide chain between residues 21-40 where the sequence in β-lactoglobulin is:

$$\overset{28}{\text{...Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ileu-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg...}}$$

This deduction was made from the sequence analysis reported for β-lactoglobulin A (Braunitzer et al., 1972) and from the amino acid analyses reported for acid hydrolysates of the tryptic glycopeptide from β-lactoglobulin Dr and the corresponding peptide from β-lactoglobulin A (Bell et al., 1970). However, the sequence shown above does not contain the necessary sequon for N-acetyl-glucosaminylation, so that the amino acid sequences of the "tryptic" peptides just mentioned cannot be quite identical. If they differed in that the Asp residue (No. 28) were in fact Asn, in β-lactoglobulin Dr there would be an appropriate asparagine sequon at positions 28-30 and this seems to be the

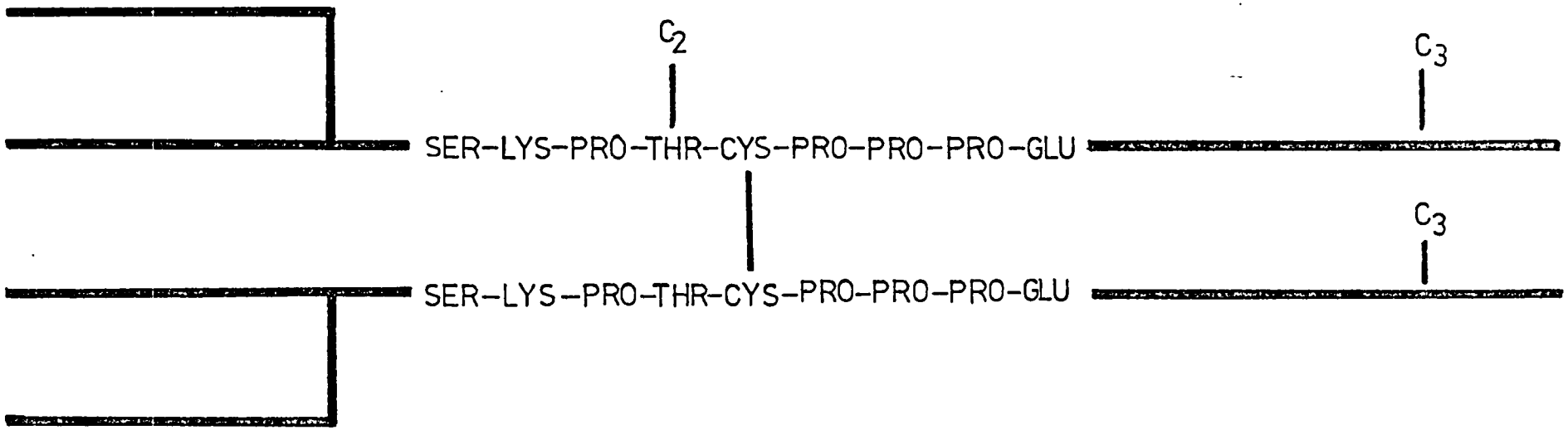


Fig. 2. A generalized representation of rabbit IgG showing asymmetric attachment of carbohydrate in the hinge region (C_2) and the carbohydrate moieties (C_3) attached to the asparagine residues in both γ-chains.

probable difference between the A and Droughtmaster variants. This difference would be genetically determined and would result from a base change in the codon for aspartic acid (GAU and GAC) to that for asparagine (AAU and AAC), that is a change of one purine to another purine. If this is indeed the case, it is perhaps not too surprising that A and Dr varients occur in equal amounts in the appropriate heterozygous animals , and the example is an extremely interesting one.

Smyth & Utsumi (1967) demonstrated that a preparation of rabbit IgG contained 0.4 residues of galactosamine per γ chain and that all the galactosamine was present as discussed below in an oligosaccharide attached in the hinge region of the γ chain. Blacklow & Marshall (1968) reported that rabbit IgG exhibited heterogeneity with respect to its carbohydrate composition and their findings may have been related, in part, to the items discussed in this section. Fanger & Smyth (1972), in a later report, confirmed that rabbit IgG, upon chromatography, could be separated into two families of molecules, one almost devoid of galactosamine (Type I) and the other containing ~ one residue of galactosamine per mole (Type II). This is attached through a threonine residue (Smyth & Utsumi, 1967), where the amino acid sequence may be represented by item 7, Table 2, but only one of the γ chains in a given molecule is substituted in this way. That is, the molecule is assymetric (see Fig. 2). Those molecules without a sequon at this point are of at least two types, one of which has the amino acid sequence shown in Table 2 (item 7) and an allotypic variant in which methionine replaces the threonine residue (Prahl et al., 1969). The latter

type lacks the appropriate sugar receptor point.

The presence of an assymetric structure in a multi-chain molecule once more focuses attention on the biosynthetic pathways and functions of these types of molecules. It is possible that the addition of a sugar in that region of the molecule causes a steric hindrance for the attachment of a second molecule to the second chain.

Variation in the sialic acid content of the carbohydrate content of a glycoprotein is also quite common. This is often easier to detect, firstly because it exerts charge differences which renders different electrophoretic and chromatographic behaviour of the glycoprotein, and secondly, sialic acid, if present, normally exists as a non-reducing terminal sugar which in most cases can be removed with neuraminidase. In fact, Schmid et al. (1962), by using starch gel electrophoresis at low pH, demonstrated seven components in a highly pure sample of α_1-acid glycoprotein. The starch gel electrophoresis was performed at pH 2.9, near the isoelectric point of the glycoprotein. Treatment of the protein with neuraminidase reduced the bands to three, which suggested that most of the heterogeneity was due to the sialic acid content of the glycoprotein. Similar reports were reported on hen ovomucoid by Beeley & Jevons (1965). They detected five components in ovomucoid upon electrophoresis at low ionic strength, three of which disappeared after neuraminidase treatment of the glycoprotein. Oshiro & Eylar (1968) reported the heterogeneity present in certain preparations of fetuin, in spite of apparent homogeneity shown by many conventional techniques. The

microheterogeneity was demonstrated by resolution of the glycoprotein into eight bands by starch gel electrophoresis at pH 4.2. Chromatography on DEAE-Sephadex of the fetuin preparation also gave several fractions, which showed similar immunological properties and sedimentation constants, but different sialic acid, hexose and hexosamine contents.

When glycoproteins contain more than one carbohydrate prosthetic group attached to their polypeptide chains, the heterogeneity might arise at a single locus or be due to differences in the composition of carbohydrate at different sites of attachment. Studies on porcine pancreatic ribonuclease (Jackson & Hirs, 1970a) revealed the presence of three carbohydrate prosthetic groups which were linked to asparagine residues 21, 34 and 76. The carbohydrate group attached to residue 34 was the simplest in the molecule and contained only mannose and glucosamine (Kabashama & Hirs, 1972), while the other two prosthetic groups attached to residues 21 and 76 were more elaborate and contained, in addition to mannose and glucosamine, fucose, galactose and N-glycollylneuraminic acid. Their compositions were found to be:

Sugar residue	Moiety attached at Asn residues		
	21	34	76
Fuc	1.0	0	1.1
Man	3.0	6.1	3.2
Gal	1.6	0	3.7
GlcNAc	6.1	2.1	10.2
NeuNGl	1.1	0	3.6

Rat[a]	Pig[b]	Goat[c]	Sheep[d]	Cattle[e]	Guinea-Pig A[f]	Guinea-Pig B[f]	Coypu[f]	Chinchilla[f]	Red deer[g]	Roe deer[g]	Aspergillus O.[h]	Bison[i]	Horse[j]
1 Gly					1 Ala	1 Ala	1 Ser						
	1 Lys	1 Lys	1 Lys	1 Lys				1 Lys	1 Lys	1 Lys		Lys	Lys
2 Glu	2 Gln	2 Glu	2 Glu	2 Glu	2 Glu	2 Glu	2 Glu	2 Glu	2 Glu	2 Glu		Glx	Glx
3 Ser	3 Ser	3 Ser	3 Ser	3 Thr	3 Ser	3 Ser	3 Ser	3 Ser	3 Ser	3 Ser		Thr	Ser
4 Arg													
5 Glu													
6 Ser		4 Ala	4 Ala	4 Ala					4 Ala	4 Ala		Ala	
7 Ser	4 Pro	5 Ala	5 Ala	5 Ala	4 Ser	4 Ser	4 Ser	4 Ser	5 Ala	5 Ala		Ala	Pro
8 Ala	5 Ala	6 Ala	6 Ala	6 Ala	5 Ala	5 Ala	5 Ala	5 Ala	6 Ala	6 Ala		Ala	Ala
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10 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys	7 Lys		Lys	Lys
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13 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg	10 Arg		Arg	Arg
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23 Lys											99 Asn		
	20 Ser	19 Ala	19 Ser	19 Ala	20 Ser	20 Ser	20 Ser	20 Ser	20 Ser	20 Ser	100 Phe	Ala	
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28 Tyr	25 Tyr	25 Tyr	25 Tyr	25 Tyr	25 Tyr	25 Tyr	25 Tyr	25 Tyr	25 (Tyr)	25 (Tyr)	1 Ala	Tyr	
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35 Arg	32 Arg	32 Ser	32 Ser	32 Ser	32 Lys	32 Arg	32 Ser	32 Gly Asp	32 Ser	32 Ser	6 Cys	Ser	
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Rat[a]	Pig[b]	Goat[c]	Sheep[d]	Cattle[e]	Guinea-Pig A[f]	Guinea-Pig B[f]	Coypu[f]	Chinchilla[f]	Red deer[g]	Roe deer[g]	Aspergillus O.[h]	Bison[i]	Horse[j]
37 Gly	34 ASN*	34 ASN	34 ASN*	34 ASN*	34 Glu	34 ASN*	34 ASN*	34 ASN*	34 Lys	34 ASN*			
38 Met	35 Met	35 Met	35 Leu	35 Leu	35 Met	35 Met	35 Met	35 Met	35 Met	35 Leu		Leu	
39 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr	36 Thr		Thr	
40 Lys	37 Gln	37 Glx	37 Gln	37 Lys	37 Lys	37 Gln	37 Gln	37 Gln	37 Gln	37 Gln	8 Ser	Lys	
41 Gly	38 Gly	38 Asp	38 Asp	38 Asp	38 Asp	38 Gly	38 Gly	38 Gly	38 Asp	38 Asp	9 Asn	Asx	
42 Ser	39 Arg	39 Arg	39 Arg	39 Arg	39 Arg	39 Arg	39 Arg	39 Tyr	39 Arg	39 Arg	10 Cys		
43 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	40 Cys	11 Tyr	Cys	
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64 Gly													
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74 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	71 Asn	36 Asn	Asx	Asx

Rat[a]	Pig[b]	Goat[c]	Sheep[d]	Cattle[e]	Guinea-Pig A[f]	Guinea-Pig B[f]	Coypu[f]	Chinchilla[f]	Red deer[g]	Roe deer[g]	Aspergillus O.[h]	Bison[i]	Horse[j]
75 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	72 Cys	37 Ser	Cys	Cys
76 His	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	73 Tyr	38 Tyr	Tyr	Tyr
77 Lys	74 Gln	74 Gln	74 Gln	74 Gln	74 Gln	74 Gln	74 Gln	74 Gln	74 Glx	74 Glx	39 Pro	Glx	Gln
78 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser	75 Ser		Ser	Ser
79 Ser	76 ASN	76 Tyr	76 Tyr	76 Tyr	76 Tyr	76 Tyr	76 Asn	76 Asn	76 Asn	76 Asn	40 His	Tyr	Ser
80 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser	77 Ser			Ser
81 Thr	78 Thr	78 Thr	78 Thr	78 Thr	78 Ser	78 Arg	78 Asn	78 Asn	78 Ala	78 Ala	41 Lys	Thr	Ser
82 Leu	79 Met	79 Met	79 Met	79 Met	79 Met	79 Met	79 Met	79 Met	79 Met	79 Met		Met	Met
83 Arg	80 His				80 His	80 Arg	80 His	80 His	80 His	80 His	42 Tyr		His
		80 Ser	80 Ser	80 Ser								Ser	
84 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	81 Ile	42 Asn	Ile	Ile
85 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	82 Thr	43 Asn	Thr	Thr
86 Asp	83 Asp	83 Asp	83 Asp	83 Asp	83 Glu	83 Asp	83 Asp	83 Asp	83 Asx	83 Asx	45 Tyr	Asx	Asp
87 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys	84 Cys		Cys	Cys
88 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	85 Arg	46 Glu	Arg	Arg
89 Leu	86 Gln	86 Glu	86 Glu	86 Glu	86 Leu	86 Val	86 Val	86 Leu	86 Glx	86 Glx	47 Gly	Glx	Leu
90 Lys	87 Thr	87 Thr	87 Thr	87 Thr	87 Thr	87 Thr	87 Thr	87 Thr	87 Ser	87 Ser		Thr	Thr
91 Gly	88 Gly	88 Gly	88 Gly	88 Gly	88 Ser	88 Ser	88 Ser	88 Ser	88 Gly	88 Gly		Gly	Ser
92 Ser	89 Ser	89 Ser	89 Ser	89 Ser	89 Gly	89 Ser	89 Asn	89 Asn	89 Asx	89 Asx	48 Phe	Ser	Gly
93 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser	90 Ser		Ser	Ser
94 Lys	91 Lys	91 Lys	91 Lys	91 Lys	91 Lys	91 Lys	91 Asp	91 Lys	91 Lys	91 Lys	49 Asp	Lys	Lys
95 Tyr	92 Tyr	92 Tyr	92 Tyr	92 Tyr	92 Phe	92 Phe	92 Tyr	92 Tyr	92 Tyr	92 Tyr	50 Phe	Tyr	Tyr
96 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro	93 Pro		Pro	Pro
97 ASN	94 Asn	94 Asn	94 Asn	94 Asn	94 ASN	94 ASN	94 ASN	94 ASN	94 Asn	94 Asn	51 Ser	Asx	Asx
98 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys	95 Cys		Cys	Cys
99 Thr	96 Ala	96 Ala	96 Ala	96 Ala	96 Ser	96 Ser	96 Ser	96 Ser	96 Val	96 Val		Ala	Ala
100 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr	97 Tyr			Tyr
101 ASN	98 Lys	98 Lys	98 Lys	98 Lys	98 Arg	98 Arg	98 Arg	98 Arg	98 Lys	98 Lys			Glx
102 Thr	99 Ala	99 Thr	99 Thr	99 Thr	99 Thr	99 Met	99 Thr	99 Thr	99 Ala	99 Ala	52 Val		Thr
103 Thr	100 Ser	100 Thr	100 Thr	100 Ser	100 Ser	100 Ser	100 Ser	100 Ser			53 Ser	73 Ser	Ser
104 Asn	101 Gln	101 Gln	101 Gln	101 Gln	101 Gln	101 Gln	101 Gln	101 Arg			54 Ser		Glx
105 Ser											55 Pro		Ser
	102 Glu	102 Ala	102 Ala	102 Ala	102 Ala	102 Ala	102 Gln	102 Glu				74 Gly	
106 Glu	103 Gln	103 Gln	103 Gln	103 Asn	103 Gln	103 Gln	103 Gln	103 Asn			56 Tyr	75 Ala	Glx
107 Lys	104 Lys	104 Lys	104 Lys	104 Lys	104 Lys	104 Lys	104 Lys	104 Lys			57 Tyr		Lys
108 His	105 His	105 His	105 His	105 His							58 Glu	76 Asp	
					105 Ser	105 Ser	105 Ser	105 Gly					
											59 Trp		
											60 Pro	77 Arg	
109 Ile	106 Ile	106 Ile	106 Ile	106 Ile	106 Ile	106 Ile	106 Ile	106 Ile			61 Ile	78 Val	
110 Ile	107 Ile	107 Ile	107 Ile	107 Ile	107 Ile	107 Ile	107 Val	107 Ile			62 Ile	79 Val	

Rat[a]	Pig[b]	Goat[c]	Sheep[d]	Cattle[e]	Guinea-Pig A[f]	Guinea-Pig B[f]	Coypu[f]	Chinchilla[f]	Red deer[g]	Roe deer[g]	Aspergillus O.[h]	Bison[i]	Horse[i]
111 Ile	108 Val	108 Val	108 Val	108 Val	108 Val	108 Val	108 Val	108 Val					
112 Ala	109 Ala	109 Ala	109 Ala	109 Ala	109 Ala	109 Ala	109 Ala	109 Ala			63 Ser 80 Phe		
113 Cys	110 Cys	110 Cys	110 Cys	110 Cys	110 Cys	110 Cys	110 Cys	110 Cys			64 Ser 81 Asn	Cys	
114 Asp	111 Glu	111 Glu	111 Glu	111 Glu	111 Glu	111 Glu	111 Glu	111 Glu			65 Gly 82 Glu	Glu	
115 Gly	112 Gly	112 Gly	112 Gly	112 Gly	112 Gly	112 Gly	112 Gly	112 Gly			66 Asp 83 Asn	Gly	
116 Asn	113 Asn	113 Asn	113 Asn	113 Asn	113 Lys	113 Asp	113 Asn	113 Asn			84 Asn	Asn	
117 Pro	114 Pro	114 Pro	114 Pro	114 Pro	114 Pro	114 Pro	114 Pro	114 Pro			85 Glu	Pro	
118 Tyr		115 Tyr	115 Tyr	115 Tyr	115 Tyr	115 Tyr	115 Tyr	115 Tyr				Tyr	
	115 Pro										86 Leu		
119 Val	116 Val	116 Val	116 Val	116 Val	116 Val	116 Val	116 Val	116 Val			87 Val	Val	Val
120 Pro	117 Pro	117 Pro	117 Pro	117 Pro	117 Pro	117 Pro	117 Pro	117 Pro				Pro	Pro
121 Val	118 Val	118 Val	118 Val	118 Val	118 Val	118 Val	118 Val	118 Val			67 Val 88 Gly	Val	Val
122 His	119 His	119 His	119 His	119 His	119 His	119 His	119 His	119 His			68 Tyr	His	His
123 Phe	120 Phe	120 Phe	120 Phe	120 Phe	120 Phe	120 Phe	120 Phe	120 Phe			69 Ser 89 Val	Phe	Phe
124 Asp	121 Asp	121 Asp	121 Asp	121 Asp	121 Asp	121 Asp	121 Asp	121 Asp			70 Gly		
125 Ala	122 Ala	122 Ala	122 Ala	122 Ala	122 Asn	122 Ala	122 Ala	122 Ala			71 Pro		
126 Ser	123 Ser	123 Ser	123 Ser	123 Ser	123 Ser	123 Ser	123 Ser	123 Ser			72 Gly		
127 Val	124 Val	124 Val	124 Val	124 Val	124 Val	124 Val	124 Val	124 Val			90 Ile		
	125 Glu	125 Ala											
	126 Pro	126 Ala											
	127 Ser	127 Ser											
	128 Thr	128 Ala											

References

(a) Beintema & Gruber (1967)
(b) Jackson & Hirs (1970b)
(c) Welling et al. (1974)
(d) Kobayashi & Hirs (1973)
(e) Smyth et al. (1963)
(f) Van Den Berg & Beintema (1975)
(g) Zwiers et al. (1973)
(h) Takahashi (1971)
(i) Stewart & Stevenson (1973)
(j) Scheffer & Beintema (1974)

TABLE 6. Amino acid sequences of ribonucleases isolated from different sources. Those from animals are the pancreatic enzymes. The residues are aligned using appropriate Braunitzer gaps. Where the full amino acid sequence is not known the residues are not numbered. Asparagine residues in "sequon" are shown by capital letters and those carrying carbohydrate are represented in a square box. ASN* demonstrates partial glycosylation.

A carbohydrate group similar to the one attached to position 34 of porcine ribonuclease is also present at residue 34 of ribonuclease B from bovine pancreatic juice. In fact, this is the only position at which a prosthetic group is attached to bovine ribonuclease (Table 6).

Another interesting example of the variations between glycoproteins of different species is found in the submaxillary mucins obtained from cattle and from the pig. The prosthetic groups of the former are predominantly of the structure 6-α-sialyl-N-acetyl-α-D-galactosaminyl (Gottschalk & Graham, 1959), whereas those in the pig glycoprotein have the structure represented as item 1, Table 5.

It is true that we know very little about the *in vivo* function of the carbohydrate group of many glycoproteins and therefore it is a very difficult task to relate heterogeneity of the structure or differences seen between species to the function of the glycoprotein.

The variations observed between analogous glycoproteins from different species may be due to different levels of activity of glycosyl transferases or of the activated sugar, or to the presence or absence of appropriate amino acid accepting sites or, of course, might in some cases be due to the presence of glycosidases. Because of these considerations, especially the nature of the activated sugar, it is important to consider in general terms the mechanism or mechanisms of glycosylation.

8. Biosynthesis of glycoproteins

So far as is known at present, it appears that polypeptide moieties of all glycoproteins are assembled on membrane-bound ribosomes (Lawford & Schachter, 1966; Molnar & Sy, 1967), although it has been suggested that the submaxillary mucins are formed initially as a large number of polypeptides which undergo polymerization and glycosylation subsequently in the Golgi (Downs & Pigman, 1969; Pigman et al., 1970). This hypothesis requires further consideration.

In general the apoproteins which are synthesized on the ribosomes are then transferred into the cysternae of endoplasmic reticulum (Palade et al., 1962; Redman et al., 1966; Redman, 1967). Most of the sugar residues are incorporated into glycoproteins following the release of the polypeptide backbone from the ribosomes, although there is evidence that N-acetyl-D-glucosamine and D-mannose residues of the core may be incorporated, to some extent, while the protein is still membrane-bound (Lawford & Schachter, 1966; Molnar & Sy, 1967; Sherr & Uhr, 1969). These findings, as also those described in the present experimental studies, are not wholly interpretable at present because of the evidence that oligosaccharide moieties may be added, largely, as a complete entity by transfer from an oligosaccharide-lipid intermediate (Waechter & Lennarz, 1976). This problem is of a controversial nature and will be discussed further

The more terminal sugars such as fucose, galactose and sialic acid are incorporated into the growing carbohydrate chain after the glycoprotein has left the endoplasmic reticulum.

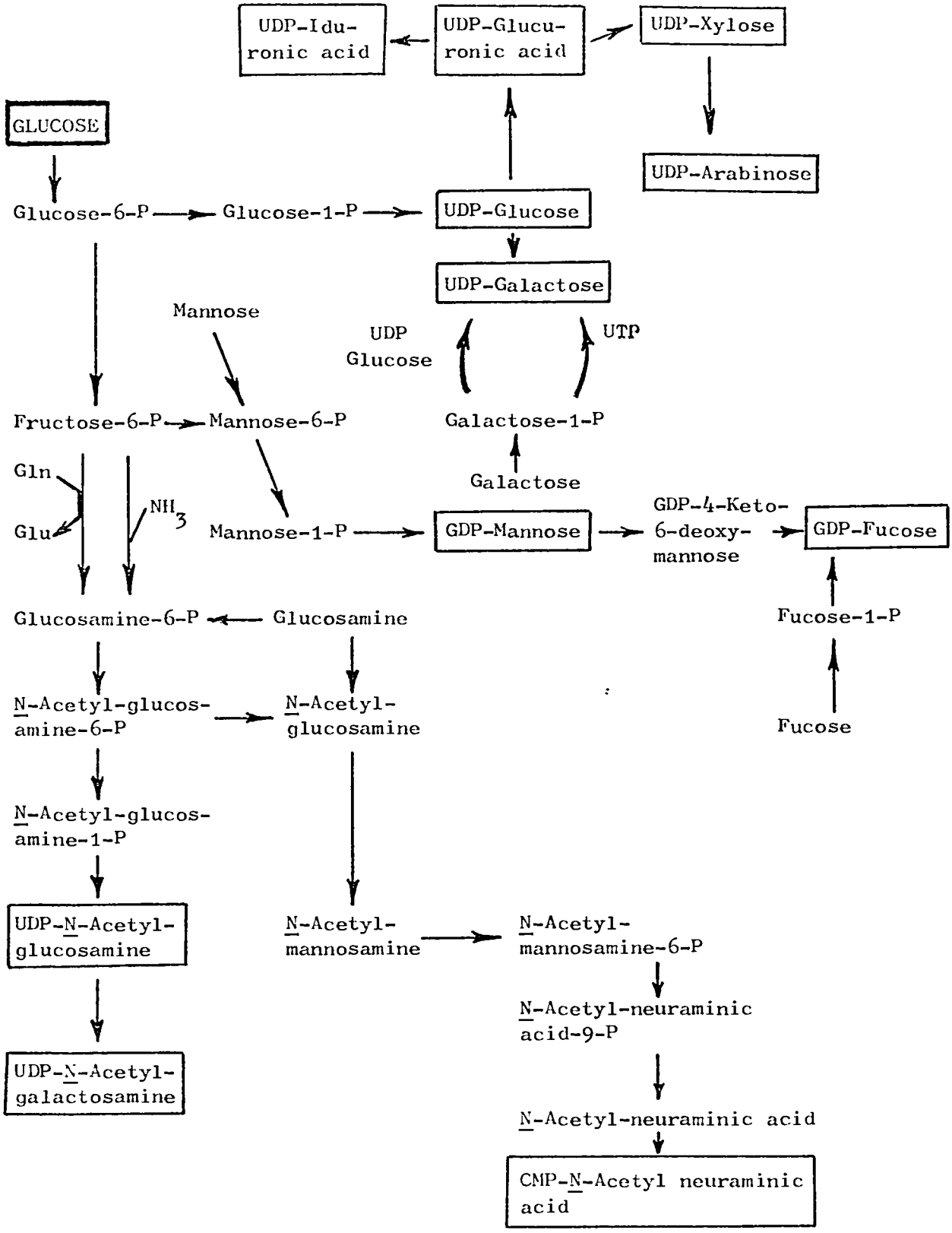


Fig.3. Representation of the pathways of nucleotide-sugar formation from glucose (from Schachter & Rodén, 1973)

The addition of sugars is controlled by the specific glycosyl transferases which are available (Ginsburg, 1964; Roseman, 1968).

(i) Biosynthesis of the protein chain of the glycoproteins

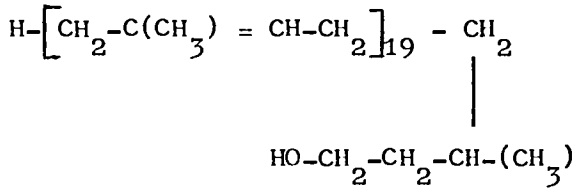
The structure of a given protein is specified by the ordered sequence of amino acids dictated by an RNA template during its synthesis (Crick, 1963). The protein structure of the glycoproteins in general follows the same pathways of synthesis as a normal protein. The validity of such a statement rests on the results of the experiments using protein inhibitors and subcellular localization of the growing polypeptide chain. Puromycin has been used extensively as an inhibitor for protein synthesis in in vitro experiments (Yarmolinsky & de la Haba, 1959; Lawford & Schachter, 1967; Molnar et al., 1965). Puromycin completely inhibits incorporation of the radioactive amino acid into glycoproteins. The site of incorporation of amino acids into glycoproteins has also been studied extensively by subcellular fractionation and histoautoradiographic methods involving the use of radioactive labelled amino acids (Lawford & Schachter, 1966; Molnar & Sy, 1967).

(ii) Biosynthesis of activated sugar

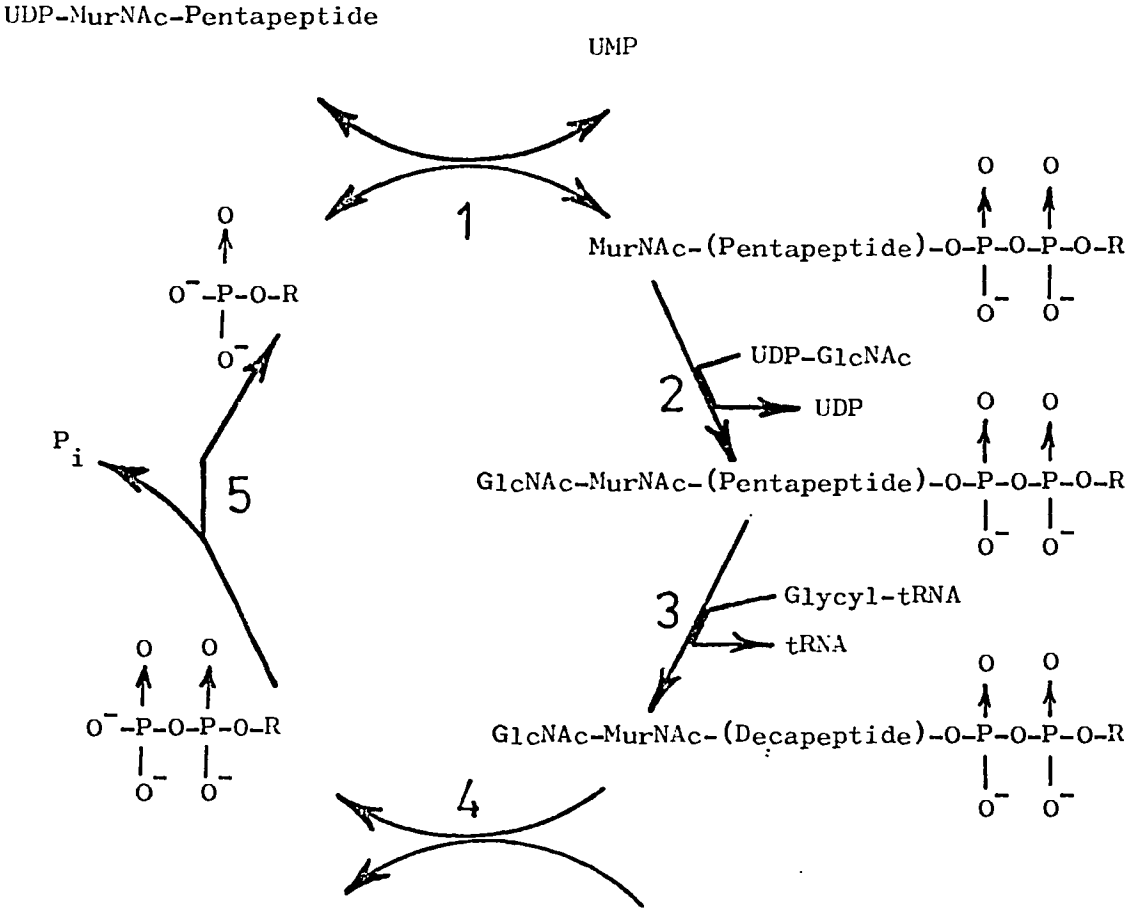
Glucose is believed to be the precursor of the sugars present in glycoproteins. The pathways by which sugar nucleotides are formed from glucose are outlined (see p. 51). The various reactions have been studied in detail by numerous workers and the reader is referred to the many review articles available on the subject (Ginsburg, 1964; Bostrom & Rodén, 1966; Neufeld & Ginsburg, 1965; Jourdian & Roseman, 1963; Sharon, 1966;

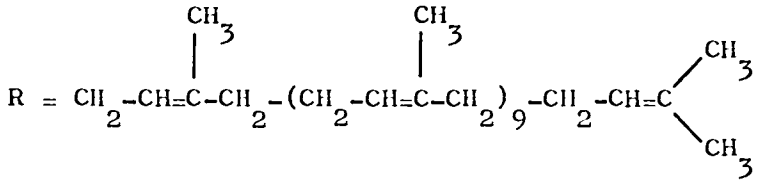
Stoolmiller & Dorfman, 1969; Rodén, 1970; Schachter & Rodén, 1973). Dietary glucosamine, mannose and other sugars may also participate directly in conversion to activated intermediates.

For reasons which will be mentioned later, it is useful to consider the evidence for subsequent conversion of the nucleotide sugars to dolichyl phosphate-linked sugars. Dolichol, isolated from pig liver and from human kidney, was shown to be an isoprenoid alcohol with the structure (Burgos et al., 1963) which may be represented as:



This compound is believed to be that (Burgos et al., 1963) isolated originally from pig liver by Channon et al. (1934) and called at that time hepene. Dolichol is normally present in liver as a fatty acyl ester (Butterworth & Hemming, 1968; see also Hemming, 1974).





Presentation of the biosynthesis of the cell wall peptidoglycan (from Lennarz & Scher, 1972).

A number of animal tissues have been shown to possess the enzymic activity needed to catalyse formation of dolichyl-phosphate linked sugars (Table 7) in reversible reactions of the forms:

$$\text{GDP - Man} + \text{DMP}^{*} \rightleftharpoons \text{DMP - Man} + \text{GDP}$$

$$\text{UDP - Glc} + \text{DMP} \rightleftharpoons \text{DMP - Glc} + \text{UDP}$$

$$\text{UDP - GlcNAc} + \text{DMP} \rightleftharpoons \text{DPP}^{*} \text{ - GlcNAc} + \text{UMP}$$

(*These abbreviations (DMP and DPP) are defined in a footnote to Table 7).

The possible participation of the lipid-linked sugars are discussed:

(iii) Lipids as intermediates in glycoprotein biosynthesis

Strominger and his co-workers (1965) discovered that a glycolipid formed from endogenous lipid in the particulate preparations of Staphylococcus aureus and Micrococcus lysodeikticus is an obligatory intermediate in peptidoglycan biosynthesis. This glycolipid contained N-acetyl-glucosamine and N-acetyl-D-muramyl pentapeptide which after addition of further glycine residues to the side chain is transferred to the growing murein. Isolation, purification and structural analysis of the lipid revealed that a pyrophosphate unit joins the reducing end of the substituted acid residue to the lipid which is a polyisoprenoid alcohol. A schematic presentation for the biosynthetic pathway for the cell wall peptidoglycan is given on p. 54.

Tissue preparation	Donor molecule	Product	Reference
Pig liver endoplasmic reticulum	GDP-Man	DMP-Man	Richards & Hemming, 1972
Rat liver microsomes[a]	UDP-Glc	DMP-Glc[b]	Behrens et al. (1971)
	UDP-GlcNAc	DPP-GlcNAc	Molnar et al. (1971)
	GDP-Man	DMP-Man	Behrens et al. (1971)
Calf pancreas microsomes	GDP-Man	DMP-Man[c]	Tkacz et al. (1974)
Red liver membrane-rich fractions	GDP-Man	Dolichyl mannosyl phosphate and mannosyl retinyl phosphate	Rosso et al. (1975)
Mouse mastocytoma microsomes	UDP-Gal	Retinol-Gal	Helting & Peterson (1972)
Rat liver smooth endoplasmic reticulum	UDP-GlcNAc	Vitamin-K-GlcNAc[d]	Johnson & Volkovich (1972)
Tumour (MOPC 46B) microsomes	GDP-Man	"DMP"-Man[e]	Baynes et al. (1973)

(a) Neither UDP-Gal or UDP-GalNAc acted as donors for the synthesis of the corresponding dolichyl phosphate-linked sugars (Behrens et al, 1971), but see text
(b) The sugar is probably of β-anomeric configuration because the compound yields 1,6 anhydroglucosan on treatment with alkali (Behrens & Leloir, 1970)
(c) The sugar was shown to be of β-anomeric configuration (Herscovics et al., 1974)
(d) Vitamin K, or a derivative thereof
(e) The authors showed that the lipid was a dolichylisoprenol with at least 18 isoprene units, one of which is saturated. It is thus at least similar to dolichol and may be identified with that lipid

TABLE 7. Examples of enzymic formation of dolichyl phosphate, and other lipid-linked sugars, catalysed by preparations from animal tissues. The reactions are reversible (DMP and DPP means dolichyl monophosphate and dolichyl pyrophosphate respectively)

The lipid acceptor was shown to be undecaprenyl phosphate. An activated lipophilic derivative is synthesised in the membrane, from UDP-N-acetyl-muramyl pentapeptide (Anderson et al., 1967) which remains intact until the first transfer of sugar from the lipid to form a glycosidic bond with the acceptor polysaccharide. Then the disaccharide-linked peptide is formed which is followed by the modification of the peptide chain (Kamiryo & Matsuhashi, 1969). The sugar is now transferred to the endogenous polysaccharide acceptor and undecaprenyl pyrophosphate is released.

Similar pathways operate in the synthesis of an antigen portion of the lipopolysaccharide of the cell envelope of Salmonella typhimurium (Weiner et al., 1965). A phospho-diester lipophilic sugar intermediate was also found to be involved in mannan synthesis catalysed by a particulate preparation from M. lysodeikticus; the system used GDP-mannose. The acceptor lipid was shown to be undecaprenyl phosphate (Lahav et al., 1969).

Intermediates of a similar nature have been observed in lower plants. Tanner (1969) discovered an acid-labile mannose-containing lipid formed from GDP-mannose, in yeast. This lipid acted in vitro as a donor in the transfer of mannose to mannan. There was metal requirement for the transfer in both reactions, the transfer to lipid required either Mg^{+2} or Mn^{+2}, while mannan production needed Mn^{+2}. Later studies by Tanner et al. (1971) showed that dolichol monophosphate isolated from both yeast and liver served as an acceptor lipid in the transfer of mannose from GDP-mannose catalysed by preparations from either of the two sources. Addition of dolichyl phosphate to the preparation

enhanced the incorporation of radioactivity into alcohol insoluble material and the addition of GDP caused a decrease in the radioactivity of dolichyl monophosphate mannose. The system is very similar to the one described above in M. lysodeikticus, and it was for this reason that the authors suggested that a similar phosphodiester link exists here.

The presence of polyisoprenols in higher plants (Wellburn & Hemming, 1966a,b), suggests that similar pathways exist in the transfer of sugar residues. Kauss (1969) observed, in a preparation of mung bean shoots, the transfer of mannose from GDP-mannose to a lipid. The reaction was reversible. Time-course studies of lipid and mannan formation led to results which suggested that the lipid was an intermediate in the mannan synthesis.

As was mentioned above (see p. 53) there is considerable evidence that animal tissues also contain dolichol and that appropriate enzymic activity is present which can catalyse formation of dolichyl phosphate-linked sugars. Some of the evidence for these reactions will be discussed in greater detail. There are a number of observations which indicate that DMP-mannose, a phosphodiester involving C-1 of the sugar may be formed.

Caccam et al. (1969), using particulate fractions of rabbit liver, hen oviduct and myeloma tumour, found incorporation of mannose from GDP-mannose into both glycoprotein and lipid. The mannolipid was closely similar in solubility and chromatographic properties to the "dolichyl" phosphate mannose (which has 55 carbon atoms in the isoprenoid chain)described by Scher et al. (1968).

The nature of the lipid, at least in the case of pig liver,

was examined more thoroughly by Richards et al. (1971) who synthesised high specific activity [^{3}H]-dolichyl phosphate and incubated it with GDP-[^{14}C]-mannose in the presence of a microsomal preparation. The sugar-lipid product was purified and was found to contain both [^{14}C] and [^{3}H] activity and had the chromatographic behaviour of mannose dolichyl phosphate. Acid hydrolysis of the compound released [^{14}C]-mannose and [^{3}H]-dolichyl phosphate.

Not only D-mannose can be transferred by liver microsomal preparations from nucleotide sugar (see also Tetas et al., 1970; Behrens et al., 1971), but N-acetyl-D-glucosamine also, as shown by the studies of Tetas et al. (1970) who showed that transfer of the sugar occurred to a lipid. Moreover, the product was identified by Behrens et al. (1971) as dolichyl pyrophosphate N-acetyl-D-glucosamine. Although the latter workers reported that transfer of galactose or of N-acetyl-D-galactosamine from their UDP derivatives did not occur under the catalytic influence of preparations of rat liver microsomes, Tetas et al. (1970) reported the production of lipid-containing derivatives of both of these sugars under the catalytic influence of microsomal preparations from rabbit. Preparations from the rat appeared to be much less stable.

Vessey et al. (1976) studied the enzymes catalysing the transfer of mannose, glucose and N-acetyl-glucosamine from GDP-mannose, UDP-glucose and UDP-N-acetyl-glucosamine respectively to endogenous dolichyl phosphate using Golgi membranes of liver. Their results suggest that all these reactions utilize the same pool of dolichyl phosphate. However, the enzymescatalysing the transfer of each sugar are different. This conclusion was

based on nucleotide inhibition studies.

Monosaccharide derivatives of isoprenoid phosphate have been found in a number of eukaryotic cells. The sugars include not only those just mentioned but also D-xylose and N-acetyl-D-mannosamine (see Waechter & Lennarz, 1976).

Lipids other than dolichol may act as acceptors of sugars from nucleotide sugars (see Table 7), but this area is not as extensively investigated.

One of the crucial unanswered questions is whether the carbohydrate moieties which are linked to asparagine residues are transferred to the protein chain, at least largely, as completed oligosaccharide moieties or whether they occur by a stepwise addition of the various sugar residues (Waechter & Lennarz, 1976). In the experiments described in Chapters 3 and 5, it has been shown that monoglycosylated pancreatic ribonuclease can be produced *in vitro* by catalytic activity present in liver and in serum. It would be presumptious at this time to make a categorical assertion as to the mechanism of biosynthesis of the types of glycoproteins in question, and it is important to consider the evidence for the involvement of transfer of an oligosaccharide moiety from a lipid-oligosaccharide to the protein chain. It is also important to consider the evidence for other synthetic mechanisms.

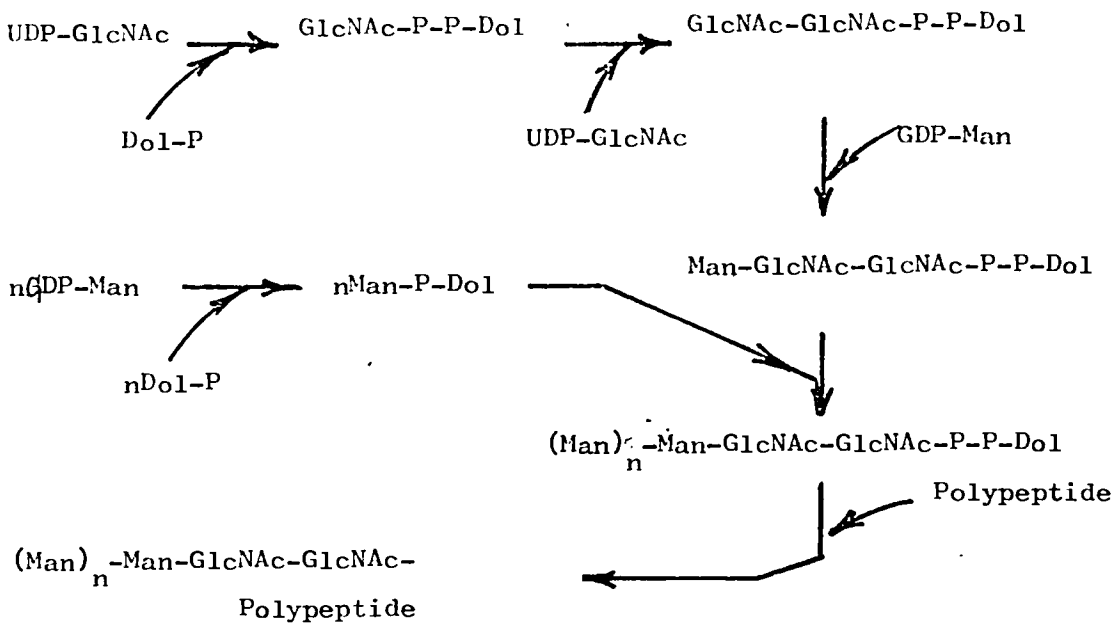
The work of Leloir *et al.* (1973) with the use of rat liver microsomal preparations led to some interesting results. These authors showed the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine to dolichyl pyrophosphate N-acetyl-glucosamine. This step did not involve a lipid intermediate. The carbohydrate

lipid, upon hydrolysis under mild conditions yielded N,N'-diacetylchitobiose. The formation of the disaccharide-containing lipid was increased by incubating with crude liver lipids. The formation of N,N'-diacetylchitobiose-containing lipid could imply that this lipid is responsible in transferring the core sugars of some glycoproteins which have N,N'-diacetylchitobiose linked to asparagine residues (Table 5, items 2, 3, 7 and 9 are examples). It was also demonstrated that preparations of rat liver microsomes can catalyse formation of the carbohydrate lipid represented by DPP-$(GlcNAc)_2$ $(Man)_x$ $(Glc)_2$ (where x = 13-16) and it was postulated that this was an intermediate in glycoprotein synthesis (Behrens et al., 1973), but the presence of glucose in the moiety was unexplained. Heath et al. (1974) showed that oligosaccharide-lipid synthesised by mouse myeloma preparations contained five mannose and two N-acetyl glucosamine residues and the structure of $(Man)_5$ -- $(GlcNAc)_2$ -P-P-dolichol was postulated for the lipid.

When preparations of hen oviduct membrane were incubated under appropriate conditions with GDP-[^{14}C]-mannose, both a labelled glycolipid and a labelled glycoprotein were produced (Chen et al., 1975). The carbohydrate moieties of each product were closely similar or even identical and were shown to have the structure represented below:

$$(\alpha\text{-Man})_n\text{-}\beta\text{-Man-}(1 \rightarrow 4)\text{-}\beta\text{-GlcNAc } (1 \rightarrow 4)\text{-GlcNAc}$$

They postulated the following scheme for the participation of the lipid intermediate in glycoprotein biosynthesis, although they pointed out that "not all the steps in this sequence have been documented in detail".



Other examples of lipid-linked oligosaccharides formed by membrane preparations from a number of tissues are listed by Waechter & Lennarz (1976).

The results of all these studies might be interpreted as an indication that incorporation of sugars into asparagine-linked carbohydrate moieties of glycoproteins occurs by a mechanism involving transfer of an oligosaccharide from a lipid intermediate to the protein chain.

The natural question to arise, however, is whether these dolichyl phosphate-linked oligosaccharides can act in eukaryotic cells as donors to asparagine residues of apoproteins or to other sugars already linked to a protein chain. So far there has been no direct evidence presented that an asparagine acts as an acceptor for such oligosaccharides, although this is sometimes assumed (see Waechter & Lennarz, 1976). However, transfer of oligosaccharides to protein from lipid intermediates has been demonstrated, even although the point of attachment is unknown.

As examples one might mention their transfer to endogenous protein acceptors in preparations of rat liver microsomes (Behrens et al., 1973), of oviduct membranes (Lucas et al., 1975), of myeloma microsomes (Hsu et al., 1974) and to a K-light chain (MOPC 46) which had been produced in a non-glycosylated form by culturing the myeloma cells in the presence of 2-deoxy-D-glucose (Eagon et al., 1975). It was not shown whether the oligosaccharide becomes linked to asparagine residue 28, the normal site of linkage in the glycoprotein (Coleman et al., 1967; Melchers, 1969).

(iv) Possible subcellular site of incorporation of N-acetyl-glucosamine residue to the asparagine residue

There is often evidence which would make one tend to believe that the oligosaccharide moieties which are linked to asparagine residues in glycoproteins are assembled one by one on the growing carbohydrate chain. In this hypothesis each assembly requires a specific sugar transferase and the product of each reaction becomes the substrate for the next sugar to be added.

The attachment of the first sugar to the polypeptide backbone has been the subject of numerous investigations. It was shown that a small amount of N-acetyl-glucosamine becomes incorporated into proteins produced by liver while the polypeptide is still attached to the ribosomes.

The subcellular particles which incorporate sugar into serum glycoproteins have been examined. When rat liver was perfused with blood containing added [^{14}C]-galactose (Sarcione, 1964) or [^{14}C]-glucosamine (Sarcione et al., 1964), the microsomal protein became labelled only to a small extent while the

membranous (deoxycholate soluble) fraction of microsomes was highly labelled. In other studies there was a definite labelling of ribosomes and deoxycholate-soluble microsomal fractions; also the specific activity of the ribosomal hexosamine was higher than that of the membrane (Molnar et al., 1965). These experiments suggest that the main site of attachment of sugar to polypeptide chains of serum proteins is the endoplasmic reticulum. However, it is difficult to interpret the results precisely because glycoproteins with different structures and rates of biosynthesis were studied. When the biosynthesis of a family of closely related α_1-acid glycoproteins of guinea-pig serum were studied it indicated that some incorporation of glucosamine occurs at the ribosomal level (Simkin & Jamieson, 1967).

The general concept that N-acetyl-glucosamine may be attached to asparagine residues while the polypeptide chains of the serum glycoproteins are still attached to the polysomes is further supported by investigation of newly formed proteins in the endoplasmic reticulum (Redman & Cherian, 1972).

It is unlikely that the glycosylation step occurs independently of peptide synthesis because of the specific requirement of an asparagine sequon as acceptor site. It may be noted also that Sinohara & Sky-Peck (1965) were unable to detect GlcNAc-Asn-tRNA in the extracts of mouse liver.

(v) Some aspects of the glycosyl transferases which may be involved in biosynthesis of glycoproteins

There are other aspects which need to be explained if biosynthesis of glycoproteins occurs by way of oligosaccharide-

lipid intermediates. One of these concerns the fact that a number of glycosyl transferases have been purified and can use *in vitro* nucleotide sugars as donors and glycoproteins or ones previously treated with glycosidases as acceptors which are relatively specific.

A thorough knowledge of the *in vivo* function of sugar transferases may be obtained by elucidating more fully their mechanism *in vitro*. A number of them need to be purified to homogeneity, their kinetics thoroughly investigated and, moreover, the natures of their binding sites shown. Do these transferases have binding sites for lipid sugars as well as for nucleotide sugars? Can the catalytic transfer by-pass the lipid intermediate step *in vitro* even if not *in vivo*? If particular isolated glycosyl transferases can use only nucleotide sugars as donors for the transfer of sugar to specific acceptor and yet there is still, *in vivo*, a need for lipid sugar intermediate, what are the functions of these types of sugar transferases?

(a) N-Acetyl glucosaminyl transferases

N-Acetyl-glucosamine appears in different positions in the carbohydrate prosthetic group of a glycoprotein (see Table 5). It occurs, linked to asparagine residues of polypeptide chains, next to galactose or with mannose. It is therefore likely that different enzyme systems would be responsible for transferring sugar to the appropriate position. In cases where endogenous acceptor(s) is used to assay the N-acetyl-glucosaminyl transferase(s), one is faced with the difficulty of

assessing which transferase is actually being dealt with.

One of the most extensively studied N-acetyl-glucosaminyl transferases was first demonstrated in goat colostrum (Johnston et al., 1966). The enzyme was purified 100- to 200-fold and found to have a metal ion requirement. The pH optimum for the enzyme was broad, practically constant from 6.0 to 8.2, the K_m values (approximate) were 8×10^{-4} M for UDP-N-acetyl-glucosamine and 4×10^{-3} M for the acceptor, which was orosomucoid previously treated with glycosidases. The enzyme appeared to be specific with respect to its requirement for a non-reducing terminal mannose residue and needed a high molecular weight acceptor as it did not transfer N-acetyl-glucosamine to any of the low molecular weight acceptors studied. The enzyme was also detected in several rat tissues (Johnston et al., 1966), large intestine, lung, kidney, mammary gland and liver. The presence of another soluble form of this enzyme in pig serum was demonstrated by Hudgin & Schachter (1971). They used α_1-acid glycoprotein pretreated with glycosidases to remove sialic acid, galactose and N-acetyl-glucosamine from the carbohydrate chain. The enzyme was precipitated mainly in the 33-55% ammonium sulphate fraction and the activity was not significantly stimulated by Triton X-100, had metal ion requirement and an effective pH range of 6.5-8.5. They also reported a similar enzyme in pig liver which was membrane-bound. They prepared a soluble form of this particulate enzyme by acetone powder extraction and compared the specificity of the three preparations. They all showed a broad optimum of Mn^{+2}, centering at about 0.025 M. The liver enzyme showed a sharp pH dependence centering around 6.5, in contrast to the

broad optimum of serum enzyme and acetone powder extract. They all showed linearity with respect to time of incubation; with serum enzyme up to 120 min, liver acetone powder extract up to 90 min and liver enzyme up to 60 min.

Mookerjea et al. (1971) reported another soluble form of this enzyme in rat and human serum. The enzyme had an absolute requirement for Mn^{+2} and neither Ca^{+2} or Mg^{+2} could replace it. It did not require Triton X-100 for its activity which was linear with respect to time and protein concentration. The optimum pH for human enzyme was ~ 7 and higher than the rat enzyme but similar to pig liver and serum enzyme. They used exogenous acceptors and found that it was most active towards α_1-acid glycoprotein pretreated with glycosidases. However, the enzyme could also catalyse the transfer of N-acetyl-glucosamine to ribonuclease B and egg albumin at a very slow rate. Although the nature of the bond between mannose and N-acetyl-glucosamine in these studies was not investigated, it seems likely that the enzyme is one of the glycosyl transferases responsible for incorporation of that N-acetyl-glucosamine which is nearest the non-reducing terminus of the oligosaccharide prosthetic groups. It is of course conceivable that the enzyme preparation contained sufficient dolichol derivatives to participate as intermediates, although so far such lipids have not been demonstrated in serum. It seems clear, however, that in one in vitro system just discussed, a monosaccharide was added and not an oligosaccharide. There are also enzymes with N-acetyl-glucosamine transfer activity in cell membrane where of course dolichol phosphate may well be present.

Schachter et al. (1970) reported the presence in rat liver of such an enzyme. Their studies showed that the enzyme is membrane bound and situated mainly in Golgi rich fractions. This enzyme needed Mn^{+2} for its full activity and was stimulated by Triton X-100; however, it has a slightly different pH optimum from that of the goat colostrum enzyme. The authors studied the enzyme activity toward both endogenous and exogenous acceptors, the exogenous acceptor used being α_1-acid glycoprotein from which the terminal trisaccharide had been removed by glycosidases. UDP-N-acetyl-glucosamine was used as the sugar donor. They found that the values obtained for endogenous acceptors were not significantly higher than those obtained with heat-treated enzyme and ranged from 2-10% of those obtained with the use of exogenous acceptor. Bosmann (1970) found, in pig liver, an enzyme system which could catalyse the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine into a defined glycoprotein acceptor and also into endogenous acceptor(s). The exogenous protein acceptor used was fetuin treated with glycosidases to expose terminal mannose residues. The enzyme transferring N-acetyl-glucosamine to this special glycoprotein needed an exposed mannose residue. The enzyme was not active towards small molecules nor, surprisingly, towards α_1-acid glycoprotein lacking three terminal sugar residues which, it will be recalled (see above) acted as a good acceptor when the enzyme from pig serum was used (Hudgin & Schachter, 1971). The exogenous and endogenous systems differed very slightly in cation effect, temperature curve and specific activity: No other major differences could be detected.

Among the other glycosyl transferases studied one might consider the isolation of the N-acetyl-glucosaminyl transferase(s) which are clearly present in rat thyroid hemilobes and are probably responsible for the addition of N-acetyl-glucosamine to thyroglobulin. Thyroglobulin has ~ 8-10% carbohydrate which consists of 19 oligosaccharide units per molecule (Spiro, 1968, 1969, 1970). These oligosaccharides are divided into three distinct units - one has a molecular weight of 1,050 and contains mannose and N-acetyl-glucosamine, the second has a molecular weight of 3,200 and contains in addition to mannose and N-acetyl-glucosamine, galactose, sialic acid and fucose (Spiro, 1965; Fukuda & Egami, 1971a,b). The third unit which has been shown in human thyroglobulin comprises galactosamine and possibly sialic acid (Arima et al., 1972). Spiro & Spiro (1968a,b) obtained from thyroid particles sialyl, galactosyl and N-acetyl-glucosaminyl transferases which have properties consistent with a role in the assembly of the second carbohydrate units of the thyroglobulin. Bouchilloux et al. (1969) reported the presence in sheep thyroid microsomes of an enzyme which transferred N-acetyl-glucosamine to an endogenous acceptor. The enzyme was preferentially located in the smooth microsomes and was extractable with Triton X-100. Their studies did not demonstrate whether the enzyme was involved in the incorporation of peptide-linked glucosamine or the more peripheral ones. Later, Bouchilloux et al. (1970) demonstrated a bimodal distribution of the enzyme in both rough microsomal fraction and Golgi fractions of sheep thyroid gland. The evidence for the presence of two enzymes came from the finding that the

rough microsomes incorporated N-acetyl-glucosamine mainly into an 8-9 S component (believed to be a precursor of thyroglobulin), while the Golgi fraction transferred the sugar to both 8-9 S component and 18 S component which was believed to be uniodinated thyroglobulin. Monaco & Robbins (1973) studied the incorporation of N-acetyl-[^{3}H]-glucosamine in rat thyroid hemilobes and also in homogenates. They showed that the extent of incorporation increased with time, reaching 0.8% of the dose in hemilobes after 12 h of incubation with the labelled sugar. The extent of incorporation into homogenate after 6 h, with a plateau to 12 h, was ~ 0.5% of the dose. The sugar was incorporated into 19 S thyroglobulin and into both soluble and insoluble precursors. The authors simultaneously studied the incorporation of N-acetyl-glucosamine, which is converted to N-acetyl-neuraminic acid in the same fractions, and found that both sialic acid and N-acetyl-glucosamine were present in soluble thyroglobulin while only the latter was found in membrane bound thyroglobulin.

(b) Galactosyl transferases

It has been suggested that the core part of a carbohydrate moiety containing mannose and N-acetyl-glucosamine is added *in vivo* as a complete entity and that the outer sugars (galactose, fucose, sialic acid and maybe N-acetyl-galactosamine) are added sequentially as monosaccharide units to the growing glycoprotein (see Waechter & Lennarz, 1976). If this is the case, the mechanism of enzymic incorporation of single sugar residues of the last-mentioned types may well differ from that involved in incorporation of the core sugars. Galactosyl transferases with a high specificity

for the nucleotide sugar and for the acceptor can be isolated from biological sources. If the nucleotide sugar acts as the donor *in vitro*, what part does dolichyl phosphate galactose play *in vivo*? And what is the meaning of the specificity of the galactosyl transferase for both nucleotide sugar and acceptor? If the assumption were made that the transfer of the sugar *in vivo* also takes place directly from the nucleotide sugar, and also that dolichyl phosphate galactose is not involved, then the function of the lipid sugar remains unexplained. And if this is the case, what functions do the other lipid sugar compounds have? This important aspect was mentioned earlier

Galactosyl transferases occur in a variety of tissues. The first report of the presence of galactosyl transferase came from the studies of Watkins & Hassid (1962), who described this enzyme in mammary glands. The enzyme was later shown to contain two protein subunits, A and B. The A protein is membrane-bound and is present in many mammalian tissues (Coffey & Reithel, 1968a,b). The A protein exhibits galactosyl transferase activity towards *N*-acetyl-glucosamine, either as free sugar or as a moiety in formation of a glycoprotein. If B protein is added to A protein, the enzyme functions as lactose synthetase. The galactosyl transferase is also present in a soluble form in milk and the B modifier protein was recognized to be α-lactalbumin (Trayer & Hill, 1971). There is also in rat serum a soluble galactosyl transferase which will catalyse *in vitro* production of glycoproteins (Fraser & Mookerjea, 1976). Again, α-lactalbumin may act as a modifier protein. The enzyme was purified to homogeneity. McGuire *et al.* (1965) showed the

presence of galactosyl transferases in a variety of rat tissues and described the presence of two enzymic activities, one utilizing N-acetyl-glucosamine as acceptor, and the other α_1-acid glycoprotein which had been digested with neuraminidase and β-galactosidase.

Sarcione & Carmody (1966) provided evidence for the presence in the deoxycholate soluble fraction of preparations of liver microsomes, of an enzyme capable of transferring galactose from UDP-galactose into liver microsomal protein. This enzyme has been purified 6-fold (Fraser & Mookerjea, 1976).

Helting & Rodén (1969) studied the transfer of galactose from UDP-galactose to the carbohydrate-protein linkage region of the chondroitin sulphate, a reaction catalysed by enzymic activity in embryonic chick cartilage. The enzyme transferred galactose into endogenous acceptor. Identification of the products resulting from incorporation revealed structures typical of the linkage region such as 4-O-β-D-galactosyl-D-xylose, 3-O-β-D-galactosyl-D-galactose and 3-O-β-D-galactosyl-4-O-β-D-galactosyl-xylose. Their studies with mixed substrates showed that the galactosylation reactions were catalysed by two different enzymes, or possibly through two independent catalytic centres on one enzyme. The transferase(s) also exhibited metal ion requirement for expression of its full activity.

(c) N-Acetyl-galactosaminyl transferases

Enzymes of this type are usually involved in catalysing transfer to carbohydrate moieties which are linked to serine and threonine residues, but it is considered useful to discuss them

briefly. The mechanism of synthesis of these types of carbohydrate moieties may not necessarily be identical with that of the system involving asparagine residues. Hagopian & Eylar (1968a,b) presented evidence for the incorporation of N-acetyl-galactosamine into the polypeptide chain of a basic protein isolated from bovine myelin catalysed by an enzyme from submaxillary gland: The donor used was UDP-N-acetyl-galactosamine. This protein normally contains no sugar residues and therefore the transferase is a carbohydrate chain initiator. Pronase digestion of the receptor led to products which were not substrates for the enzyme, which indicates that the enzyme requires a large receptor molecule. The enzyme is membrane-bound, requires Mn^{+2} for activity, and is present in Hela cells (Hagopian et al., 1968). This enzyme was released from membrane by treatment with Triton X-100 and it was purified 70-fold (Hagopian & Eylar, 1969a,b). The sugar becomes linked, to the polypeptide chain, with α-anomeric configuration.

An N-acetyl-D-galactosaminyl transferase was reported to be present in human milk from donors of blood type A or AB (Kobata & Ginsburg, 1970). The enzyme was purified 55-fold and was found to catalyse the following reaction:

$$\text{UDP-GalNAc} + \beta\text{-Gal} \rightarrow R \xrightarrow{Mn^{+2}} \alpha\text{-GalNAc-}(1 \rightarrow 3)\text{-}\beta\text{-Gal} \rightarrow R$$

$$\uparrow 1,2 \qquad\qquad\qquad\qquad\qquad\qquad \uparrow 1,2$$

$$\alpha\text{-Fuc} \qquad\qquad\qquad\qquad\qquad\qquad \alpha\text{-Fuc}$$

where R represents the rest of the carbohydrate moiety.

The product is blood type A substance. Hearn et al. (1968) found a similar enzyme in human submaxillary gland preparations. Tuppy & Schenkel-Brunner (1969) have reported an enzyme with similar properties in human stomach mucosal linings.

Another soluble form of N-acetyl-galactosaminyl transferase was found in human sera (Ko & Raghupathy, 1972). This enzyme was able to transfer N-acetyl-galactosamine from UDP-N-acetyl-galactosamine to exogenous acceptors. The acceptors used were basic myelin proteins from beef brain and spinal cord, and bovine submaxillary mucin previously freed of its sialic acid and galactosamine residues. Alkaline borohydride treatment of the products showed that 80% of the sugar was covalently linked to the protein moiety. This enzyme behaves very similarly to the one studied by Hagopian & Eylar (1968a,b) and mentioned above, and is present in sera of all individuals irrespective of their blood group. It is therefore different from the one responsible for production of blood group A antigenicity.

The studies of Whitehead et al. (1974b) show that an N-acetyl-galactosaminyl transferase present in the plasma of A and AB blood groups has an affinity to bind to agarose gel beads and is eluted by UDP. The presence of the transferase in soluble form has also been reported in cerebrospinal fluid (Den et al., 1970) and amniotic fluid (Nelson et al., 1973). The reason for the presence of enzymes of this nature in these fluids is not wholly clear, but Mookerjea et al. (1972) and Kim et al. (1972) have suggested that tissue damage may lead to release of the enzymes.

(d) Fucosyl transferases

L-Fucose is known to occur in human blood group substances and human milk oligosaccharides in several types of linkages, $\alpha(1 \rightarrow 2)$ to galactose, $\alpha(1 \rightarrow 4)$ to N-acetyl-glucosamine, $\alpha(1 \rightarrow 3)$ to either glucose or N-acetyl-glucosamine and $\alpha(1 \rightarrow 6)$ to galactose. $\alpha(1 \rightarrow 2)$ linkage also occurs in porcine submaxillary mucin and $\alpha(1 \rightarrow 3)$ linkage is present in human α_1-acid glycoprotein. Some of the enzymes with the necessary specificities for producing linkages of these types are in soluble form, while others are membrane-bound. The occurrence and function of these enzymes are described below.

Watkins & Morgan (1959) proposed that the genetically determined presence or absence of the specific sugar transferases is responsible for the differences between the blood groups of the individuals and included fucosyl transferases. The hypothesis has now been confirmed by experiment.

The A and B genes in the ABO system, the H gene in the Hh system and the Le gene in the Lele systems are structural genes producing enzymically active products. The two genes, H and Le, producing two types of fucosyl transferases, will be discussed in detail here.

The fucosyl transferase produced by H gene catalyses the following reaction:

$$\text{GDP-Fuc} + \beta\text{-Gal} \rightarrow \text{R} \longrightarrow \beta\text{-Gal-R} + \text{GDP}$$

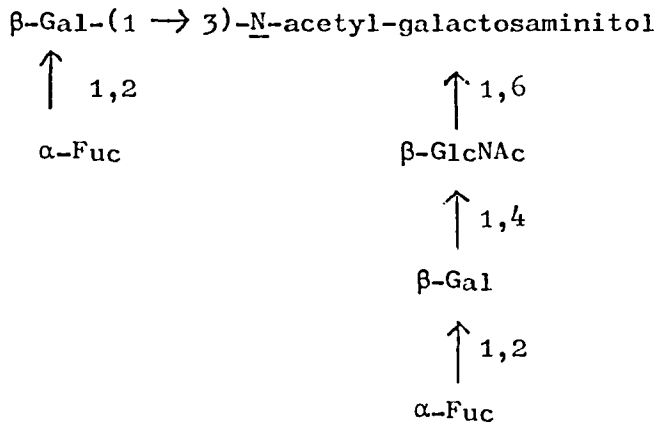
$$\uparrow 1,2$$

$$\alpha\text{-Fuc}$$

This enzyme was first characterized in human milk by

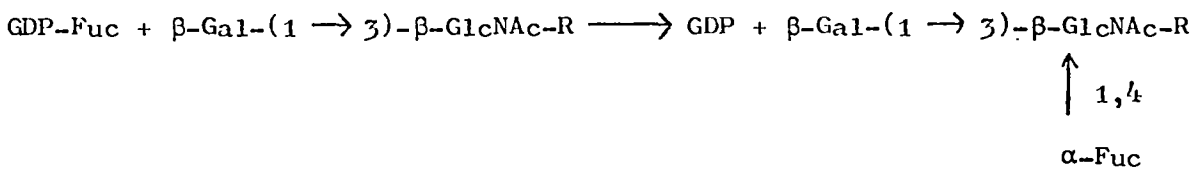
Shen et al. (1968). These authors, with their studies on secretors and non-secretors, revealed the presence of several L-fucosyl transferases, one of which is responsible for the formation of all the O-α-L-fucopyranosyl-(1 → 2)-O-β-D-galactopyranosyl structures found in milk oligosaccharides. Chester & Watkins (1969) studied the presence of this enzyme in human submaxillary gland and stomach mucosal linings from persons who were secretors (of ABH). Their studies showed that in the tissues in which secreted blood group glycoproteins are synthesised, particle bound α-L-fucosyl transferases are present. Chester (1971) found the H gene specified transferase at a very low level in the stomach mucosal preparations of most non-secretors. The enzyme is also present in the serum of both secretors and non-secretors (Schenkel-Brunner et al. (1972), except in the case of the very rare Bombay O_h phenotype.

Rovis et al. (1973) derived from human H-active glycoprotein a product of the following structure:



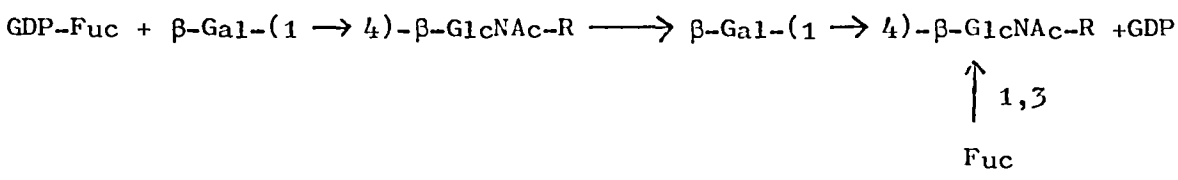
This structure suggested that the acceptor requirement for the α-2-L-fucosyl transferase is that of a β-D-galactosyl residue possibly linked (1 → 3) or (1 → 4) to the next sugar.

The 4-α-L-fucopyranosyl transferase which is the Le gene specified enzyme was first detected in milk (Shen et al., 1968; Grollman et al., 1969). Chester & Watkins (1969) demonstrated the presence of the enzyme in submaxillary gland and stomach mucosa of human donors having Le gene. This enzyme catalyses the reaction



The enzyme does not transfer L-fucose to free N-acetyl-glucosamine or to oligosaccharides with a non-reducing terminal N-acetyl-glucosamine residue (Chester, 1971).

A third fucosyl transferase has been shown to be involved in the biosynthesis of blood group specific glycoproteins. The reaction catalysed by the enzyme could be presented as:



Although the structure formed is an analogue of the Le^a-active trisaccharide, it does not have Le^a specificity. The enzyme has been found in human submaxillary gland and stomach mucosal tissue (Chester & Watkins, 1969), in human milk (Shen et al., 1968) and also in human serum (Schenkel-Brunner et al., 1972).

Aston et al. (1968) isolated, from an H Le^b substance, a serologically active fragment with a fucosyl residue linked

(1 → 6) to galactose. The formation of such a linkage probably needs another fucosyl transferase. So far no other similar case has been reported in blood group specific glycoproteins, but oligosaccharides with linkages of fucose (1 → 6) to galactose have been reported in human urine (Strecker et al., 1973).

Bosmann et al. (1968) showed the presence of two fucosyl transferases in Triton X-100 extracts of HeLa cells. One of the enzymes utilized as acceptor pig submaxillary mucin from which sialic acid and fucose had been removed, leaving an available galactose residue for substitution. This enzyme was shown to have an optimum pH of 6.8, with no apparent need for metal ions. The authors suggested that the enzyme could be involved in the formation of the α-L-fucosyl-(1 → 2)-O-D-galactose linkages found in blood group substances and porcine submaxillary glycoprotein. The other fucosyl transferase found in the HeLa cells utilized as acceptor fetuin from which sialic acid and galactose residues had been removed, thus leaving N-acetyl-glucosamine residues available for substitution. This enzyme was shown to have a pH optimum of 6.0 and also a metal ion (Mg^{+2}) requirement for expression of its full activity. This enzyme is probably involved in the formation of (1 → 4) linkages to N-acetyl-glucosamine residues. Both enzymes were membrane-bound, mainly located in smooth internal membrane and had highly specific macromolecular receptor requirements.

Bella & Kim (1971) isolated from rat small intestinal mucosa a fucosyl transferase which was found to catalyse the transfer of L-fucose from GDP-fucose to glycoprotein or oligosaccharide acceptors. This microsomal enzyme attaches fucose in an

α(1 → 2) glycosidic bond to a terminal β-galactosyl residue of the oligosaccharide chain. The structure formed is the determinant for human blood group H activity. The enzyme showed a pH optimum of 6.0 in the presence of 40mM Mg^{+2} or 20mM Mn^{+2}. The enzyme was inhibited by GDP, GMP, GTP or GDP-mannose. The apparent K_m for GDP-fucose (10^{-6}M) was the same whether a glycoprotein or an oligosaccharide was the acceptor.

Jabbal & Schachter (1971) reported the presence in pig liver of two fucosyl transferases which catalyse the transfer of L-fucose from GDP-fucose to various derivatives of α_1-acid glycoprotein. One of the enzymes requires a galactosyl residue as the accepting moiety. The other one requires an N-acetyl-glucosamine residue. The former is believed to be involved in the biosynthesis of glycoproteins of the serum. The pig enzyme could also transfer fucose into the disaccharide, galactosyl-(1 → 4)-N-acetyl-β-D-glucosamine, but is incapable of incorporating fucose into lactose, galactosyl-(1 → 6)-N-acetyl-β-D-glucosamine, galactosyl-(1 → 3)-N-acetyl-β-D-glucosamine and the α- and β-methyl-D-galactopyranosides. It is a membrane-bound enzyme, requires Triton X-100 and divalent cation for optimum activity. The other fucosyl transferase could incorporate L-fucose into a position on the oligosaccharide prosthetic group of α_1-acid glycoproteins at which L-fucose does not normally occur in the native glycoprotein. The function of this enzyme in pig liver is not known.

(e) Sialyl transferases

Most mammalian tissues contain sialyl transferase activity and *in vitro* studies have been carried out with CMP-sialic acid

as the donor. The credit for the discovery goes to Jourdian et al. (1963) who reported the presence of this enzyme activity in lactating mammary gland. Later works on this type of transferase by Carlson et al. (1973a,b) and Bartholomew et al. (1973) revealed the presence of at least four separate enzymes in the rat mammary gland, sheep submaxillary gland, embryo chicken brain and goat colostrum. An enzyme preparation isolated from rat liver (Hickman et al., 1970) was shown to be activated by low levels of deoxycholate and had no metal ion requirement. By examining a variety of naturally occurring glycoproteins, which contained sialic acid residues, they found that the intact glycoproteins were not efficient acceptors; however, removal of sialic acid with the use of neuraminidase from these glycoproteins turned them into potential acceptors. Among the acceptors they used asialo-caeruloplasmin was shown to be the best. The enzyme is very specific because removal of galactose from the terminal position of the asialo-glycoprotein or conversion of carbon 6 of the terminal galactose residue to the aldehydo moiety results in products without acceptor activity.

Studies by Hudgin & Schachter (1971) on pig liver particulate and serum sialyl transferases suggest that in each case only a single enzyme is responsible for transferring sialic acid to α_1-acid glycoprotein, fetuin and N-acetyllactosamine. The enzyme is specific and the transfer occurs only if a non-reducing terminal galactose residue is linked (β-1 $\rightarrow$ 4) to a penultimate N-acetyl-glucosamine.

Spiro & Spiro (1968b) reported the presence of a sialyl transferase in calf thyroid particulate preparations which was shown to

catalyse the transfer of sialic acid from CMP-sialic acid to a number of glycoproteins and glycopeptides including asialo fetuin and glycopeptides therefrom, as well as asialo thyroglobulin glycopeptides. Their studies with different acceptors showed that sialic acid-free bovine submaxillary mucin and galactose containing glycopeptides isolated from earthworm cuticle collagen were inactive. The enzyme could be solubilized by ultrasonic treatment. This enzyme is similar in many of its substrate specificity requirements to the one described in pig liver (Hudgin & Schachter, 1971), but it differed from the latter in that it showed no activity towards low molecular weight substrates such as N-acetyllactosamine, galactose or lactose.

Studies by Bosmann (1972) on platelets led to the localization of an enzyme, CMP-sialic acid:asialo-fetuin sialyl transferase, as well as the hydrolytic enzyme neuraminidase, on the outer surface of platelet plasma membrane. The sialyl transferase purified 54.3-fold showed a metal requirement (Mn^{+2}), a pH optimum of 7.5 and was shown to be active towards asialo-fetuin and asialo-prothrombin.

There are soluble forms of sialyl transferase in human amniotic fluid (Nelson et al., 1973), in normal and pathological human sera (Mookerjea et al., 1972) and in pig serum (Hudgin & Schachter, 1971).

(f) Xylosyl transferases

These enzymes are not usually involved in the formation of carbohydrate moieties in which asparagine acts as the linkage point to the glycoproteins, although the prosthetic group of

bromelain does contain a xylose residue (item 7, Table 5). More commonly, xylose links the polypeptide chain of many of the glycosaminoglycans to the peptide core.

Since the original studies of Muir (1958) inplicating the presence of serine in the linkage of chondroitin-4-sulphate to protein, it has been established (see Lindahl & Roden, 1972) that the linkage region between protein and polysaccharide in heparin, chondroitin-4-sulphate and dermatan sulphate has the following structure:

GlcUA-Gal-Gal-Xyl-Ser

Xylose is bound to the hydroxyl group of serine via a glycosidic bond (Rodén, 1970).

Xylose has been reported to be a constituent of mucopolysaccharides of human and veal brain membrane (Wardi et al., 1966), and of glycoproteins isolated from human lung (Masamune et al., 1957), cancerous ascitic fluid (Sugimoto, 1956) and aorta (Klemer & Burnbaum, 1965). The precursor of such glycosidically bound xylose used in in vitro studies was UDP-xylose and the transfer of the sugar from its precursor to the acceptor(s) has been the subject of a large number of investigations. Robinson et al. (1966) showed the presence in a 10,000 g supernatant fraction of embryonic chick cartilage, which was capable of transferring xylose from UDP-xylose to an endogenous acceptor. The transfer was catalysed by divalent cations (Mn^{+2}, Mg^{+2}) and had a pH optimum of 6.5. Mild alkaline treatment of the labelled protein yielded xylitol.

Xylosyl transferase activity was also found in cell-free preparations of hen oviduct (Grebner et al., 1966a). The enzyme,

which was activated by Mn^{+2} and glutathione, transferred xylose from UDP-xylose to an endogenous acceptor(s). Although the incorporation of xylose into protein was established by acid hydrolysis of the product, the authors were unable to separate any xylosyl-serine compound from their products. The same authors presented evidence that a preparation of ascites mast cell tumour, a tissue known to produce heparin and its precursors, catalyses the transfer of xylose from UDP-xylose to a serine residue of an endogenous acceptor. Reductive alkaline treatment of the product produced xylitol as the only radioactive material (Grebner et al., 1966b) when UDP-[^{14}C]-xylose was used as donor. Horwitz & Dorfman (1968) studied the subcellular location of xylosyl transferase in chick embryonic cartilage and showed that all of the activity necessary for production of the linkage to the protein, as well as the completion of the polysaccharide chain, were present in preparations of rough and smooth microsomes, the xylosyl transferase being present predominantly in the rough microsomal fraction.

Baker et al. (1972) made a thorough investigation aimed at finding an exogenous acceptor for xylosyl transferase. They tested several small molecular weight compounds, including serine, serine derivatives and serine containing peptides. They also obtained, upon tryptic digestion of proteoglycan, a chondroitin sulphate-peptidoglycan, which was then subjected to Smith degradation and the product was used as acceptor. They found that serine and the tripeptide Ser-Gly-Gly exhibited the same acceptor activity ($K_m \sim 20mM$), while the Smith degraded peptidoglycan prepared from tryptic peptides appeared a much

better acceptor. The Smith-degraded proteoglycan itself was the best acceptor of all with a K_m value of 0.064mM, expressed in terms of concentration of serine residues. The enzyme system used by the authors was a particulate preparation of chick embryonic cartilage and the enzyme assays were performed where incorporation of activity from UDP-[^{14}C]-xylose to the acceptor was linear with respect to both time of incubation and enzyme concentration. The product of xylosyl transfer to Smith-degraded proteoglycan was characterized by (a) paper chromatographic identification of radioactive xylose after hydrolysis, (b) formation of [^{14}C]-xylitol upon treatment with alkali in the presence of borohydride and (c) isolation of xylosyl-serine. The result of these analyses all confirmed that xylose had become covalently bound to serine residues of the Smith-degraded proteoglycan. The use of exogenous acceptors revealed that xylosyl transferase exhibited specificity with regard to the acceptor, as the proteoglycan when treated with testicular hyaluronidase or bacteria chondroitinase yielded products which did not act as acceptors.

Stoolmiller et al. (1972) studied the xylosyl transferase level in different tissues of embryonic chick, using both endogenous and exogenous systems. The cartilage had the highest specific activity towards both endogenous and exogenous acceptors. The specific activity of the brain enzyme appeared to be $\sim$8% of that of the cartilage. Intestine and liver extracts showed no activity towards exogenous acceptor. The authors also attempted to purify the enzyme. The homogenate of cartilage in buffers containing 0.2 - 1M KCl was centrifuged at 105,000 g to

yield a supernatant containing 65-95% of the original activity. The rest of the activity was in the pellet. The supernatant was fractionated by ammonium sulphate and enzyme activity collected from the fraction precipitated between 26-78% saturation.

The ammonium sulphate fraction of the enzyme was subjected to further purification with density gradient centrifugation and column chromatography on a Sephadex G-200 column. A complete separation from other glycosyl transferases was obtained by column chromatography. The elution pattern of the xylosyl transferase showed a moderate retardation of a small portion of the enzyme activity, while the bulk of it was greatly retarded. The molecular weight of the latter was 110,000-120,000 based on its position on the column. The column chromatography revealed two interesting facts, (a) xylosyl transferase operated in the absence of transfer of other types of sugar residues, and (b) the enzyme activity was not dependent on the intact membrane. The kinetic studies on the enzyme using exogenous acceptor (Smith-degraded proteoglycan) showed an apparent K_m value of 240 µg/ml for the proteoglycan and 2.5×10^{-5} M for UDP-xylose. The pH optimum for both endogenous and exogenous acceptors was 6.5-7 and the activity fell quickly at higher pH.

From the studies of these authors (Stoolmiller et al., 1972) on the distribution of the transferase in different tissues, one may conclude that the level of the enzyme is high in tissues where the rate of mucopolysaccharide formation is high. It is postulated by the authors that these transferases control the initiation of the chondroitin sulphate chain on the core protein.

9. Glycosyl transferases as possible mediators for cellular adhesion

In all the studies mentioned above the sugar donor used has been a nucleotide sugar; an unanswered question is whether the enzyme preparations contain lipids which might participate in transfer of sugar from nucleotide sugar to the various acceptors. If this indeed be the case there are various other aspects which need investigating such as whether specific enzymes are needed for converting a nucleotide sugar to a lipid phosphate sugar, enzymes which might be different from those involved in transferring sugar from the lipid intermediate to the acceptor. There are other possible mechanisms which can be explored only after extensive purification of the various sugar transferases. It may however be that the nucleotide sugars can act as sugar donors *in vitro*, but that lipid intermediates are functioning *in vivo* (Marshall, 1974).

Additionally, glycosyl transferases have been suggested to function directly as part of the cell surface receptor complex involved in intercellular adhesion. The mechanism which was first postulated by Roseman (1970) involved the ability of glycosyl transferases on the surface of one cell to bind the appropriate sugar acceptor(s) on the adjacent cells. The theory has been further extended by Bosmann (1971) and by Jamieson *et al.* (1971) to explain the platelet-platelet and platelet-collagen interactions respectively. Much more work is needed to explain the validity of this hypothesis and, in fact, there is a considerable controversy as to whether the appropriate glycosyl transferases occur on the exterior surface of the plasma membrane (Shur & Roth, 1975).

10. Multiglycosyl transferase system

This concept was put forward by Roseman (1970): He envisaged a system whereby glycosyl transferases are organized in a regular sequence leading from rough endoplasmic reticulum to smooth endoplasmic reticulum, Golgi and finally to the plasma membranes. As the carbohydrate moiety is undergoing synthesis within the cell, the product of each step of reaction becomes the substrate for the appropriate glycosyl transferase which will be needed for the addition of the next sugar residue. This concept which Roseman called a "cooperative sequential specificity" would not necessarily be invalidated even if it were eventually shown that a carbohydrate moiety was synthesised as a lipid-oligosaccharide moiety.

11. The present work

The present studies were concerned with examining whether there is enzymic activity in various tissues capable of catalysing addition of N-acetyl-glucosamine to the asparagine residue of an asparagine sequon. Ribonuclease A was used as a convenient substrate in the studies and UDP-N-acetyl-glucosamine was used as the sugar donor. Enzyme activity was indeed demonstrated to be present in liver, serum and preparations of yeast cell membranes.

The enzyme will be referred to as UDP-N-acetyl-glucosamine —— L-asparagine sequon N-acetyl-β-D-glucosaminyl transferase, and it has been suggested by Professor E.C. Webb, in his capacity as a member of the Commission on Biochemical Nomenclature (Enzyme Nomenclature, 1972), that the enzyme should be listed as belonging to the 2.4.1 group.

CHAPTER 2

1. Materials and General Methods

(i) Sources of materials used

Uridine diphospho-N-acetyl-glucosamine (Grade 1, Na^+ salt, Cat. No. U-4375) and uridine diphosphate (Cat. No. U-4125), and CDP-choline (Cat. No. C-0256) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. UDP-N-acetyl-D-[U-^{14}C]-glucosamine (> 200 Ci mole^{-1}; NH_4^+ salt) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. It ran as a single substance, its location being assessed by radioactive scanning and under ultraviolet light, when chromatographed on Whatman No. 1 paper (descending) in ethanol-1M ammonium acetate-acetic acid (50:20:33, by vol). A reference sample of cold UDP-N-acetyl-glucosamine chromatographed identically (R_F = 0.28). Spermidine (Batch No. 5382-8) and Spermine (Batch No. 41466) were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A. and Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. respectively. Phospholipase A was a Boehringer product (Cat. No. 15057).

Ribonucleic acid (Yeast; Cat. No. 15332) purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. was dialysed exhaustively against sodium acetate (0.05M in acetate) buffer, pH 5.5, to remove nucleotides and the solution was freeze-dried. Ribonuclease A (Cat. No. 15410), also obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., was chromatographed on a column of CM-cellulose (Taborsky, 1959) before use. Hen egg albumin was prepared by Dr. R.D. Marshall (Warner, 1954).

CM-cellulose (Whatman CM 70 and CM 23, 0.6 mequivalent/g;

fibrous), DEAE-cellulose (Whatman DE 23, 1.0 mequivalent/g; fibrous) were obtained from Modified Cellulose Ltd., Maidstone, Kent, U.K. Sephadex G-25 (fine), G-100, G-200, Sulphopropyl Sephadex C-25 (2.3 $\pm$ 0.3 mequivalent/g; particle size 40-120 μm), were bought from Pharmacia, Uppsala, Sweden. Amberlite CG-120 (200-400 mesh), IR CG-50 (200-400 mesh), British Drug Houses Ltd., Poole, Dorset, U.K., were used throughout the chromatographic procedure.

Wheat germ agglutinin-Sepharose was obtained either as a gift from Professor Michel Monsigny, C.N.R.S., 54045 Orléans, Cedex, France (L'Industrie Biologique Française, 92115 Clichy, France), or was bought from Miles Laboratories Ltd., Stoke Poges, Bucks., U.K. (Lot AWGA 10).

Crystalline trypsin (Boehringer Corp.; Cat. No. 15330) was purified before use (Maroux et al., 1962) in order to free it from extraneous proteases. The rest of the chemicals were all analytical grade reagents.

Rabbit liver was frozen solid immediately after removal from the animal (Honee Bun Farm Products Ltd., Little Venn, Wollsery, Bideford, Devon, U.K., or Buxted Rabbit Co., Great Totiase Farm, Buxted, Sussex, U.K.). In some experiments fresh liver from rabbit was used. The animal was killed by cervical dislocation and the liver was removed immediately. It was rinsed in ice-cold, 0.25 M sucrose solution and kept cold ($4^{\circ}C$) until used, within 0.5 h.

Plasma and serum samples: In the experiments in which whole serum was used, fresh blood was taken from a subject (Z.K.) immediately before use. It was allowed to clot at room temperature

and the serum was collected after centrifugation. For ammonium sulphate fractionation of plasma, outdated blood samples (AB, RH-positive) were used. These had been kept at $4^{o}C$ for 20 days in the presence of anticoagulant citrate-phosphate and were obtained from the Haemotology Department, St. Mary's Hospital, London, W.2. Red cells were removed by centrifugation.

The yeast mutant x 2180-1A-5 mnn2 was supplied by Dr. C.E. Ballou, Department of Biochemistry, University of California, Berkeley, CA 94720, U.S.A. and tunicamycin was kindly provided by Dr. G. Tamura, Laboratory for Microbiology, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku 113, Tokyo, Japan.

Wistar strain female rats (200-250 g) were used.

(ii) Treatment of chromatographic material prior to use

CM-Cellulose (Whatman CM 70 or CM 23, 0.6 mequivalent/g; fibrous) was prepared for use by swelling the commercial product in 0.5M HCl. It was recycled by successive treatments with 0.5M HCl and 0.5M NaOH. It was then washed with water to neutrality and suspended in the appropriate buffer (see below). DEAE-Cellulose (Whatman 23, 1.0 mequivalent/g) was prepared for use by swelling it in 0.5M NaOH, recycled by successive washing with 0.5M NaOH and 0.5M HCl, washed with water to pH 7.0 and suspended in an appropriate buffer. Toluene was added to prevent bacterial growth.

Lectin affinity columns: Sepharose-bound lectin was equilibrated with appropriate buffer before use. The Sephadex chromatographic materials were all made to swell before use by soaking in an

appropriate buffer for the time suggested by the manufacturers. The ion exchange resins Amberlite IR 120 (mesh size 200-400) and Amberlite IR CG-50 (mesh size 200-400) were regenerated before use.

(iii) Counting of radioactivity

This was carried out in 10 ml of scintillating fluid. A mixture of two parts of toluene containing 0.5% 2,5-diphenyl-oxazole and 0.02% of 1,4-bis-(5-phenyloxazol-2-yl) benzene and one part of Triton X-100 was used in most of the experiments. In those carried out with preparations from rat liver (Chapter 6) the scintillator consisted of 6 ml 2-methoxy-ethanol plus 10 ml of scintillator fluid referred to, but without Triton X-100. A Nuclear Chicago scintillation counter was used (efficiency 85% with the first of the scintillation fluids, 61% with the second) and corrections were made where necessary for quenching by protein.

Counting of radioactive strips was done with the Panax gas-flow strip scanner.

(iv) Absorbance measurements and electrophoretic methods

Spectrophotometric measurements were done with the use of a Unicam SP 500, SP 600 or SP 700.

High voltage electrophoresis: The cooled plate apparatus was made in the Department of Chemical Pathology, St. Mary's Hospital Medical School, London, W.2., U.K. The electrophoretic technique used was basically the one described by Atfield & Morris (1961) unless otherwise stated. Electrophoresis was performed on Whatman 3 MM paper (22 x 56 cm) in 2.5% formic acid,

7.8% acetic acid, pH 2.0, at 3 KV and 65 mA for 30 min to 1 h.

Disc gel electrophoresis: This was performed in the presence of sodium dodecyl sulphate according to the procedure of Marshall & Zamecnik (1969) in 9.4% polyacrylamide gels. The running buffer contained 0.1% of sodium dodecyl sulphate, as it did in the original experiments. The need for detergent in this buffer should be obvious, although this was not made clear in the description of the original procedure. 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 (Goodman & Matzura, 1971). The solutions were evaporated to dryness and the residues were counted for radioactivity, after addition of scintillation fluid.

(v) Amino acid analysis

Glycoprotein was hydrolysed in vacuo in 6N-HCl at 110°C for 16 h, glycopeptide in 4N-HCl at 100°C for 4 h. The 6N-HCl was constant boiling HCl, twice glass distilled and the 4N-HCl was prepared by appropriate dilution from it. Hydrolysis was followed by evaporation of the HCl at room temperature in vacuo over NaOH.

Analysis of the dried sample was performed with the Locarte analyser (Locarte Co., Emperor's Gate, London, S.W.7., U.K.). The amino acids, as well as glucosamine in acid hydrolysates of glycopeptides, were assessed with two programmes. One which was used to separate the amino acids was pH 3.25 (0.2$\underline{M}$ Na^+), 50 min, pH 4.25 (0.2$\underline{M}$ Na^+) 80 min, pH 6.65 (1.0$\underline{M}$ Na^+), 140 min (prog.I). The second programme allowed the separation of glucosamine from

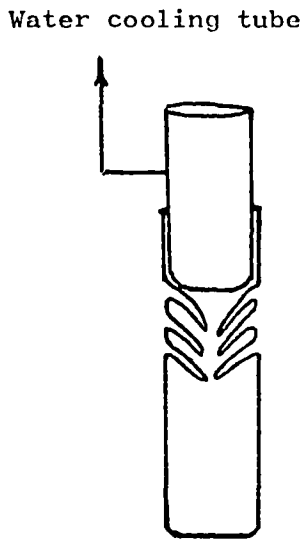


Fig. 4. Specially constructed tubes for glucosamine analysis, used in the Kraan & Muir (1957) modification of the Elson-Morgan procedure

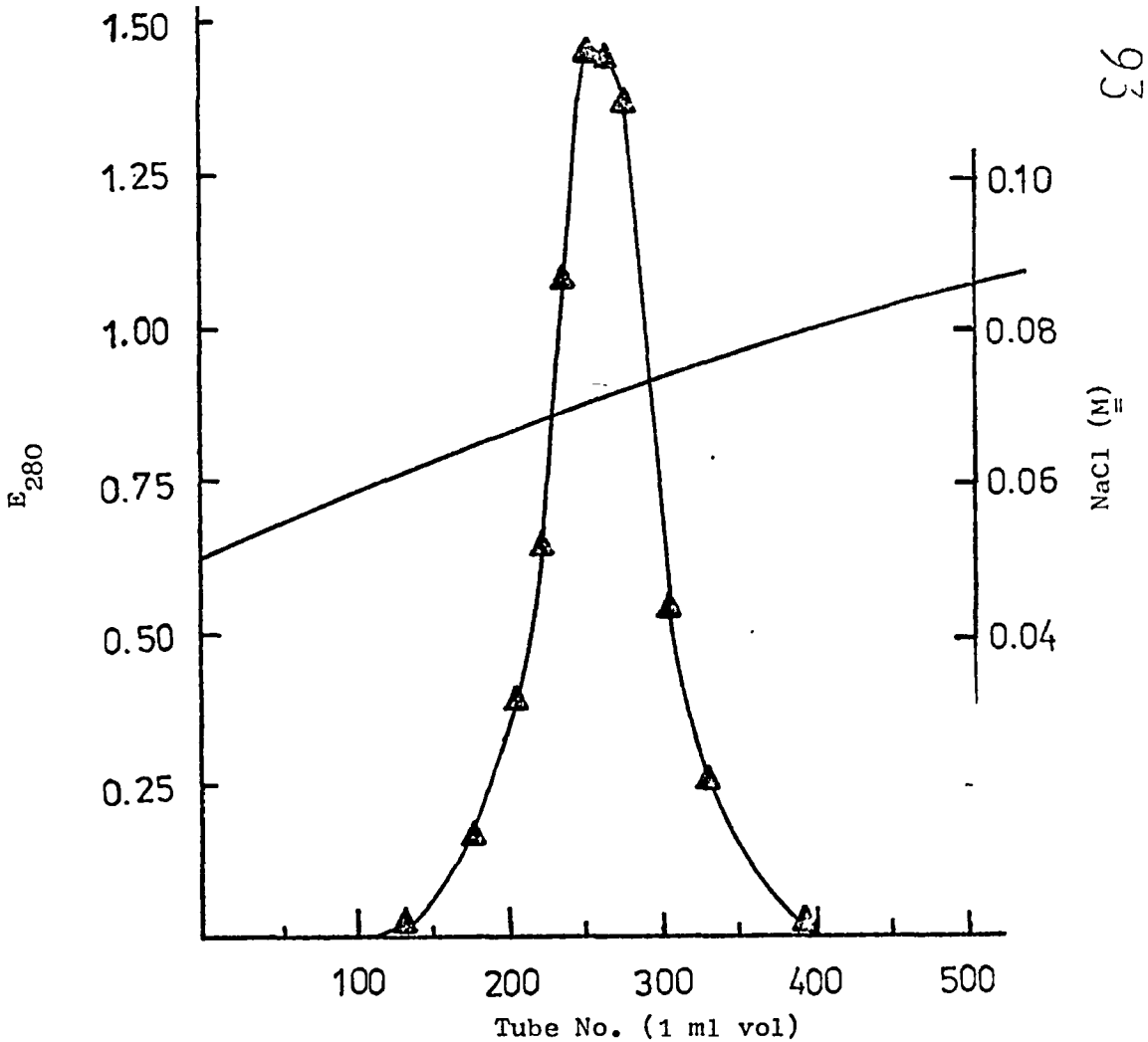


Fig. 5. Purification of ribonuclease A on a CM-cellulose column (40 x 0.9 cm).

leucine, pH 4.25, 100 min, pH 5.28 (0.35M Na^+), 140 min (Prog.II).

(vi) Hexosamine analysis

Recognition and estimation of the hexosamine was made by the chromatographic procedure just described. The hexosamine was also quantitated by an Elson-Morgan procedure (Kraan & Muir, 1957).

Samples were hydrolysed in vacuo at 100°C, 4M-HCl, 6 h and the hydrolysate was evaporated to dryness. The residues were taken up in water. The required amount was transferred to the especially constructed tubes as illustrated in Fig. 4. It is extremely important that the steam does not escape from the tubes because the chromogen, 2-methyl pyrrol is steam volatile (Schloss, 1951; Cessi & Piliego, 1960). To this was added 0.1 ml of acetyl acetone reagent (2% v/v in 0.5N Na_2CO_3). The tubes were kept for 30 min in boiling water with the solution just below the surface of the water in the bath and then removed and cooled. Ethanol (0.4 ml) was added, followed by 0.1 ml of Ehrlich's reagent. The intensity of the red colour was measured at 530 nm using a blank with no glucosamine. The glucosamine content of the samples were estimated with the use of a standard curve obtained by using different quantities of glucosamine.

2. Preparation of substrate as acceptors for N-acetyl-glucosamine

(i) Purification of Ribonuclease A

The procedure used was based on that described by Taborsky (1959).

A column (40 x 0.9 cm) was used for the purification of the commercial ribonuclease A. Amounts of 100 mg of the sample were dissolved in 2 ml of Tris-HCl buffer, pH 7.0 (0.01M in Tris), and applied to the column which had been pre-equilibrated with

the same buffer. Elution was effected with a linear gradient of NaCl in Tris buffer. The gradient was prepared from 2 x 250 volumes, one containing buffer only and the other 0.1M NaCl in the starting buffer. Chromatography was performed at 4°C and fractions of 3 ml were collected and each was assayed for absorbance at 280 nm. The fractions containing protein were assessed for ribonuclease activity (Kalnitsky et al., 1959). The ribonuclease peak which eluted at 0.07M NaCl concentration (Fig. 5) was collected, desalted on a column of G-25 and freeze dried. Glucosamine analysis by Elson-Morgan reaction (Kraan & Muir, 1957) on this fraction revealed the absence of glucosamine. This purified ribonuclease A sample was used throughout the entire experiment.

(ii) Preparation of S-cyanoethylated ribonuclease A

Ribonuclease A was cyanoethylated according to the method described by Plummer & Hirs (1964). The solvent was 8M urea (urea was recrystallized from water-ethanol before use) freshly prepared and adjusted to pH 8.5 using 0.1% methylamine solution (Anfinsen & Haber, 1961). The solution was kept under an atmosphere of nitrogen. Ribonuclease A (20 mg) was added to make a final concentration of 0.5 mM (2.9 ml). A 50-fold excess theoretical amount of mercaptoethanol was added with stirring. The pH was adjusted to 7.9 by the addition of 0.1 ml methylamine (0.1%) and sufficient 0.2N NaOH. The solution was kept under nitrogen and stirred for $4\frac{1}{2}$ h. A 2-fold excess of acrylonitrile was added to the mixture, the pH of which was then adjusted to 8.0 with HCl (0.02M). The solution was stirred for a further 2 h. The pH was lowered to 4 with glacial acetic

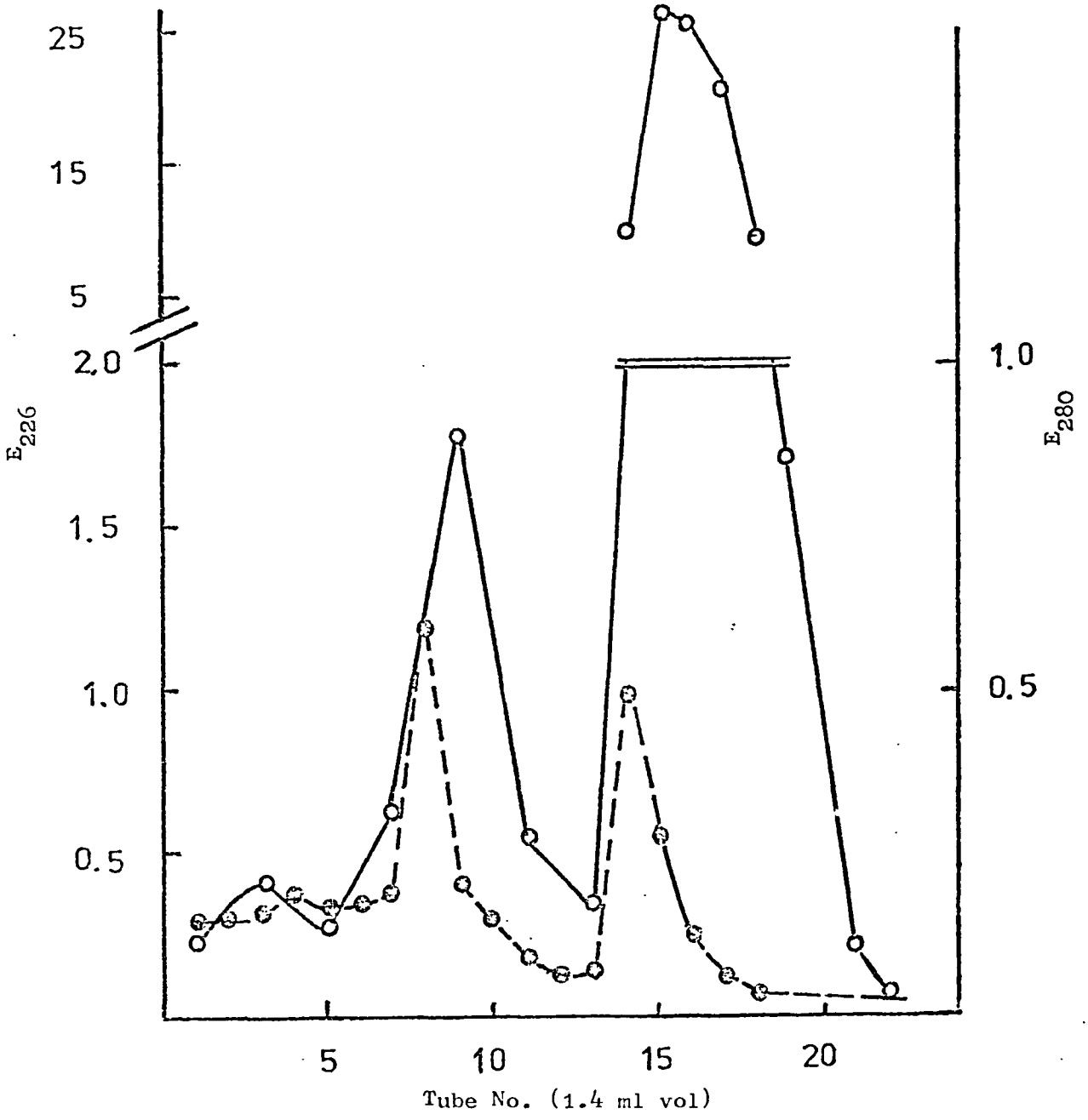


Fig. 6. Elution profile of the CNBr-cleaved ribonuclease A on a Sephadex G-10 column (0.9 x 27 cm)

E_{280}

E_{226}

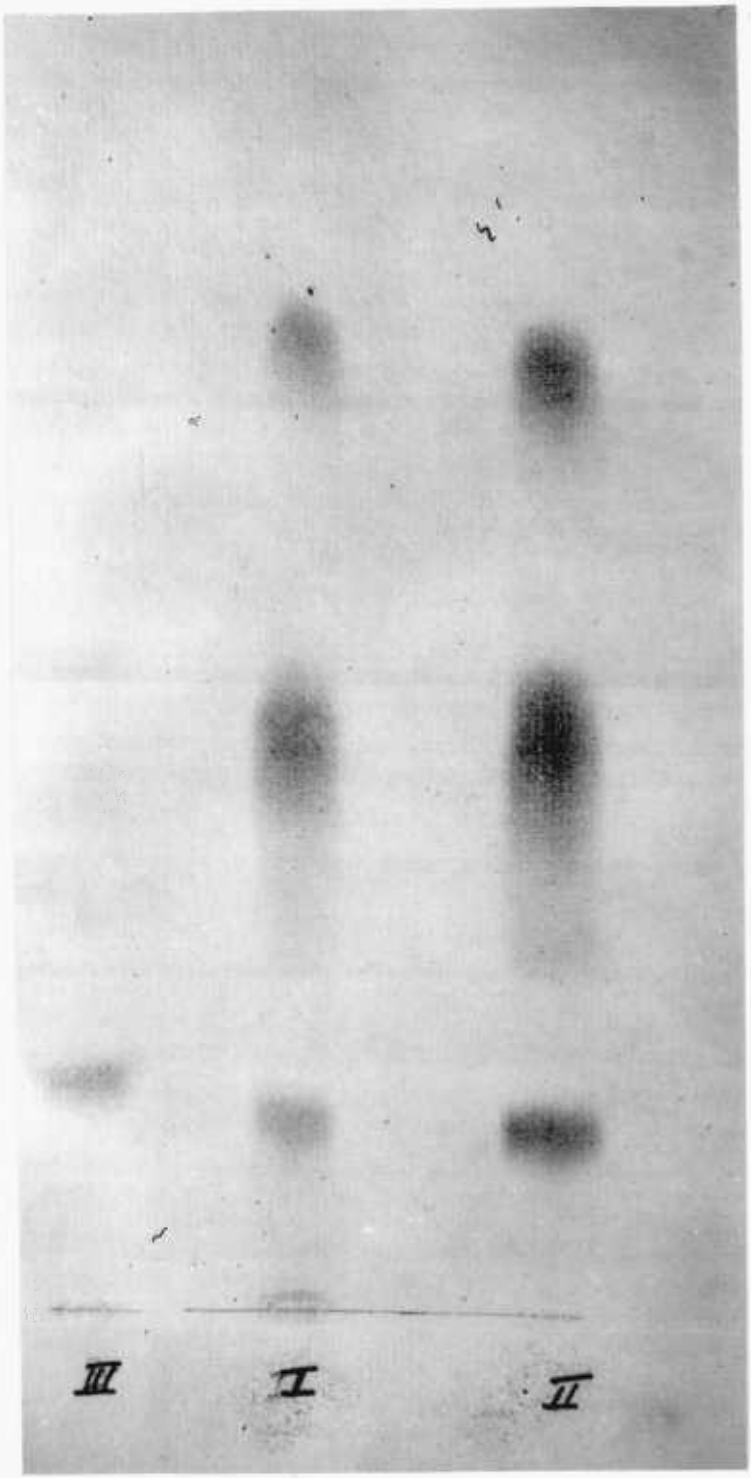


Fig. 7. High voltage electrophoresis of the peptide fragments obtained by CNBr cleavage of ribonuclease A.

Cyanoethylated ribonuclease A (19mg) was incubated with CNBr in 70% formic acid overnight (see p.98) A portion of the mixture was taken for electrophoresis, shown as II on the picture. The rest was chromatographed on CG-10 column and a portion of tube 9 (see Fig. 6) was used for electrophoresis designated as I on the picture. When the mixture was dialysed against water most of the peptides were lost, shown as III on the picture.

acid and the solution was put on a column (35 x 3 cm) of Sephadex G-25, pre-equilibrated with 0.2M acetic acid. The column was eluted with the same solvent. The protein separated from the other reagents and eluted in the void volume. The protein was measured at 280 nm and the fractions containing protein were combined and freeze dried. The product formed a suspension in water.

(iii) Isolation of peptides after cyanogen bromide cleavage of S-cyanoethylated ribonuclease A

Ribonuclease A has methionine residues at positions 13, 29, 30 and 79. Cleavage with cyanogen bromide in 70% formic acid might be expected to yield four peptides (residues 1-13, 14-29, 31-79 and 80-124) with C-terminal homoserine or homoserine lactone residues.

The lyophilized, cyanoethylated ribonuclease A was dissolved in 70% formic acid (19 mg protein/2ml). A 50-fold excess (Steers et al., 1965) crystalline cyanogen bromide was added and the flask was stoppered tightly and left at room temperature overnight.

The preparation was filtered on a Sephadex G-10 column (27 x 0.9 cm) which was eluted with water. There were two u.v. absorbing peaks (Fig. 6), but ninhydrin staining material was present only in the first (Fig. 7) of these. Most of the material was lost upon dialysis (Fig. 7). Fractions containing the peptides were combined and evaporated to dryness.

3. Isolation of pure trypsin from commercial crystalline trypsin

The method of Maroux et al. (1962) was used for the further purification of the crystalline trypsin. Trypsin (17 mg) was

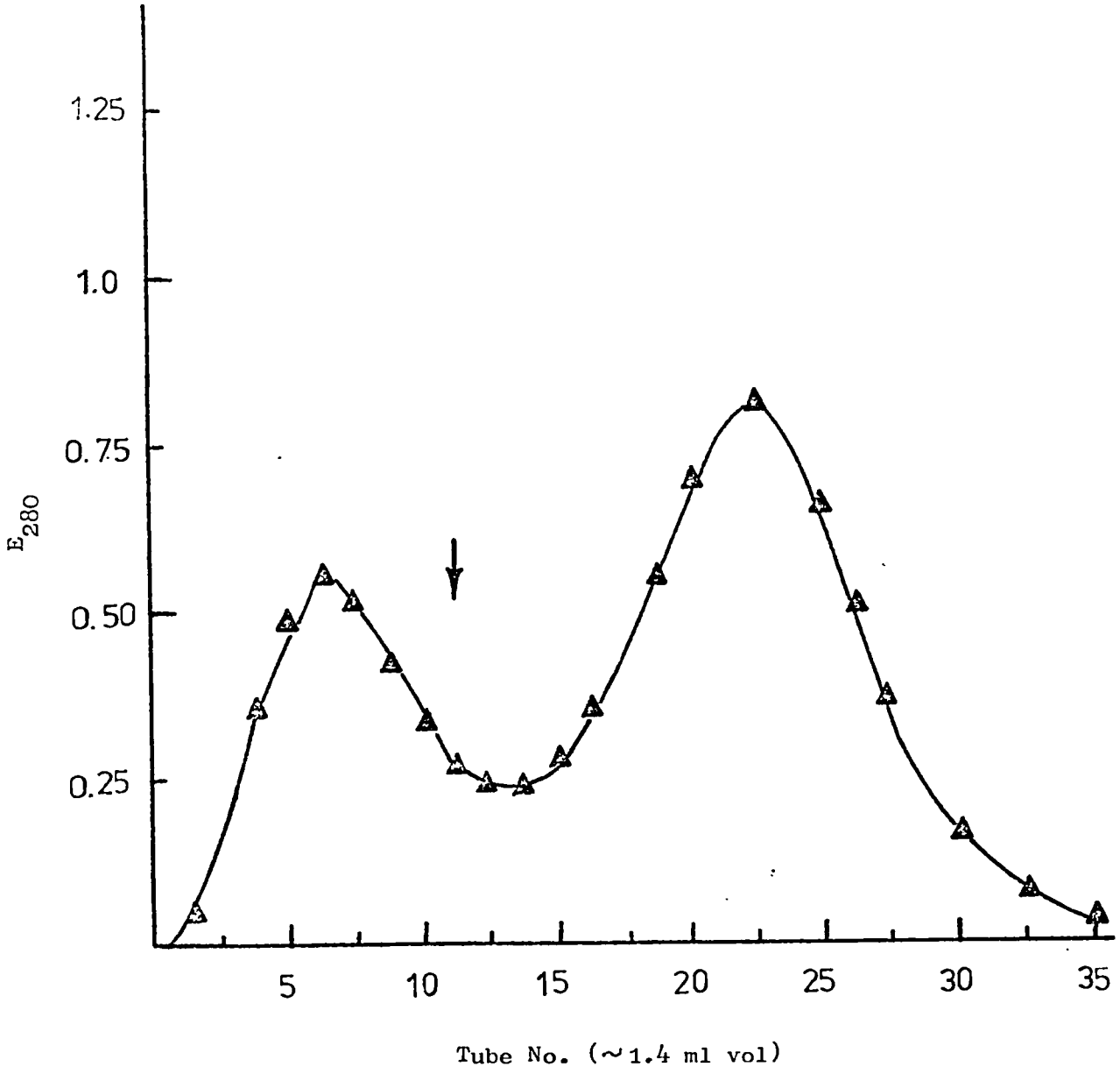


Fig. 8. Elution profile of trypsin (10 mg) on a CM-cellulose column (0.9 x 9.0 cm). The arrow indicates the change of the eluting buffer. Fractions 15-25 containing trypsin were combined, desalted and freeze-dried.
The arrow indicates the change of buffer.

dissolved in 1.5 ml of sodium citrate buffer (0.05M in citrate) containing 0.013M $CaCl_2$, pH 6.0. The solution was placed on a column of CM-cellulose (0.9 x 9 cm). The column was eluted with 14 ml of this buffer followed by 30 ml of 0.1M sodium citrate buffer, pH 6.0, containing 0.013M $CaCl_2$. The flow rate was 5 ml/h. The eluate was collected in 2 ml vol. Two u.v. absorbing peaks appeared (Fig. 8), the second of which (fractions 15-25) contained trypsin and was desalted on a G-25 Sephadex column (30 x 2.5 cm) using 0.2M acetic acid as eluting agent. The eluate was freeze-dried and kept for use. About one-third of the commercial crystalline trypsin consisted of proteases other than trypsin.

4. Preparation of enzyme fractions

(i) Liver extracts

(a) Rabbit liver homogenates were used directly in some experiments

Either frozen or fresh liver was cut up finely and homogenized at 2°C in a Potter-Elvehjem homogenizer with buffer (0.05M Tris-HCl, pH 7.4, 4°C). Usually 2 ml buffer were used per g wet weight of liver.

(b) Microsomal preparations from rabbit and rat liver.

These were prepared according to the method of Keller & Zamecnik (1956). Eight g liver was homogenized at 4°C in 2.3 volumes of buffer containing 0.35M sucrose, 0.035M $KHCO_3$, 0.004M $MgCl_2$ and 0.025M KCl with five strokes of a motor-driven glass teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15,000 g for 10 min at 5°C. The supernatant was

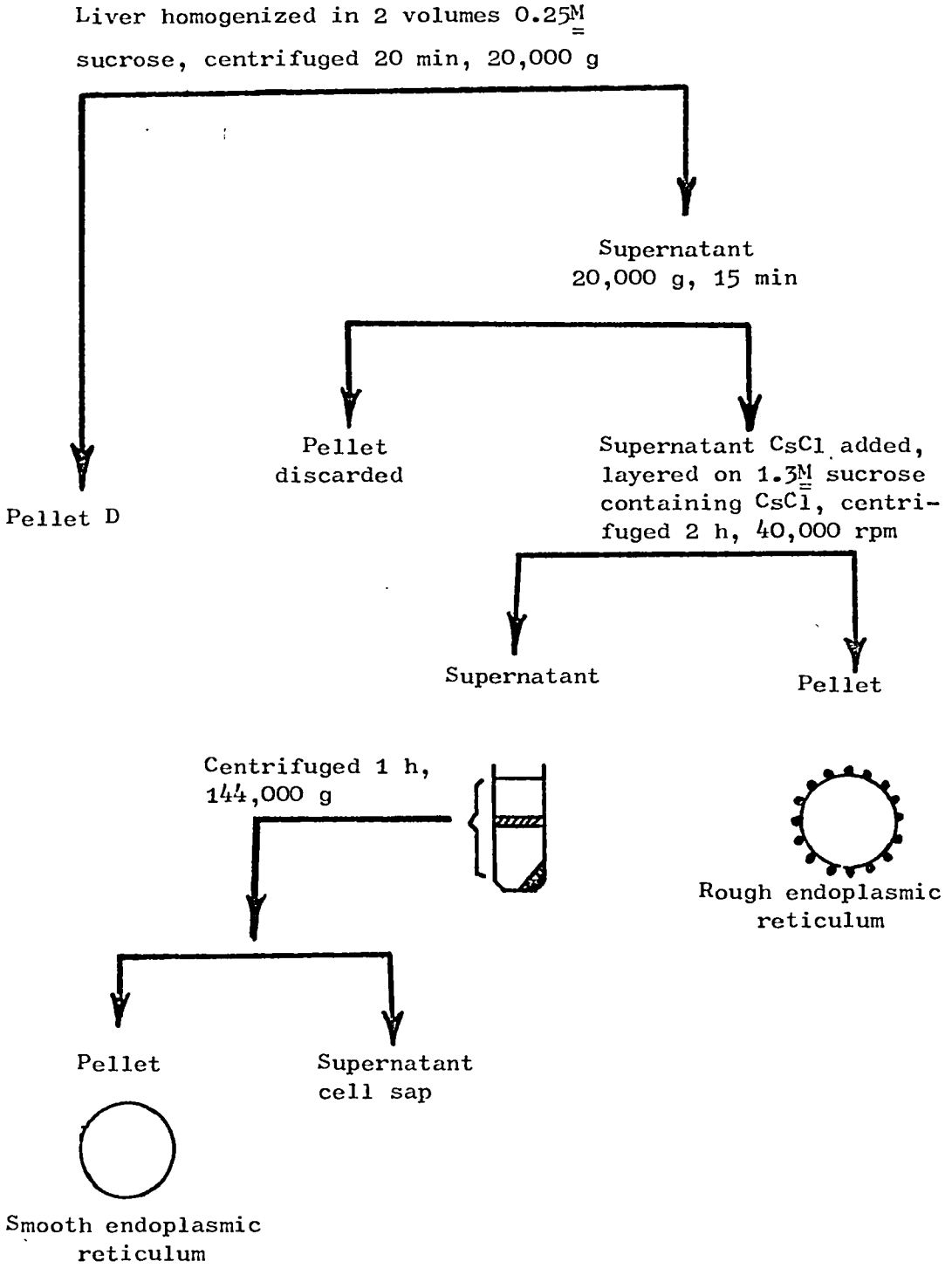


Fig. 9. Schematic representation of the isolation procedure for rough and smooth endoplasmic reticulum.

diluted with 3 vol of 0.9M sucrose, 0.004M $MgCl_2$ and 0.025M KCl and centrifuged at 105,000 g for 1 h. The microsomal pellet was washed in the buffer in which incubations were subsequently performed (see p. 181) and centrifuged at 105,000 g for 30 min at 5°C. The pellet was then suspended in the incubation buffer to a concentration of 1 g wet weight liver per ml. The protein content of the suspension was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

(c) Preparation of rough and smooth endoplasmic reticulum from rabbit and rat liver

The procedure of Dallner (1963) as described by Lawford & Schachter (1966) was used (see Fig. 9). Twelve g liver was finely cut and homogenized at 2°C in 24 ml of 0.25M sucrose with five strokes of a motor driven glass teflon Potter-Elvehjam homogenizer. The homogenate was centrifuged for 20 min at 20,000 g. The supernatant (post mitochondrial supernatant) was recentrifuged at 20,000 g for 15 min to ensure complete removal of heavy material. To the supernatant was added 0.24 ml 1.5M CsCl solution and the mixture was then layered in 6 ml volumes over 6 ml aliquots of 1.3M sucrose, 15mM CsCl in 12.5 ml centrifuge tubes. The preparation was centrifuged at 40,000 rpm and 5°C in a Spinco type 40 rotor (~105,000 g) for 2 h. The pellet contained rough endoplasmic reticulum. The upper 8 ml volumes of each tube were removed and combined. The pooled fraction was centrifuged at 144,000 g for 1 h to yield smooth endoplasmic reticulum as pellet. The preparation of rough and smooth endoplasmic reticulum was washed with 0.9% KCl and recentrifuged at 144,000 g for 30 min. The preparations were suspended in 2 ml of 0.05M Tris-HCl (0.05M in Tris) (pH 7.1 for rat liver, pH 7.4 for rabbit liver).

Fraction	Total protein mg/g wet weight	Total RNA mg/g wet weight	RNA/protein *	nmoles GlcNAc incorporated/mg enzyme protein/h Endogenous & Exogenous	Endogenous	Difference
Homogenate	231 ± 23.5	13.95 ± 2.35	0.06	5.71 ± 0.43	4.247 ± 0.44	1.47
10,000 g supernatant	158 ± 5.8	6.58 ± 1.20	0.069			
Rough endoplasmic reticulum	7.89 ± 1.59	4.51 ± 0.4	0.53	7.32 ± 0.32	4.40 ± 0.54	2.92
Smooth endoplasmic reticulum	3.14 ± 0.47	0.33 ± 0.08	0.089	4.75 ± 0.16	4.05 ± 0.24	0.70
Pellet D	34.6 ± 3.0	2.70 ± 0.005	0.07	4.68 ± 0.36	3.59 ± 0.37	1.09

The statistical variations are calculated from four different experiments.

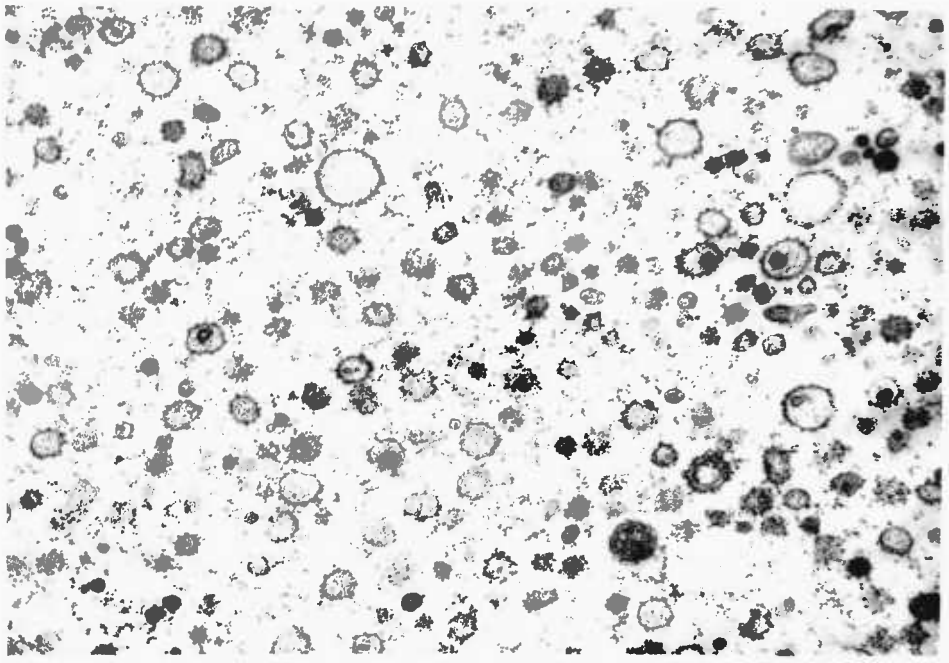
* The ratio of RNA/protein is obtained from one of the four preparations of liver with the following protein and RNA values:

RER: Protein = 8.4 mg, RNA = 4.51 mg; SER: Protein = 3.34 mg, RNA = 0.30 mg; Pellet D: Protein = 37.6 mg, RNA = 2.7 mg; Homogenate: Protein = 216 mg, RNA = 14.66 mg; 10,000 g Supernatant: Protein = 80 mg, RNA = 5.45 mg.

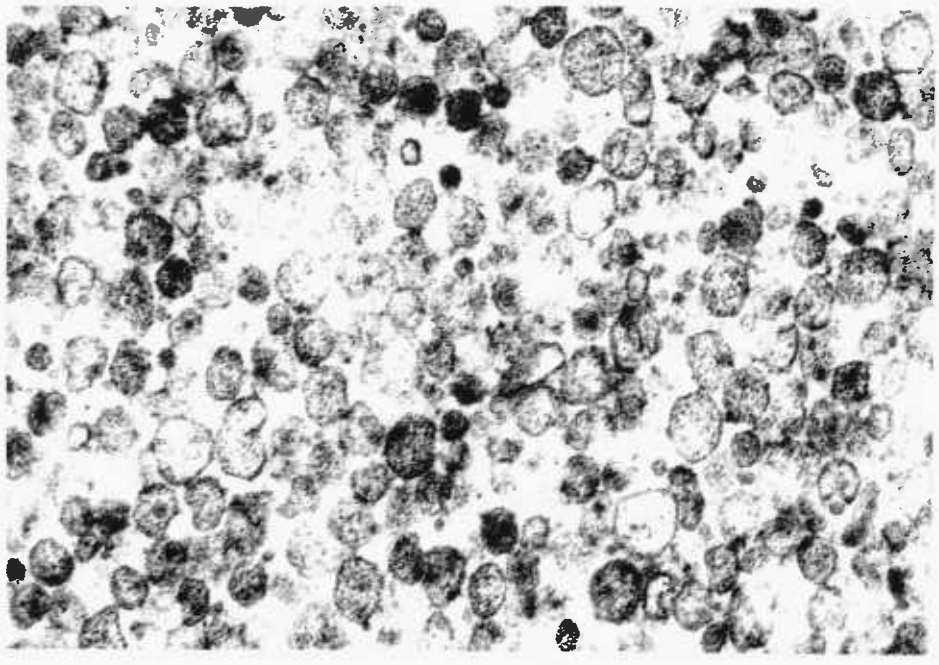
TABLE 8. Amounts of protein and ribonucleic acid (mg/g wet weight of liver) and of enzyme activity (transfer of N-acetyl-glucosamine) in subcellular fractions of rabbit liver (for assay conditions see text). The ratios of RNA/protein in the fractions are also given.

The rough and smooth endoplasmic reticulum preparations were usually characterized by measuring the ratios of ribonucleic acid/protein. The protein estimation was carried out by the method of Lowry et al. (1951), bovine serum albumin being used as standard. Estimation of ribonucleic acid, calculated from ribose measurements, were done only after both rough and smooth endoplasmic reticulum had been freed of the considerable amounts of sucrose in them. The suspension (0.5 ml) of either rough, smooth or pellet D in buffer was taken for ribonucleic acid estimation. The ribonucleic acid was precipitated by addition of four volumes of 10% trichloracetic acid. The precipitate was collected by centrifugation at 6,000 g for 10 min at $5^{\circ}C$ and washed first with the same volume of 10% trichloracetic acid as used in precipitation, followed by a similar volume of water and finally with the same volume of cold 2% $HClO_4$. The precipitate was extracted twice with 10% $HClO_4$ at $70^{\circ}C$ for 40 min (1 ml $HClO_4$ per 3 to 4 mg liver), the volume of the combined extracts was made to a standard volume and the ribonucleic acid estimation was done according to the method of Ceriotti (1954), using ribose as standard. The ratio of ribonucleic acid/protein in the preparations of rough endoplasmic reticulum was normally around 0.5 (see Table 8).

In one case, where rat liver was used for subcellular fractionation, a morphological analysis, using electron microscopy was also performed (Fig. 10). These electron micrographs were carried out by Mr. K.L. Sikri in the Department of Cellular Biology and Histology, St. Mary's Hospital Medical School, London, W.2., U.K.

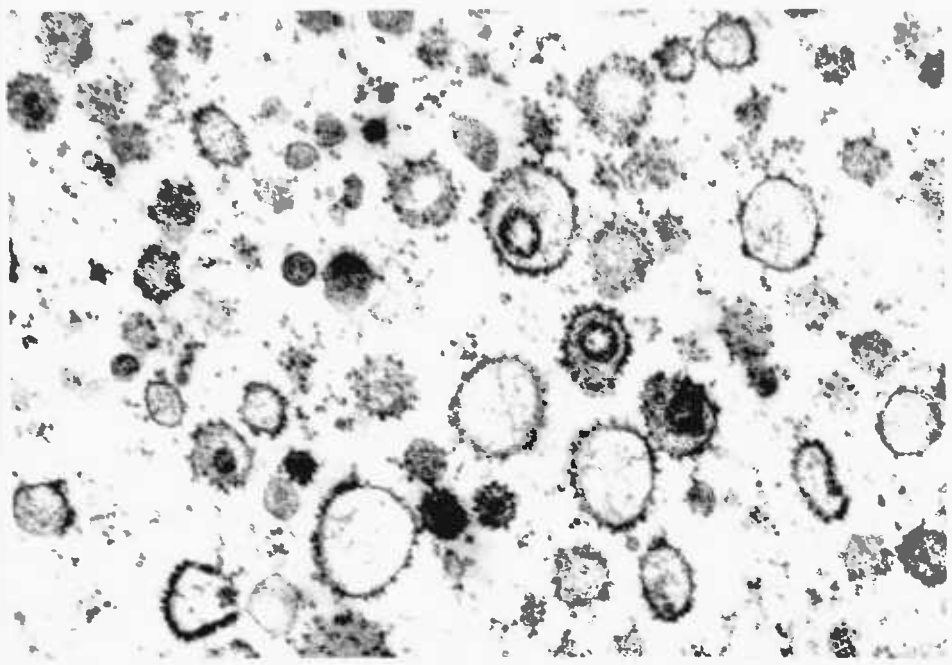


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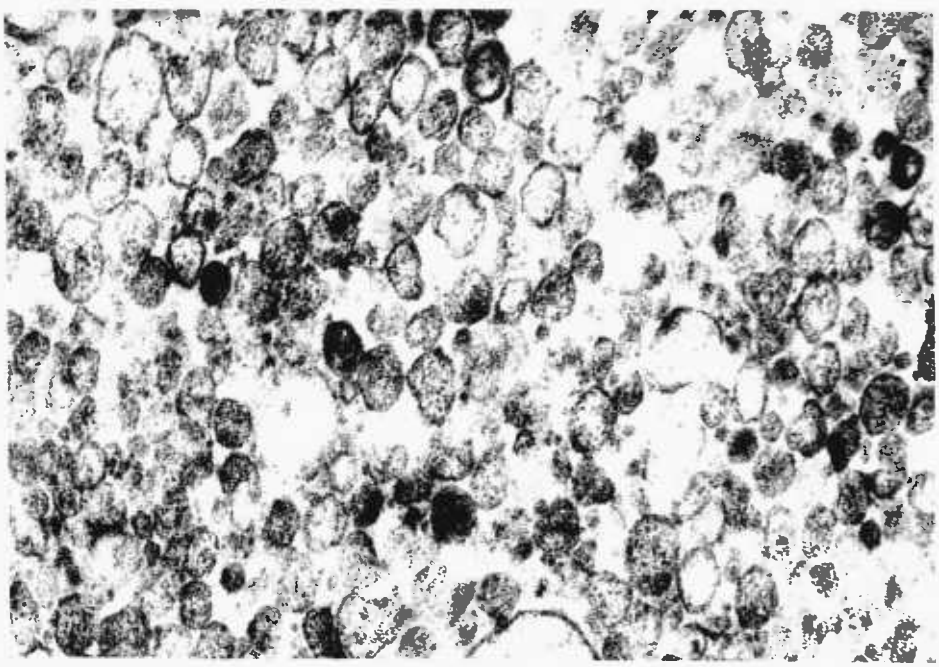


B

Fig. 10. Electron micrographs of rough and smooth endoplasmic reticulum obtained from rat liver by Dallner's method (Dallner, 1963). The pictures shown as A & C are from rough endoplasmic reticulum at magnifications of 2 x 15,000 and 2.5 x 20,000. Those shown as B & D are of smooth endoplasmic reticulum at magnifications of 2.75 x 20,000 and 4.5 x 15,000.



C



D

Fraction No.	Degree of saturation by ammonium sulphate (%)	Total[b] E_{280}	Enzyme activity	
			Specific	Total[c] x 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 17, column 6. The specific activity is expressed per unit of E_{280} also.

TABLE 9. Recoveries of total protein (absorbances at 280nm) 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]

(ii) Human plasma fractions

Plasma was dialysed against 2 x 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°C) and dissolved in 10 ml of 0.04M Tris-phosphate buffer (pH 8.6). The solution was dialysed against the same buffer (2 x 2 l) overnight at 4°C 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°C shown in Table 9, p. 107) wherein are also shown the recoveries of protein, assessed as total absorbance units at 280 nm.

(iii) Preparation of yeast cell membranes

These were prepared by Dr. Frans Reuvers and Mrs. Christina Habets-Willems in Dr. Pieter Boer's laboratory (Laboratory for Physiological Chemistry, University of Utrecht, Utrecht, The Netherlands) and were sent by air to London where I carried out the investigations concerned with the presence of the enzyme (see Chapter 5). The method used by the Dutch workers was as follows:

Saccharomyces cerevisiae, mutant x 2180-1A-5mnn2, was grown at 30°C in a medium similar to that described previously (Van Rijn et al., 1972). Adenine (0.37mM) was added and half the amount

of $(NH_4)_2SO_4$ was replaced by the addition of 40 ml of phosphate-free casamino acid solution for each litre of medium. The casamino acid solution was prepared by dissolving 40 g of casamino acids (Difco Laboratories, Detroit, MI, U.S.A.) and 6 g of $MgSO_4$, $7H_2O$ in 300 ml of water. The pH of the preparation was adjusted to 8.0 (25% v/v, saturated NH_3 in water, specific gravity 0.88) and the volume made up to 400 ml. The mixture was kept at 0°C for 3 h and filtered. The solution contained less than 0.1 mM phosphate.

Cells were harvested in the late exponential phase and 1.5×10^{10} cells/20 ml of Tris-HCl buffer (0.05 M in Tris), pH 7.0, containing 1 mM mercaptoethanol, were mechanically disrupted at 0°C in a cell homogenizer (MSK, Braun, Melsungen, German Federal Republic), by shaking them for 5 min in the presence of 25 ml of Ballotini beads (0.25-0.30 mm diameter). Cell debris was removed as a precipitate after centrifuging the homogenate (10 min, 1,000 g, 4°C). The supernatant was centrifuged (30 min, 40,000 g, 4°C), and the pellet, consisting of membranes, was used as a suspension in the Tris-HCl buffer, pH 7.0, containing 1 mM 2-mercaptoethanol. It contained 21 mg of protein/ml, the protein being assessed by the procedure of Lowry et al. (1951), with bovine serum albumin as standard.

5. Partial hepatectomy of rats

The latent ability of the liver for growth is greater than that of any other mammalian organ. The main lobes comprising 68 ± 2% of the total weight of the liver may be removed and regenerate rapidly. Partial hepatectomy refers to complete surgical removal of these two lobes (see Bucher & Malt, 1971).

Time after partial hepatectomy (H) or sham operation (S) (h)	Weights of liver (g)	Amount of rough endoplasmic reticulum in terms of protein (mg/g liver)
0 (untreated rat)[a]	11.2	7.84[b]
	11.8	7.0
	10.9	5.0
24 (H)	3.2	4.41
	3.5	7.39
48 (H)	7.2	
	7.7	9.92
72 (H)	8.4	5.20
24 (S)	9.4	
	7.7	5.16
48 (S)	8.6	
	9.4	7.52
72 (S)	10.1	4.88

(a) Values of the amount of the protein in rough endoplasmic reticulum preparation from normal Wistar rats were reported to be 6.31 mg/g wet weight of liver by Lawford & Schachter (1966), and 12 mg/g by Dallner (1963) and by Depierre & Dallner (1976)

(b) The ratio of RNA:protein was found to be 0.59

TABLE 10. Weights of livers of normal, partially hepatectomized and sham operated rats during the period of regeneration. The amounts of rough endoplasmic reticulum prepared from the livers are also given.

Partial hepatectomy was performed under ether anaesthetic (Higgins & Anderson, 1931; Waynforth, 1969). The animal was laid on its back and an incision of about 2 cm was made posteriorly from the edge of the xiphoid cartilage, first through the skin and then through the abdominal muscles. The median and left lobes of the liver were moved out of the abdominal cavity. The lobes were gently pulled out and the ligaments connecting the left lobe to blood vessels, stomach and to the dorsal peritoneum were cut. A ligature was tied around the lobes and these were cut. The muscle incision was sutured and the skin incision was closed by Michel wound clips. The mortality was extremely low. Sham operated animals had the lobes of the livers exposed through the surgical incision. They were pushed back into the abdominal cavity and the animal sutured.

The rats were killed at intervals of 24, 48 and 72 h after hepatectomy. The livers were removed immediately and rinsed in cold buffer (0.05M Tris maleate, pH 7.0) and weighed. Table 10 shows the weights of the livers after hepatectomy. After 72 h the liver had practically regained its original weight, but much fat was present.

6. Enzymic assays

The enzyme preparation was incubated with ribonuclease A, UDP-N-acetyl-glucosamine, a metal ion or a polyamine and other appropriate additives such as Triton X-100 and CDP-choline. The precise conditions differed from one experiment to another and the details are given at various points in the later text.

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 usually collected on DAWP 02500 millipore filters, carefully washed with the precipitating acid (10 x 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 were applied. In all cases, control experiments were performed in which ribonuclease A was not added to the incubation, 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 characterize glycosylated ribonuclease, the reaction was terminated by cooling the incubation mixture to 4°C rather than by addition of acid.

7. Isolation of monoglycosylated ribonuclease (ribonuclease-GlcNAc)

(i) Chromatography on CM-cellulose

An incubation mixture, after centrifugation (2,000 g; 10 min; 4°C), was applied to a column (9 x 0.9 cm) of CM-cellulose previously equilibrated with 0.01M Tris-HCl (0.01M in Tris, pH 7.0) at 4°C. Elution was effected with a linear gradient of NaCl in the Tris buffer in the manner previously described in Section 2 (i) of this Chapter. 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 (Kalnitsky et al., 1959).

(ii) Chromatography on Amberlite CG-50

A column (45 x 0.9 cm) of Amberlite CG-50 in 0.2M sodium phosphate buffer (pH 6.02) was prepared. A mixture of ribonuclease A and [^{14}C]-glycosylated ribonuclease (~ 5 mg of total protein) was applied to the column and eluted with sodium phosphate buffer (0.2M, pH 6.02). The fractions were assayed for protein (220 nm) and ribonuclease activity (260 nm; Kalnitsky et al., 1959).

(iii) Chromatography on wheat-germ agglutinin-Sepharose

A column (2 x 0.3 cm) was prepared at 4°C 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 x 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 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 of the Morgan-Elson reaction (Reissig et al., 1955). 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

(Reissig et al., 1955). The residual solution of glycosylated ribonuclease was freeze-dried.

8. Isolation of glycopeptides from glycosylated ribonuclease

Glycosylated ribonuclease (1-18 mg) was cyanoethylated (Plummer & Hirs, 1964; see Chapter 2, p. 95). A solution of cyanoethylated, glycosylated ribonuclease (1%, w/v) was disgested 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 0.02M 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 (10,000 g, 15 min, 4°C). The pellet was taken up in 0.2M acetic acid and the mixture was recentrifuged under the same conditions. The supernatant was added to the previous (pH 3.3) supernatant and chromatographed on a column (250 x 0.9 cm) of Sephadex G-25 (fine) and eluted with 0.2M acetic acid. Volumes of 1 ml were collected and assayed for radioactivity and absorbancy at 226 nm.

Some of the procedures described above were modified in order to accommodate the various experiments done. The modifications made are indicated at the appropriate points in later Chapters.

CHAPTER 3

THE GLYCOSYLATION OF RIBONUCLEASE A CATALYSED BY RABBIT LIVER EXTRACTS

In the Spring of 1973 when this work was begun, there was little if any direct indication that lipid intermediates might be involved in the production of carbohydrate moieties of glycoproteins in animals. Data available were mostly concerned with the direct transfer of the sugar from nucleotide sugars to the endogenous or exogenous acceptors. It seemed reasonable, therefore, to find an appropriate protein which contains the necessary asparagine sequon to undergo glycosylation. Bovine pancreatic ribonuclease A seemed to be a reasonable substrate as it contains near position 34 the amino acid sequence ^{34}Asn-Leu-Thr-Lys. In the approach taken it was assumed that an asparagine residue rather than aspartic acid was the acceptor amino acid for the formation of the carbohydrate-peptide linkage of the GlcNAc-Asn form.

In the first series of the experiments, ribonuclease A was incubated with UDP-N-acetyl-[U-^{14}C]-glucosamine in the presence of homogenates of rabbit liver.

1. Incorporation of N-acetyl-β-D-glucosamine into ribonuclease A, catalysed by rabbit liver homogenate

Liver (2 g) was homogenised in 4 ml of Tris-HCl buffer (0.05M in Tris, pH 7.4) with six strokes of a motor driven Potter-Elvehjem homogeniser. The homogenate was used as the source of the enzyme. Incubations were performed in glass tubes pre-washed with chromic acid. The incubation mixture contained in the final volume of 1.05 ml the following components: UDP-N-acetyl-glucosamine 0.042mM containing 2240 cpm/nmole, $MnCl_2$ 10mM, buffer Tris-HCl

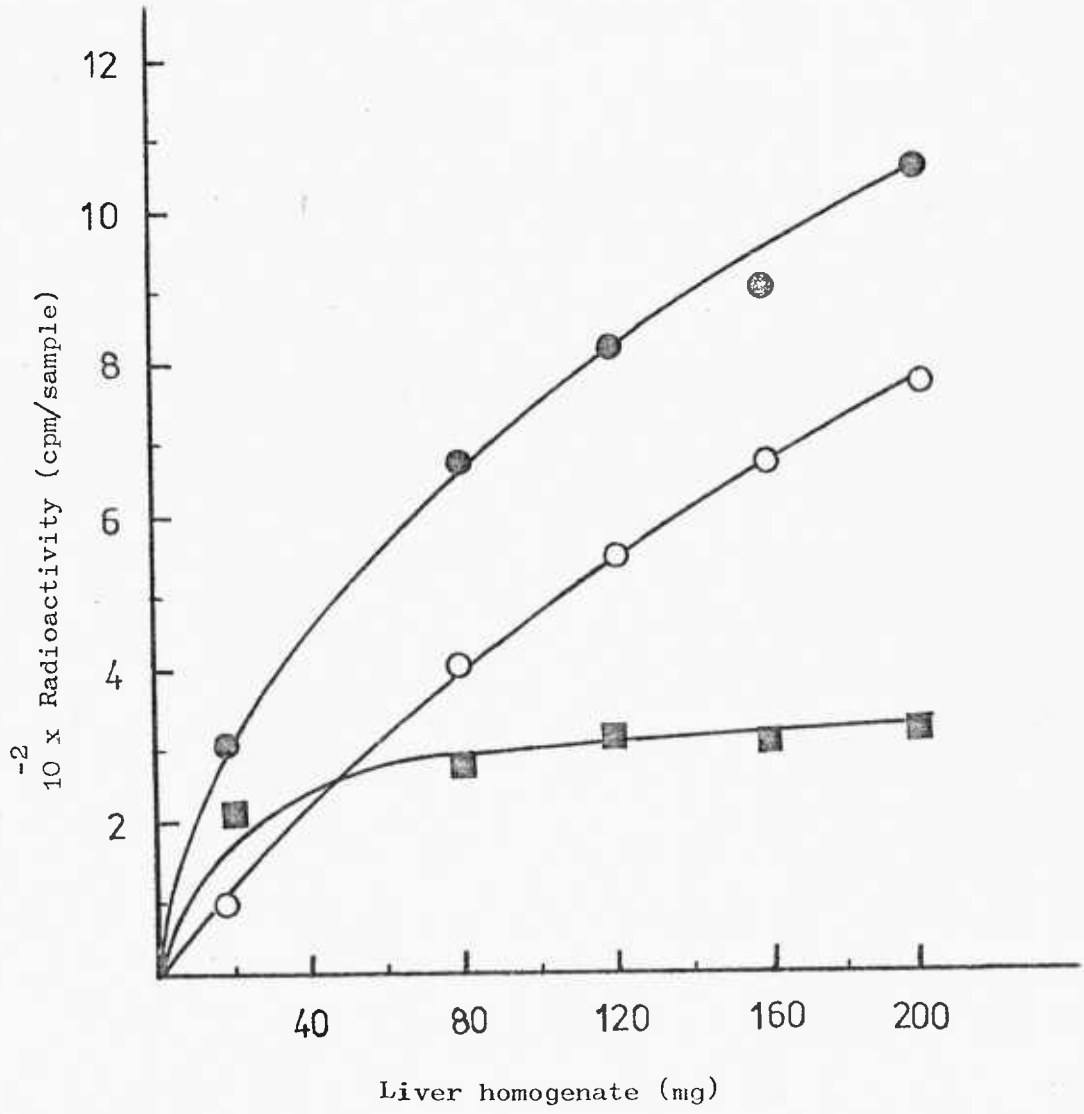


Fig. 11. Relationship between the amount of N-acetyl-glucosamine transferred from UDP-N-acetyl-glucosamine to bovine pancreatic ribonuclease A in the presence of increasing amounts of rabbit liver homogenate. Experiments were carried out in the presence ● —— ● and absence O —— O of added ribonuclease A. The difference curve is thus ■ —— ■

143mM and ribonuclease A 6.8 μM. The enzyme protein was varied from 20-200 mg. The incubation was performed for 30 min at 30°C. The reactions were terminated by addition of 1% phosphotungstic acid in 5% TCA. The precipitates were collected on millipore filters (DAWP 02500), washed successively with more of the precipitating reagent and dried in air. The filters were transferred to scintillation vials and 10 ml scintillation liquid (see Chapter 2, p. 91) were added. The radioactivity incorporated into acid precipitable material was measured using a Nuclear Chicago scintillation counter and corrections for protein were applied. There was considerable incorporation into endogenous protein, but the amounts of radioactivity incorporated in those experiments in which ribonuclease A was used were greater (Fig. 11).

The results of these experiments suggested that there was in homogenates of rabbit liver an enzyme or enzymes capable of catalysing the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine to ribonuclease A. However, further examination was needed as to whether glycosylation of ribonuclease A had really occurred and the following experiment was devised to test this.

A homogenate of liver was prepared as stated above in Tris-HCl buffer (0.05M), to a final concentration of 1 g liver/2 ml buffer. Ribonuclease A (1.0 mg) was incubated with unlabelled UDP-N-acetyl-glucosamine (0.2 mg, 0.570mM) in the presence of 59mM $MnCl_2$ and enzyme protein (100 mg) in a final volume of 0.5 ml. The final buffer concentration was 50mM, and the temperature of the incubation was kept at 37°C. The reaction was terminated after 1 h by cooling the mixture to 2°C, followed

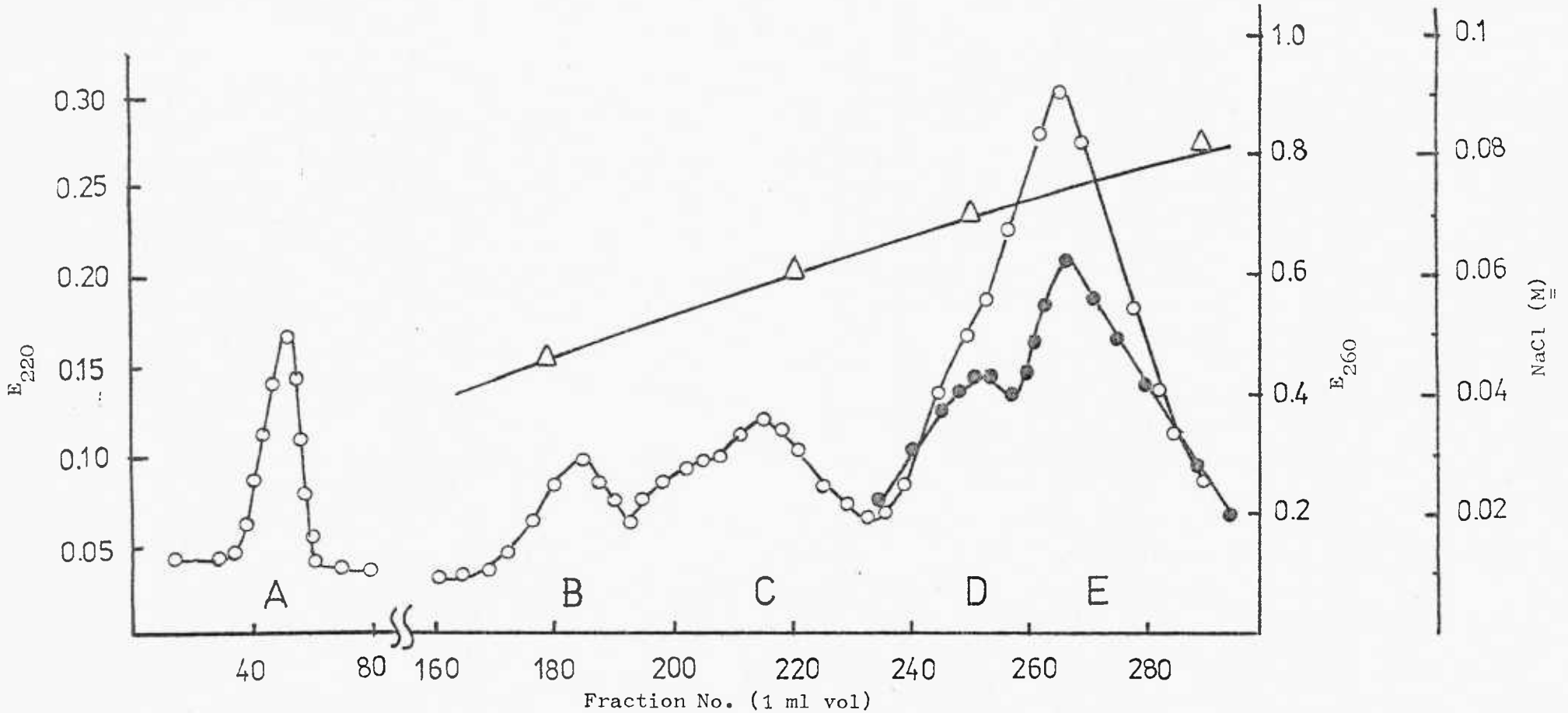


Fig. 12a. Elution profile of an incubation mixture containing ribonuclease A (1 mg) and rabbit liver homogenate (100 mg protein) on a column of CM-cellulose (0.9 x 9 cm). For conditions of incubation see text

o—o E_{220}

●—● E_{260}

△—△ NaCl concentration (M)

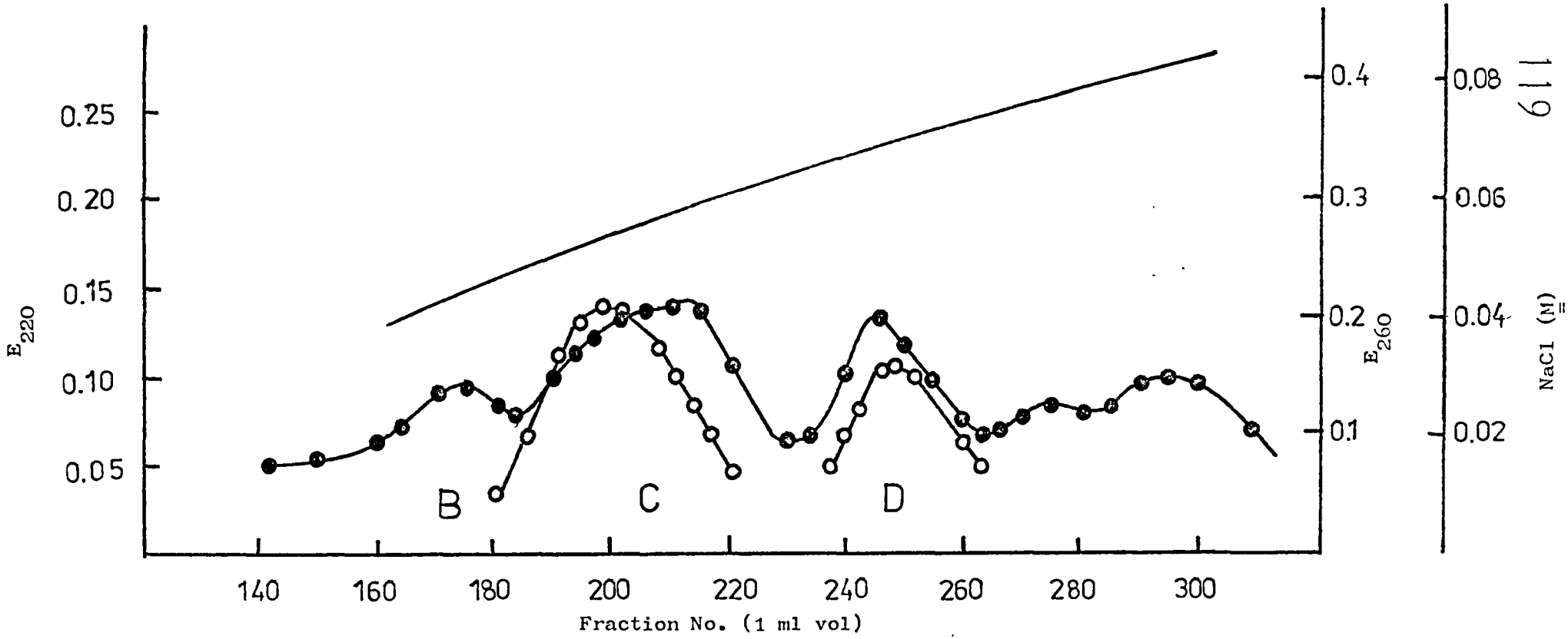


Fig. 12b. Chromatography of rabbit liver homogenate (100 mg protein) on a CM-cellulose column (0.9 x 9 cm)

●—● E_{220}

○—○ E_{260}

—— NaCl concentration (M)

by centrifugation at 2,000 g for 5 min at 4°C. The supernatant was applied to a column of CM-cellulose (0.9 x 9 cm)(see p. 94). The column was eluted with a gradient of NaCl in the starting buffer. The gradient was 150 ml of 0.01M Tris-HCl, pH 7.0 to which 150 ml of 0.1M NaCl in 0.01M Tris flowed. Fractions of 1 ml were collected and absorbance at 220 nm was measured (see Fig. 12a). The protein peaks in the region where bovine pancreatic ribonuclease might be expected to be eluted (0.074M NaCl; Taborsky, 1959) were analyzed for ribonuclease activity according to the method of Kalnitsky et al. (1959). There were two enzymically active peaks in this region, D and E. Peak D appeared at a NaCl concentration of 0.07M, with peak E at 0.074M NaCl. Before any conclusions could be reached as to the origin and nature of the ribonuclease activities in peaks D and E (Fig. 12a), it was deemed necessary to carry out similar chromatography with preparations of rabbit liver homogenate, without addition of bovine pancreatic ribonuclease A. The results of these experiments (Fig. 12b) show that there are liver ribonucleases eluting from the column at concentrations of NaCl of 0.055M and 0.069M. There is clearly a hepatic ribonuclease which chromatographs close to bovine pancreatic ribonuclease A under the conditions used, and due consideration had to be given to this. A similar type of experiment was next done in which larger amounts of ribonuclease A were used.

Ribonuclease A (7.8 mg, 0.652mM) was incubated with unlabelled UDP-N-acetyl-glucosamine (1.53 mg, 2.55mM), $MnCl_2$ 59mM. The enzyme protein concentration was 100 mg in the final volume of 0.85 ml and the Tris-HCl buffer concentration was 50mM.

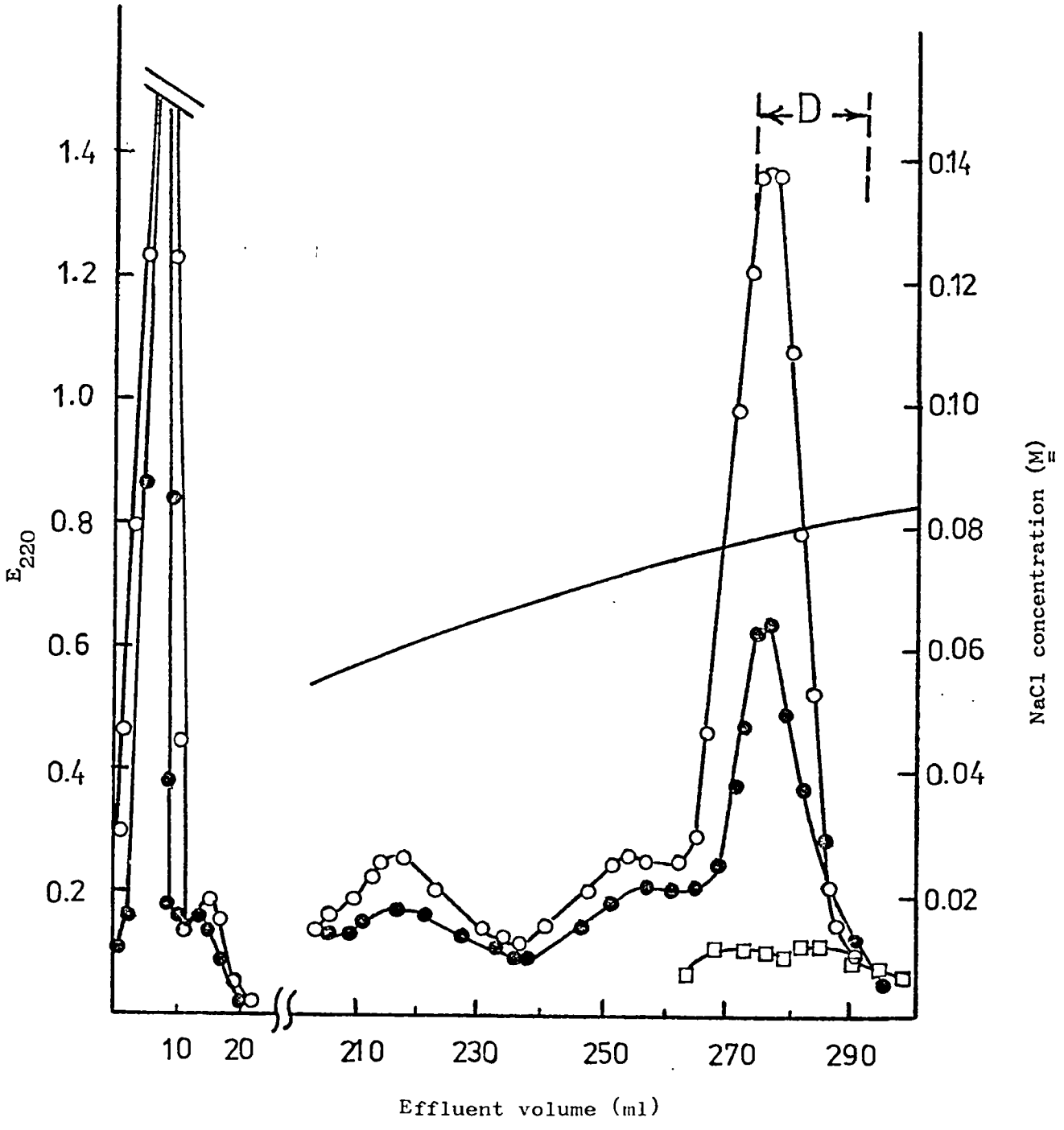


Fig. 13. Chromatography on CM-cellulose column (9.0 x 0.9 cm) of an incubation mixture of ribonuclease A, UDP-N-acetyl-glucosamine, $MnCl_2$ and liver homogenate as described in the text (see p. 120)

O —— O E_{220} ribonuclease A was present in the mixture.

_____ NaCl (M)

□——□ E_{220} no ribonuclease A was added to the mixture

●——● E_{220} ribonuclease A was added only after the incubation

Incubation was performed for 6 h at 37°C. A control mixture containing all the components, but without the addition of ribonuclease A, was also incubated.

When the incubation mixture containing added ribonuclease A was chromatographed on CM-cellulose (Fig. 13), the pancreatic ribonuclease appeared as the last peak of protein. This peak occurred at a region of the chromatograph where the NaCl concentration was 0.07$\underline{4}$M as in that experiment described in Fig. 12. This protein contained bound glucosamine: Fractions 272-290 were combined (Fraction D, Fig. 13) and dialysed against water, and the retentate was evaporated to dryness. The residue was hydrolysed in $\underline{4}$M HCl, 6 h, 100°C $\underline{\text{in vacuo}}$. The hydrolysate was analyzed by the Elson-Morgan reaction and it was shown to contain 0.14 moles glucosamine/mole ribonuclease, based on the amount of ribonuclease used. If it is assumed that ribonuclease-GlcNAc co-chromatographed with native ribonuclease, the extent of incorporation would be 0.23 mole/mole. To confirm that the peak was indeed due to ribonuclease A, the incubated mixture obtained in the control experiment was divided into two parts. One part was directly chromatographed on CM-cellulose. As shown in Fig. 13, there is a relatively small amount of protein eluted at the region where ribonuclease A is eluted. To the other part was added 2.6 mg ribonuclease A and the mixture was chromatographed. In neither of these control experiments was there glucosamine (Elson-Morgan reaction) in those regions of chromatograms corresponding to fraction D.

The conclusion drawn from the results of these experiments was that a catalytic incorporation of $\underline{\text{N}}$-acetyl-glucosamine into

ribonuclease A could be demonstrated *in vitro*. What was equally clear also was that there were small amounts of proteins from the liver homogenate chromatographing with ribonuclease A and its glycosylated product. A reasonable approach to resolving this problem seemed to be to fractionate the homogenate in order to increase the purity of the enzyme(s) fraction.

2. Distribution of UDP-N-acetyl-D-glucosamine-asparagine sequon N-acetyl-β-D-glucosaminyl transferase in subcellular fractions of liver

(i) Detection of enzyme in rough and smooth endoplasmic reticulum

It is often accepted that N-acetyl-β-D-glucosaminylation of the nascent apoprotein occurs at least largely, while the latter is associated with the polysomes (Molnar *et al.*, 1965; Molnar & Sy, 1967; Lawford & Schachter, 1966; Simkin & Jamieson, 1967; Redman & Cherian, 1972; see also p. 64.

In order to investigate the location of this enzyme in rabbit liver, the method of Dallner (1963), as described by Lawford & Schachter (1966) was used to prepare rough and smooth endoplasmic reticulum (see p. 101). The pellets obtained were suspended in buffer (Tris-HCl 0.05M, pH 7.4) to give a protein concentration equivalent to 1 g liver/0.2 ml buffer, as assayed by Lowry's method. Each pellet was assayed for enzyme activity as follows: Ribonuclease A, o.1 mg, was incubated with UDP-N-acetyl-glucosamine, 0.042 μmoles containing 238 cpm/nmole, 59mM $MnCl_2$ and 50mM buffer. The enzyme protein was either smooth endoplasmic reticulum (see Fig. 9), rough endoplasmic reticulum or pellet D (0.5 mg) in the final volume of 200 μl. Incubations were performed at 37°C for 1 h and terminated by addition of 2 ml 1% phosphotungstic acid in 0.5M HCl.

Assay conditions	2	3	4	5	6	7	8	9	10	11	12	13	14
Tris-HCl buffer (mM)	50[a]	50	50	50	50	50	50	50	50	50	50	50	50
Ribonuclease A (mM)	0.035	0.035	0.035	0.035	0.035	0-0.35	0.035	0.035	0.035	0.035	0.035	0.992	0.629
Amounts mg	0.1	0.1	0.1	0.1	0.1	0-1.0	0.1	0.1	0.1	0.1	0.1	41.7	20
UDP-GlcNAc (mM)	0.28	0.28	0.28	0.28	0.28	0.28	0-0.56	0.28	0.28	0.28	0.28	3.83	2.81
cpm/nmole	247	247	247	247	247	247	189	247	247	247	210	77.3	145
CDP-choline (mM)	0	0	0	0	0	0	0	0	0-8.0	0	0	0	0
UDP (mM)	0	0	0	0	0	0	0	0	0	0-0.5	0	0	0
Triton X-100 %	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0-1.0	0.3	0.3
N-acetyl-glucosamine (mM)	0	0	0	0	0	0	0	0-20	0	0	0	0	0
Enzyme protein (mg)	0.25	0.25	0.25	0.25	0-1.0	0.25	0.25	0.25	0.25	0.25	0.25	300[b]	300[b]
pH	5-9	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Temperature °C	37	37	20-40	37	37	37	37	37	37	37	37	37	37
$MnCl_2$ (mM)	50	50	50	0-180	50	50	50	50	50	50	50	20	26
Time (h)	1.0	0-6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	6	6
Total volume (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	3.0	2.27

(a) In this experiment the buffer used was Tris-maleate 0.05M in both;

(b) This value is the equivalent amount of liver in mg from which the preparation has been derived

TABLE 11. Conditions of incubation used in the enzymic N-acetyl-glucosaminylation of bovine pancreatic ribonuclease A with preparations of rabbit liver.

Control experiments were also carried out by omitting ribonuclease from the mixture. Results of the experiment reveal that there is radioactivity incorporated into both endogenous protein and ribonuclease A when rough endoplasmic reticulum is used as the source of the enzyme. The incorporation, however, is greater when ribonuclease is present in the mixture (Table 8).

There is also incorporation when smooth endoplasmic reticulum is used.

Pellet D, which contains nuclei, mitochondria, rough and smooth endoplasmic reticulum and other cellular material (Lawford & Schachter, 1966) is quite rich in the N-acetyl-glucosaminyl transferase in question. It seems likely that the enzyme is present in fractions additional to rough and smooth endoplasmic reticulum (Table 8).

(ii) Factors affecting enzymic activity

Having established the presence of the enzyme in preparations of rough endoplasmic reticulum from rabbit liver, attempts were focused on finding optimum conditions for the enzymic incorporation of N-acetyl-glucosamine into ribonuclease A. The factors needing investigation were pH, time, enzyme and substrate concentration. Accompanied with these studies, the effects of various inhibitors and activators were also investigated.

(a)pH

Incubations were set up containing the components listed in Table 11, column 2. The buffer used in this case was Tris-maleate (0.05M in each) and the pH range selected (adjusted with NaOH) was 5-9. Incubations were performed for 1 h at 37°C and

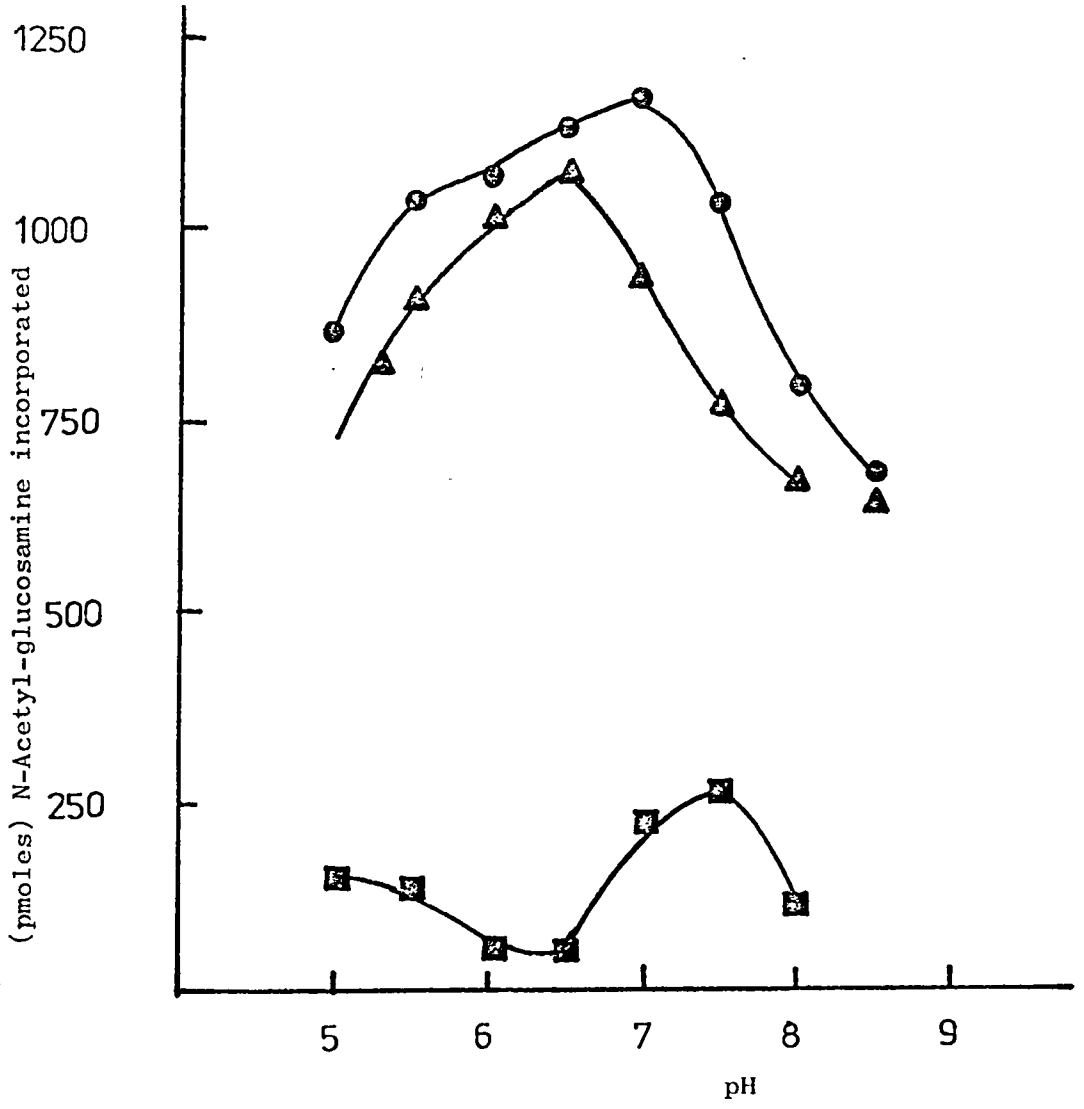


Fig. 14. Effect of pH on the incorporation of N-acetyl-glucosamine into ribonuclease A catalysed by preparations of rough endoplasmic reticulum from rabbit liver.

●——● Ribonuclease A present in the mixture

▲——▲ Incubations performed in absence of ribonuclease

■---■ The difference curve

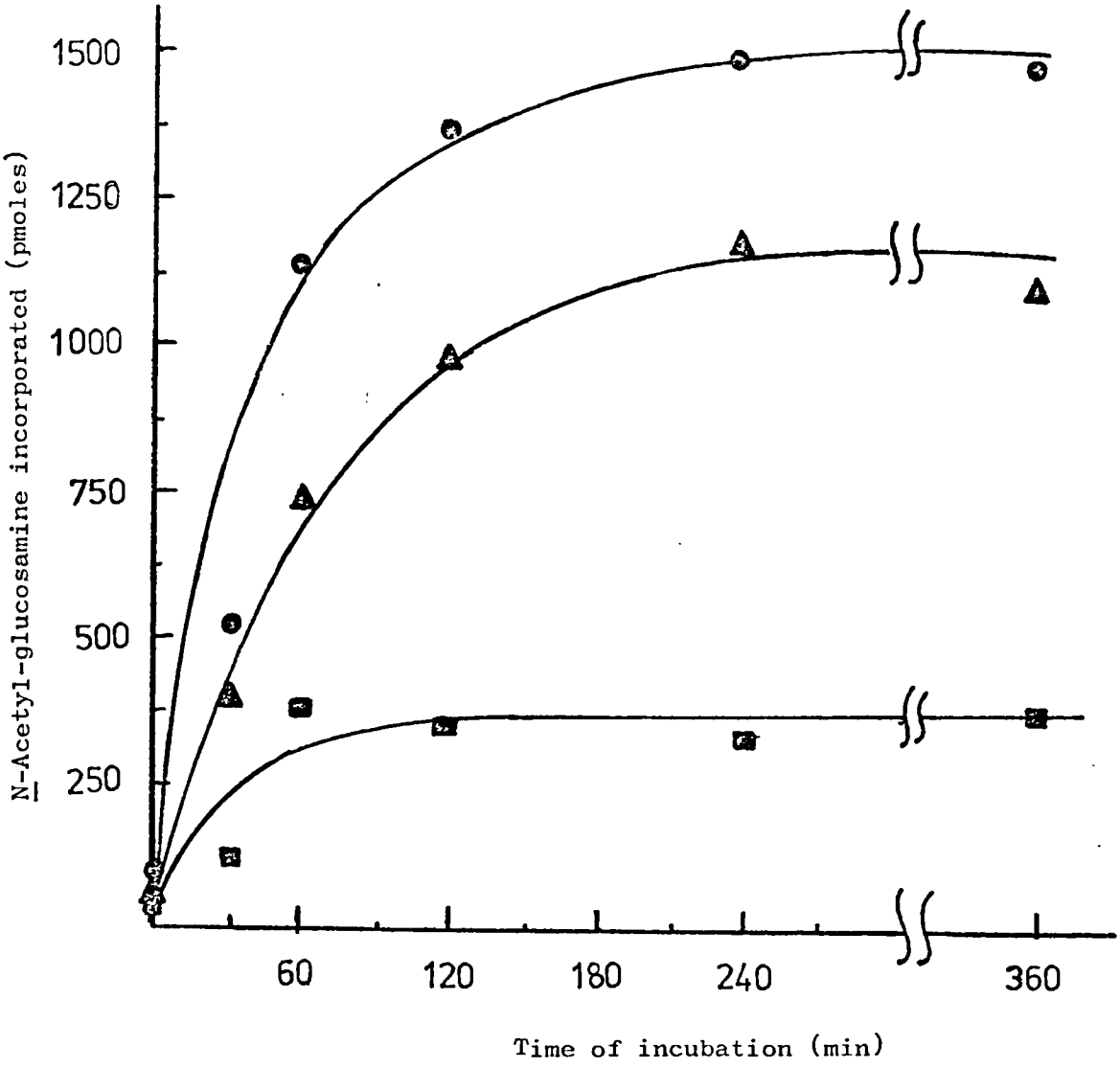
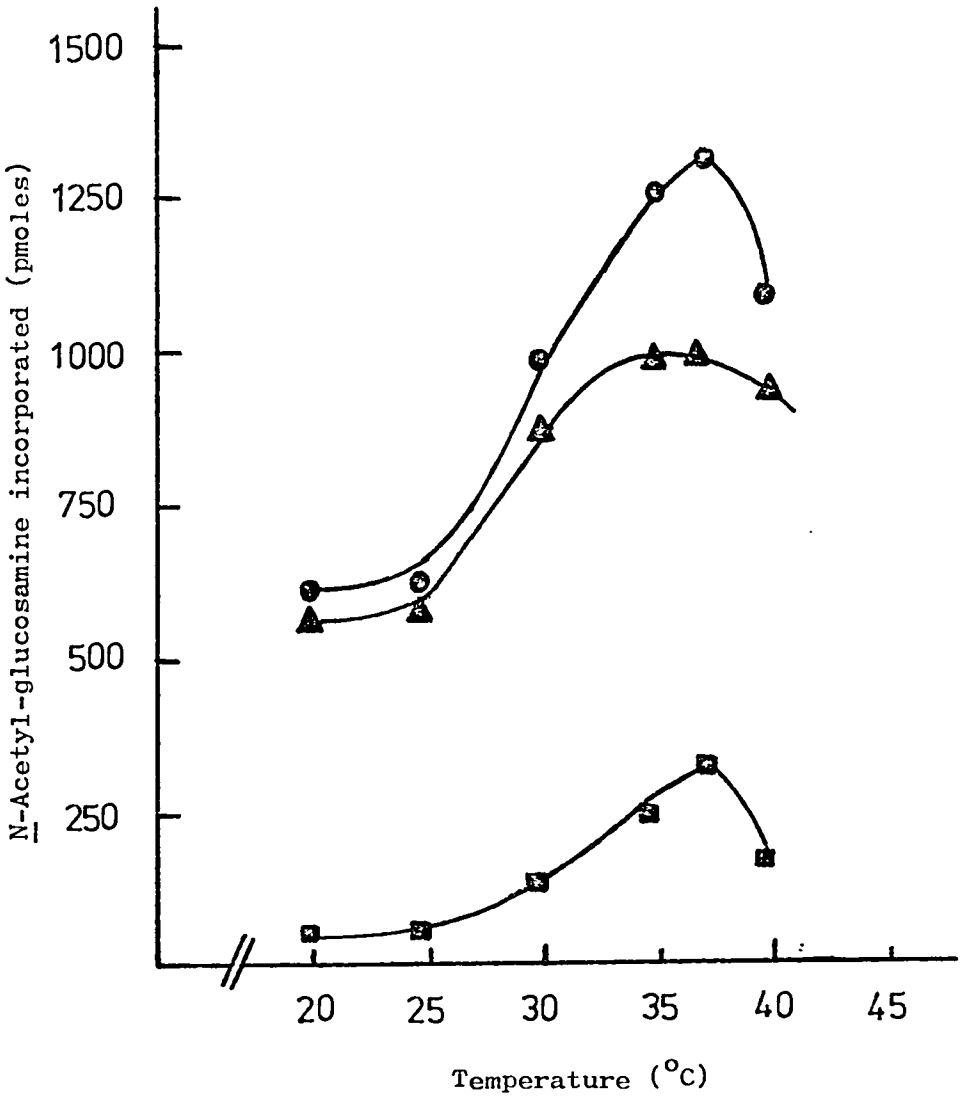


Fig. 15. Rate of incorporation of N-acetyl-glucosamine into ribonuclease A.

●—● Incubation performed in the presence of ribonuclease A

▲—▲ Incubations carried out in the absence of added ribonuclease

■—■ Difference curve which represents incorporation into ribonuclease



●——● Incubations performed in the presence of ribonuclease A

▲——▲ Incubations performed in the absence of ribonuclease A

■——■ represents the difference

Fig. 16. Effect of temperature on the incorporation of N-acetyl-glucosamine into acid precipitable material using preparations of rough endoplasmic reticulum from rabbit liver (for conditions of incubation see Table 11, column 4).

terminated by acid precipitation as stated before. The results presented in Fig. 14 indicate that the incorporation into endogenous protein has a pH optimum of 6.5, while the incorporation into exogenous protein (ribonuclease A) had an optimum value of 7.4. The maximum extent of incorporation at this pH amounted to $\sim$4%.

(b) Time course of incorporation of N-acetyl-glucosamine

The rate of incorporation of N-acetyl-glucosamine into ribonuclease A was studied by incubating ribonuclease A with UDP-GlcNAc under the conditions given in Table 11, column 3. The pH of the incubation mixture was taken from the previous studies as 7.4 and control experiments devoid of ribonuclease A were performed simultaneously. The results presented in Fig. 15 indicate that reaction was practically complete within about 2 h, but only about 6% of the ribonuclease had undergone glycosylation.

(c) Temperature

The effect of temperature on the activity of the enzyme was studied over the range 20-40°C. The conditions of incubation are given in Table 11, column 4. At temperatures below 30°C the rate of incorporation is very small and it increases with increasing temperature, reaching a maximum around 35-37°C, and then it falls off. The incorporation into endogenous protein also follows a similar pattern (Fig. 16).

(d) Metal ions

Incubations were set up containing the components described in Table 11, column 5, but with varying concentrations of the

following metal ions (the salt used was a chloride): Co^{+2}, Mn^{+2}, Ni^{+2} and Mg^{+2}. Control experiments were also carried out in the identical manner except ribonuclease was omitted from the mixture. The reaction was terminated by the addition of 1% phosphotungstic acid in 0.5M HCl. The precipitates were filtered on millipore, washed and dried. The radioactivity incorporated into acid precipitable material was measured by scintillation counting. The results (Fig. 17) show that Co^{+2} and Mn^{+2} are effective in increasing the rate of incorporation of N-acetyl-glucosamine into endogenous protein as well as into ribonuclease A. The optimum concentration is around 50mM. Ni^{+2} ions are less effective; Mg^{+2} has no effect.

(e) Protein concentration

The conditions of incubation are given in Table 11, column 6. The enzyme protein was rough endoplasmic reticulum suspended in buffer at protein concentrations from 0-1.0 mg per total incubation mixture. Results indicate that the incorporation rate is not directly proportional to the amount of the enzyme protein used (see Fig. 18).

(f) Ribonuclease A concentration

This factor was investigated using the enzyme preparation described immediately above. The conditions of incubation are given in Table 11, column 7. Ribonuclease A was the variable factor ranging in concentration from 0-0.35mM. The incorporation rate was measured as usual by acid precipitation of the radioactive products formed, followed by scintillation counting. As Fig. 19 shows, under the assay conditions used, the incorporation

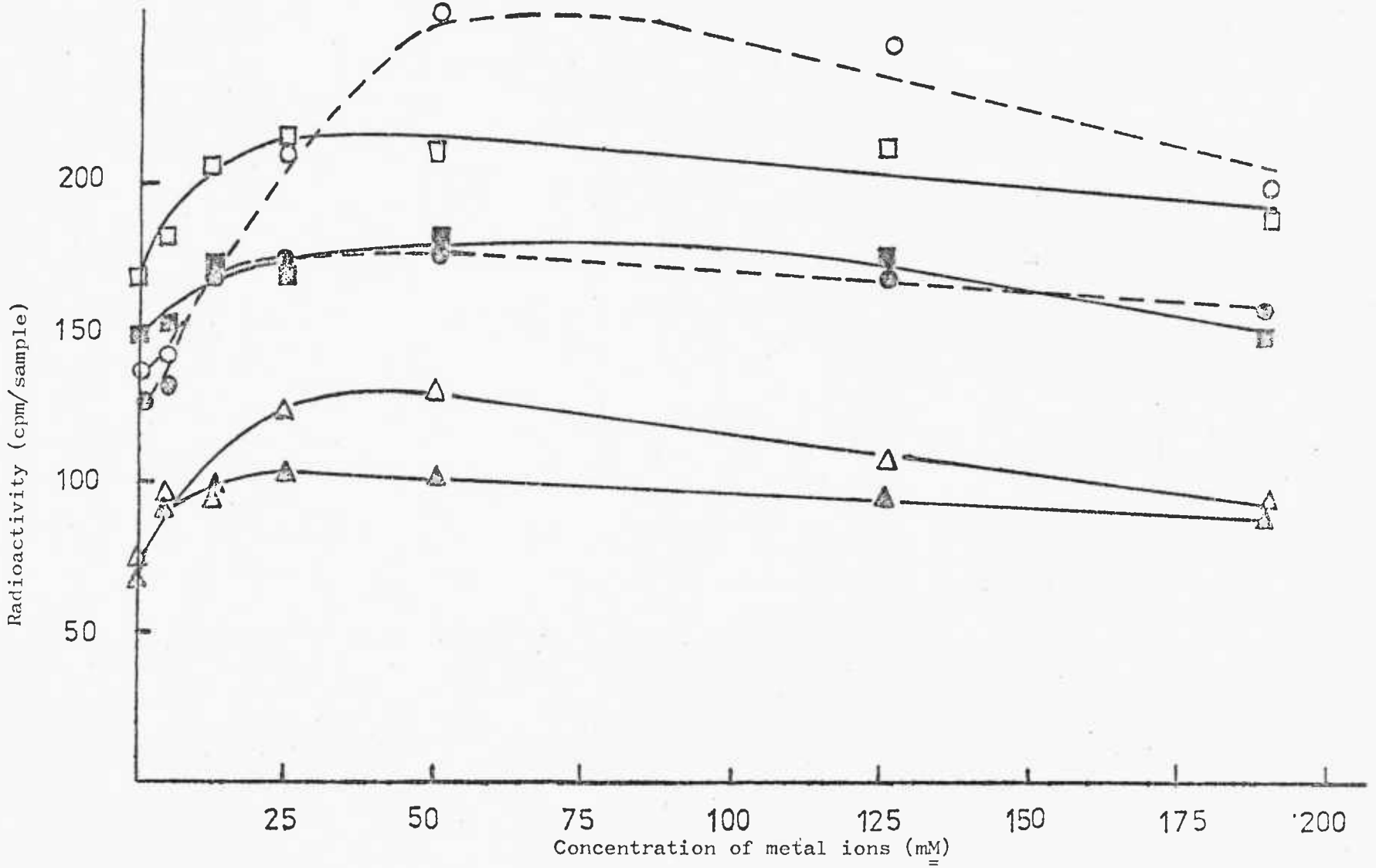


Fig. 17. Effect of metal ions on the incorporation of N-acetyl-glucosamine into ribonuclease A.

O—O Co^{+2} with added ribonuclease A: ●—● without added ribonuclease: □—□ Mn^{+2} with added ribonuclease A:
■—■ Mn^{+2} without added ribonuclease: △—△ Ni^{+2} with added ribonuclease A: ▲—▲ without added ribonuclease

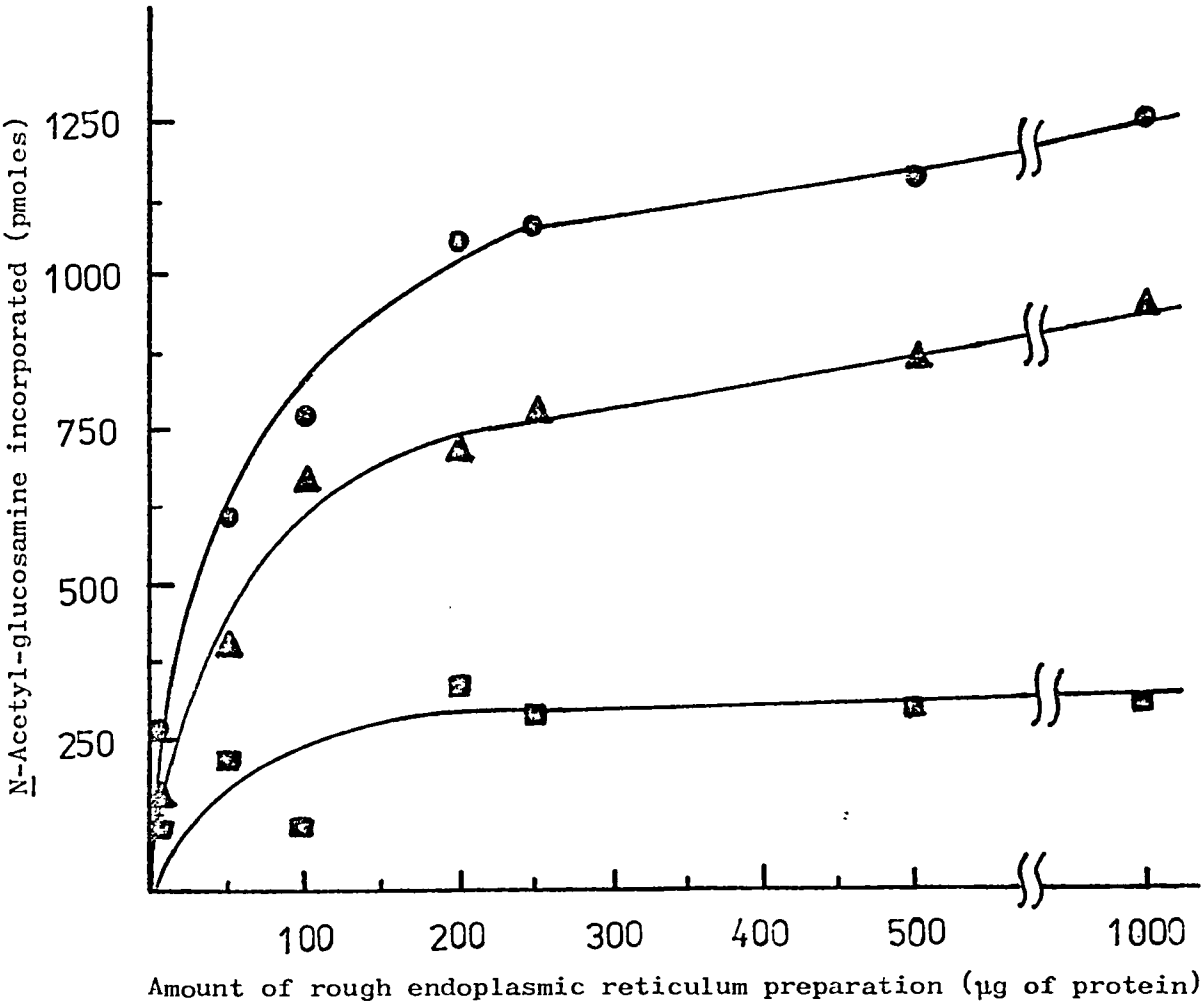


Fig. 18. Incorporation of N-acetyl-glucosamine into ribonuclease A catalysed by preparations of rough endoplasmic reticulum from rabbit liver. For conditions of incubation see Table 11, column 6.

●—● Ribonuclease was added to the incubation mixture

▲—▲ Incubation was performed in the absence of ribonuclease A

■—■ presents the difference

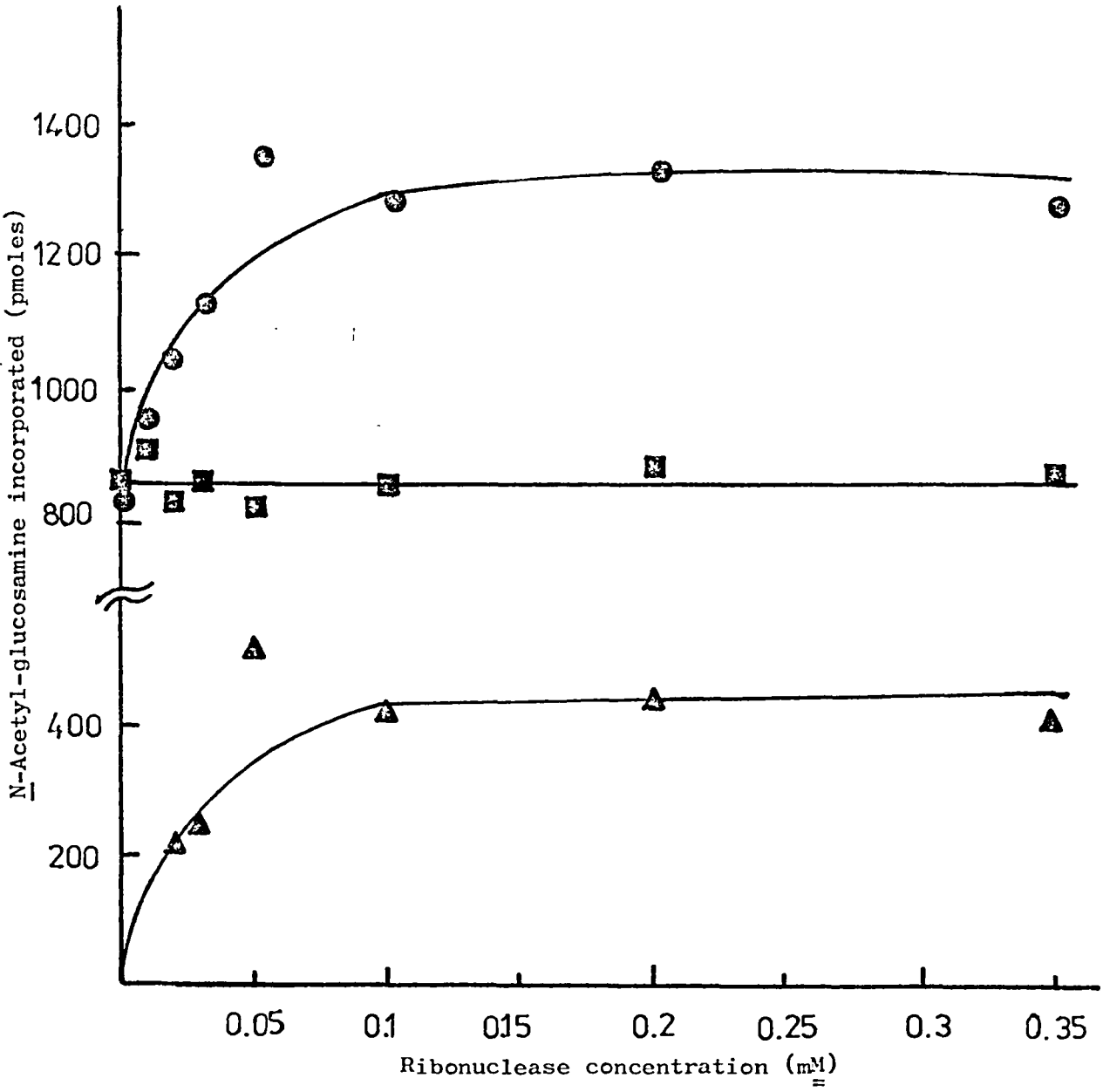


Fig. 19. Relationship between the extent of incorporation of N-acetyl-glucosamine and the concentration of ribonuclease A. Incorporation into acid precipitable material when ribonuclease A was present in the mixture, ●—●; incorporation when ribonuclease A was omitted from the mixture ■—■ ; the difference curve is shown as ▲—▲

rate is at a maximum at ribonuclease concentrations of 0.05mM and above.

(g) UDP-N-Acetyl-glucosamine concentration

Conditions of this experiment were similar to the one mentioned above, with the difference that there were variations in the concentration of UDP-N-acetyl-glucosamine; the ribonuclease A concentration was in all cases 0.035mM. Control experiments devoid of ribonuclease A were carried out simultaneously (for conditions of incubation see Table 11, column 8). Fig. 20 presents the results. The incorporation rate increased with the UDP-N-acetyl-glucosamine concentration over the range employed.

(h) N-Acetyl-glucosamine

Conditions of incubation are described in Table 11, column 9. The variable factor in these experiments was the concentration of N-acetyl-glucosamine (0-20mM). Again, the radioactivity incorporated into acid precipitable material was measured. It seems that the presence of N-acetyl-glucosamine, even up to a concentration of 20mM, exerts no significant effect on the rate of incorporation into either endogenous protein or ribonuclease A.

(i) CDP-Choline

The effect of CDP-choline on the extent of glycosylation of ribonuclease A was investigated by incubating mixtures containing the components listed in Table 11, column 10, but with varying amounts of CDP-choline (0-8mM). The radioactivity incorporated into acid precipitable material was estimated as before. CDP-choline exhibited a small stimulatory effect on the incorporation

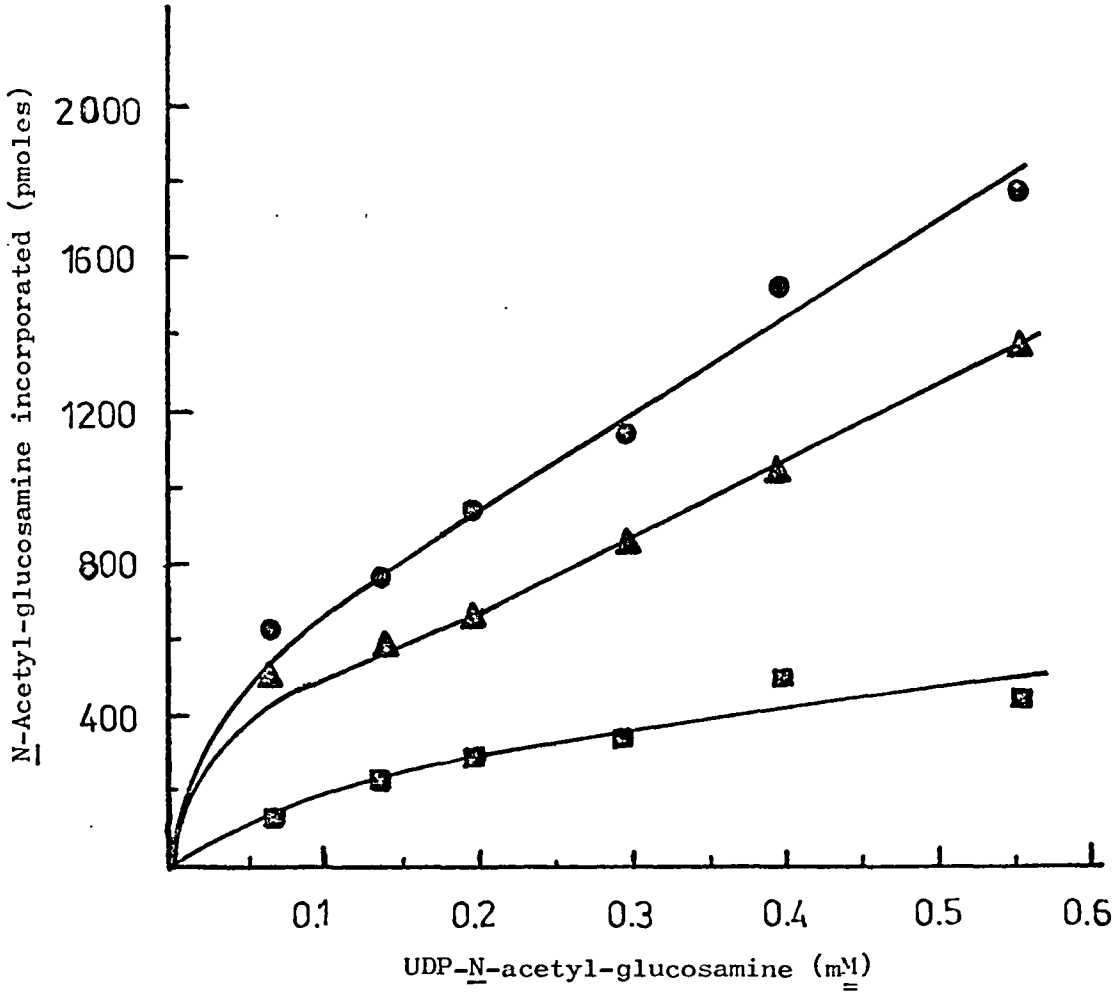


Fig. 20. Effect of UDP-N-acetyl-glucosamine concentration on the activity of N-acetyl-glucosaminyl (→ asparagine sequon) transferase. Enzyme protein amount was 250 µg per tube.

●—● Ribonuclease A present in the incubation mixture

▲—▲ Ribonuclease A was omitted from the mixture

■—■ represents the difference curve

of the radioactivity into ribonuclease A (Fig. 21a). The effect is almost linear with respect to the increasing concentration of CDP-choline.

(j) Effect of UDP on the rate of incorporation

The conditions of this experiment were similar to the one described above (Table 11, column 11), except that CDP-choline was replaced by uridine diphosphate. The concentration of UDP varied from 0-0.5m$\underline{M}$. Fig.21b shows that UDP acts as an inhibitor for the enzymic incorporation of $\underline{N}$-acetyl-glucosamine into ribonuclease A. At a concentration of 0.5m$\underline{M}$, UDP exhibits $\sim$50% inhibition of the enzyme activity.

(k) Effect of membrane modifiers on the activity of the enzyme

The activities of several enzymes which are membrane bound appear to depend on the structure of the lipid phase of the membrane. Some of the earlier works by others will be discussed later (p. 169), and in view of those findings, the effects of phospholipase A_2 (see below), of the non-ionic detergent Triton X-100 and of sonication were assessed.

(i) Effect of Triton X-100

Rough endoplasmic reticulum prepared by Dallner's method from 12 g of liver was suspended in 0.05$\underline{M}$ Tris-HCl buffer, pH 7.4, to a final concentration of 10 mg protein per ml.

Incubations were performed under conditions given in Table 11, column 12. Triton X-100 concentration varied from 0-1%. The radioactivity incorporated into acid precipitable material increased by a factor of 1.7 in the presence of 0.04% detergent,

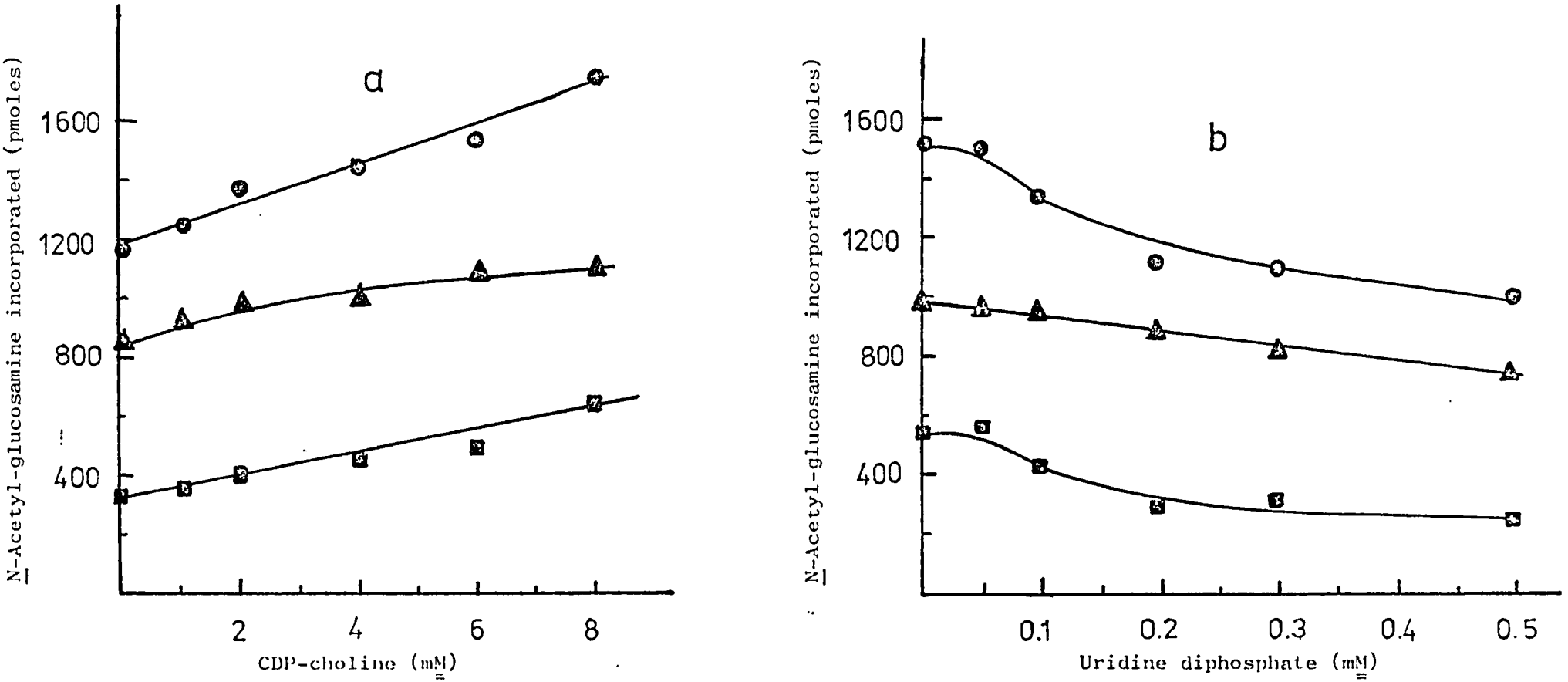


Fig. 21. Effect of (a) CDP-choline and (b) uridine diphosphate on the N-acetyl-glucosaminyl (——> asparagine sequon) transferase activity in the preparations of rough endoplasmic reticulum from rabbit liver.

●—● Incubation mixtures containing added ribonuclease A

▲—▲ Incubation mixtures devoid of added ribonuclease A

■—■ represents the difference curve

1.68 (0.08%), 1.56 (0.1%), 1.67 (0.2%), 1.56 (0.3%), 1.52 (0.5%) and 1.4 (1%). The radioactivity incorporated was largely unaltered in the experiments where ribonuclease A was omitted from the mixture.

(ii) Sonication

An MSE Ultrasonic Power Unit was used for these experiments. Aliquots (0.2 ml) of the suspension of rough endoplasmic reticulum in the buffer were taken and sonicated at 1 Amp for 0, 15, 30, 60, 120 and 240 seconds. The sonication was performed at 0°C. The sonicated preparations were assayed for enzyme activity by incubating 250 µg enzyme protein with the following components: Ribonuclease A 0.035mM, UDP-N-acetyl-glucosamine 0.28mM containing 247 cpm/nmoles, $MnCl_2$ 50mM, buffer (Tris-HCl, pH 7.4) 50mM and Triton X-100 0.4%. The incubation was performed for 1 h at 37°C. The activity incorporated into acid-precipitable material was measured and plotted against the time of sonication. As can be seen from Fig. 22, a prolonged sonication inhibits the incorporation both into endogenous and exogenous acceptors. However, there is little effect on the activity at 0-30 sec of sonication time.

(iii) Phospholipase A

0.4 ml of the suspension of rough endoplasmic reticulum in buffer (10 mg/ml) was incubated with phospholipase A at 25°C. The ratio of phospholipase A to protein was 1/100 (Zakim et al., 1973). Aliquots were taken at 0, 0.5, 1, 10, 15, 30, 60 and 120 min intervals. The reaction was stopped by the addition of EDTA (5mM final concentration). The enzyme activity after

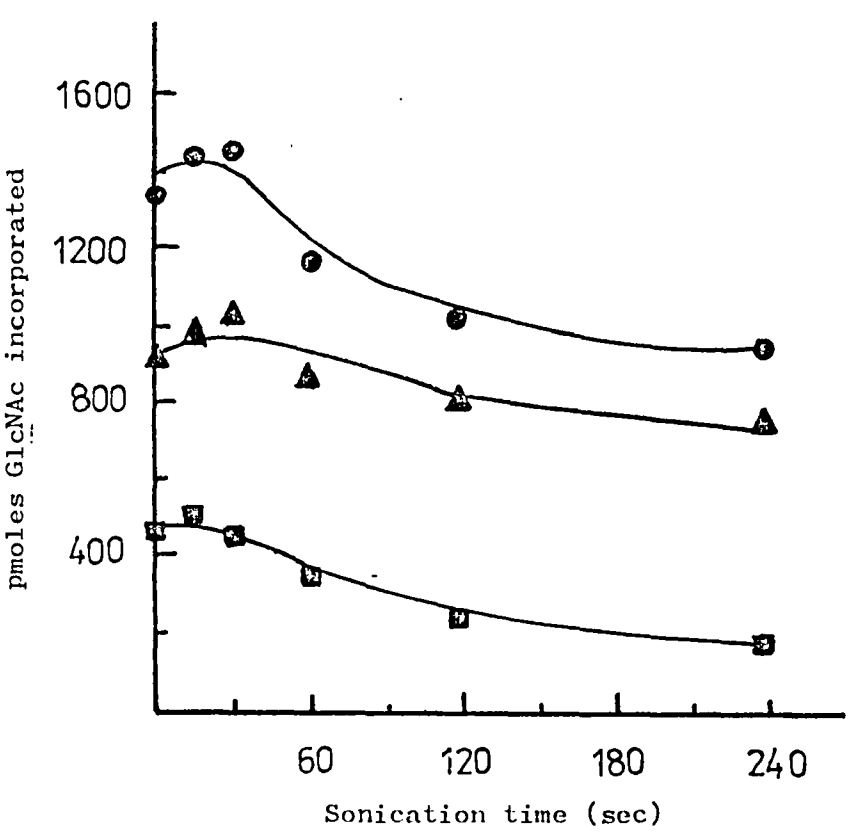


Fig. 22. Effect of sonication time on transferase activity of preparations of rough endoplasmic reticulum from rabbit liver. 0.2 ml vols of a suspension of rough endoplasmic reticulum (10 mg protein/ml) in 0.05M Tris-HCl buffer were sonicated at 2°C at 1 Amp
●—● ribonuclease A present in the mixture
▲—▲ ribonuclease A was omitted from the mixture
■—■ the difference curve

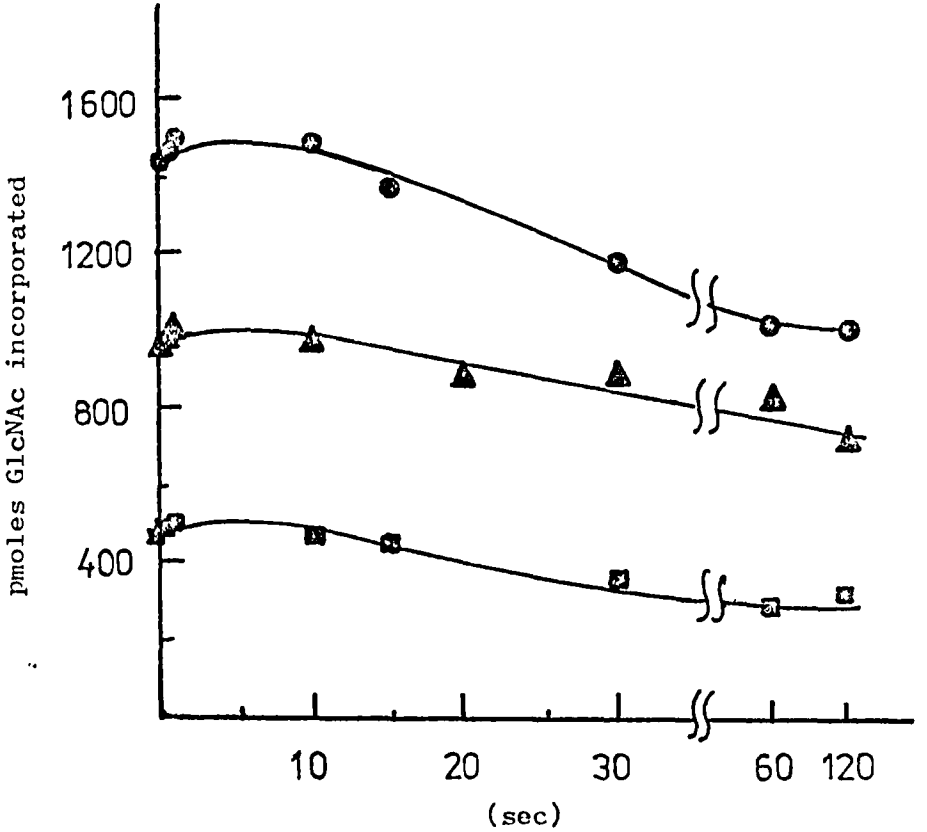


Fig. 23. Effect of treatment of membrane with phospholipase A_2 on the enzyme activity. 4 mg enzyme protein in 0.4 ml Tris-HCl buffer, pH 8.0 containing 40 µg (8 I.U.) phospholipase A_2 were incubated at 25° C. Reaction was stopped by adding EDTA to aliquots removed for assay to a final concentration of 5mM: EDTA at the concentration used causes a loss of ~7% of the transferase activity

phospholipase A treatment was measured by the usual assay method. The results obtained are presented in Fig. 23. The incorporation rate appears to be unaltered when short periods of incubation are used.

3. Identification of the product of glycosylation of ribonuclease A

It was reported in 1973 that both ribonuclease A and S-carboxymethylated ribonuclease A could potentiate *in vitro* the transfer of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine to endogenous acceptors, catalysed by preparations of microsomes from rat liver (Letts & Schachter, 1973). In view of this report it was of greatest importance to demonstrate that glycosylation of ribonuclease A was indeed occurring.

(i) Chromatographic separation (CM-cellulose) of a mixture of ribonuclease A and glycosylated ribonuclease from appropriate incubation mixtures

Ribonuclease A (41.7 mg) was incubated with a portion of rough endoplasmic reticulum (corresponding to 300 mg liver homogenate) in the presence of Mn^{+2} and UDP-N-acetyl-glucosamine (for assay conditions see Table 11, column 13). The reaction mixture was chromatographed on CM-cellulose (15 x 0.9 cm). There was radioactivity in the earlier part of the ribonuclease peak (Fig. 24). There was about 0.072 moles glucosamine per mole of ribonuclease A (7.2% conversion). The ribonuclease present in the latter part of the peak could also be converted into a glycosylated form. Fractions 62-82 (Fig. 24) were combined, dialysed, evaporated to dryness and glycosylated again under the conditions given in Table 11, column 14. The mixture

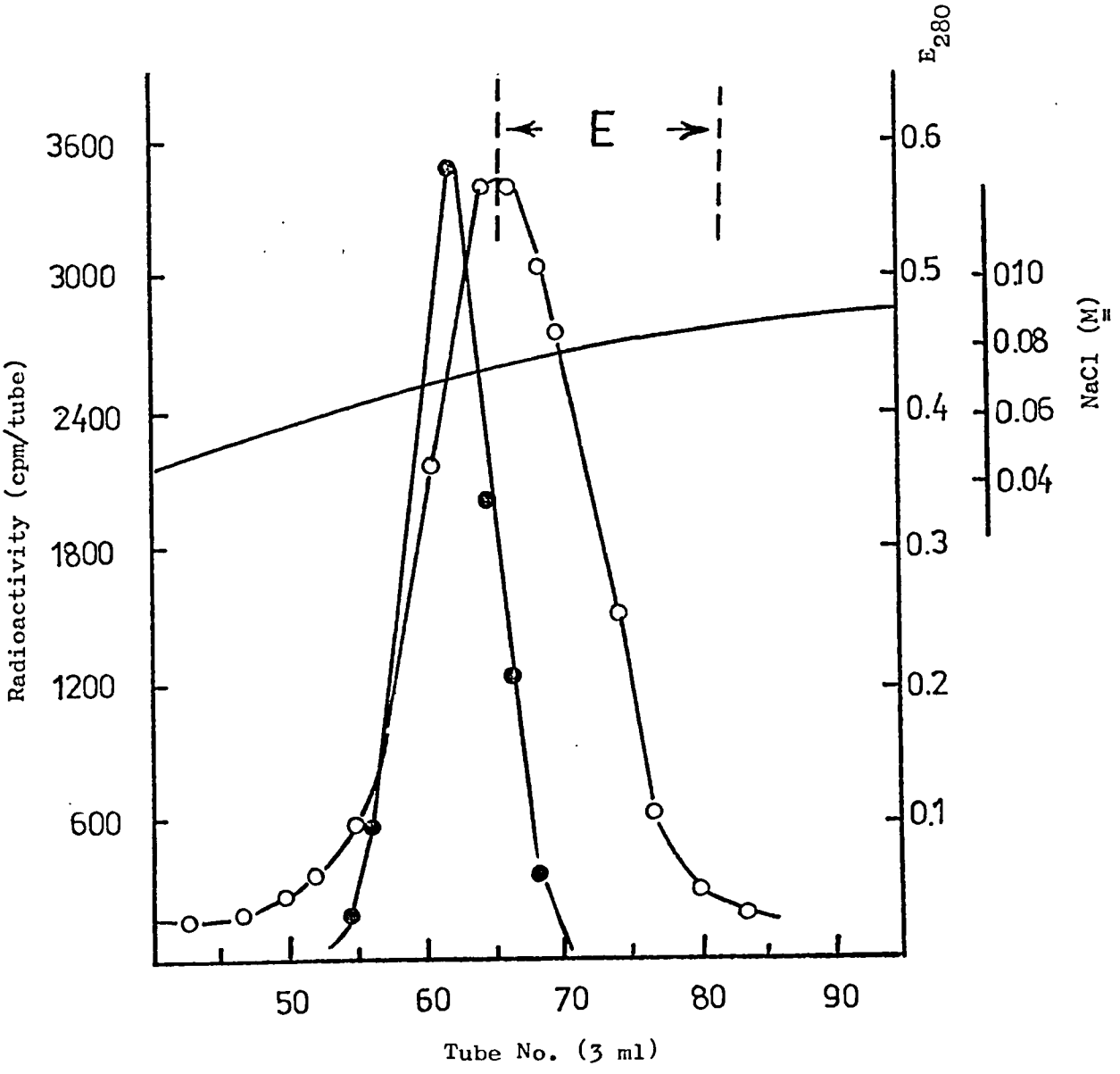


Fig. 24. Partial separation of monoglycosylated ribonuclease by chromatography on CM-cellulose column.

o —— o E_{280}

● —— ● Radioactivity

_____ NaCl gradient

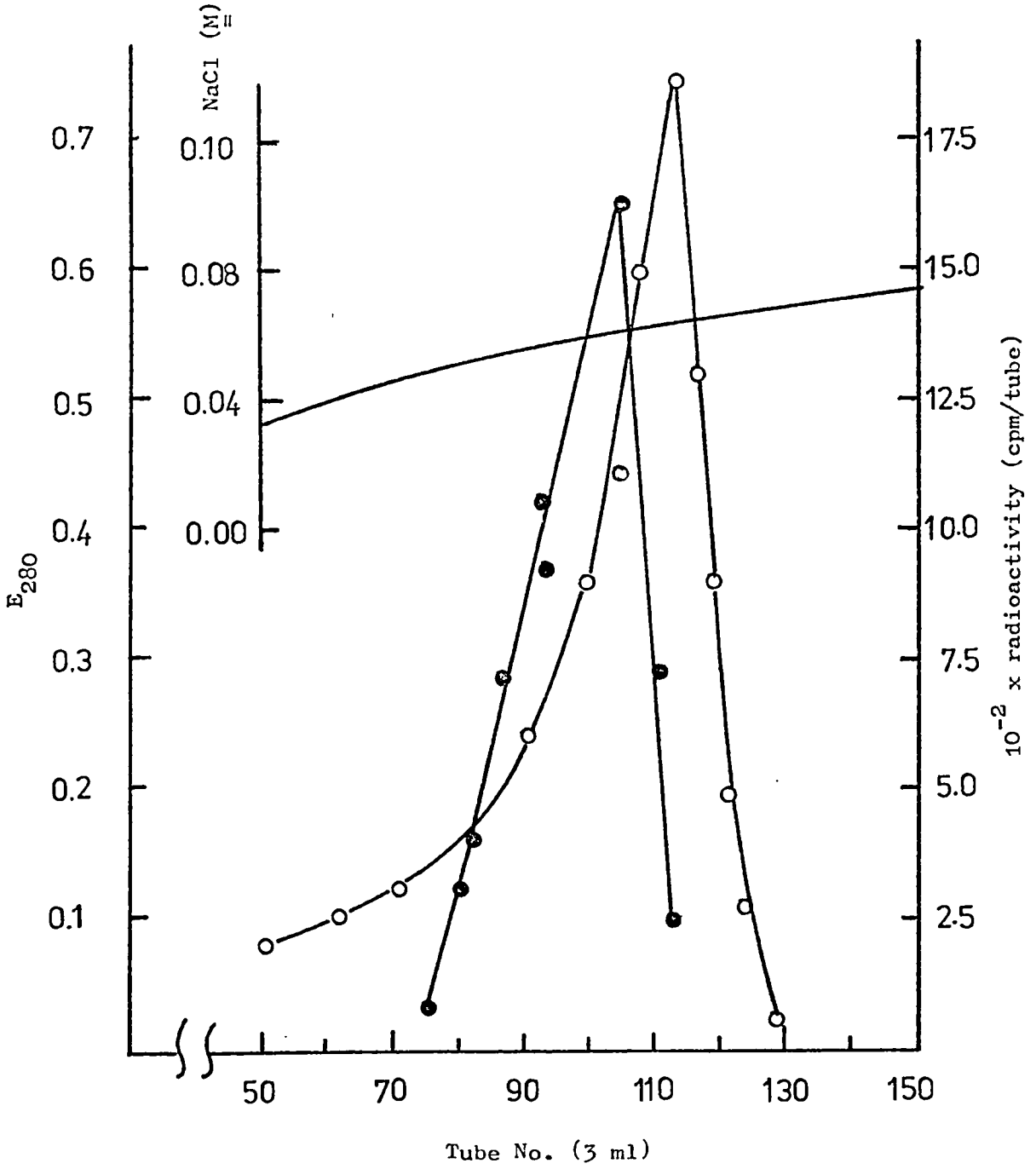


Fig. 25. Partial purification of $[^{14}C]$-labelled monoglycosylated ribonuclease on CM-cellulose column using a shallower NaCl gradient.

o —— o E_{280}

● —— ● Radioactivity

_____ NaCl gradient

was chromatographed on CM-cellulose using a slightly shallower gradient (Fig. 25) in the hope of separating glycosylated and non-glycosylated ribonuclease: This hope was not fulfilled. It is apparent that the glycosylated form of ribonuclease produced under the conditions described is less tightly bound to CM-cellulose, but the chromatographic conditions used do not allow complete separation of the two forms. In this experiment 0.162 moles of glucosamine were incorporated into one mole of ribonuclease A.

(ii) Attempted separation of glycosylated ribonuclease and ribonuclease A by chromatography on Amberlite CG-50

A column of CG-50 (45 x 0.9 cm; 200-400 mesh) was equilibrated with sodium phosphate buffer (0.2M in phosphate, pH 6.02) and was calibrated as follows: 2.51 mg ribonuclease A was dissolved in 1 ml sodium phosphate buffer (0.2M, pH 6.02) and applied to the column. The elution was performed using the same buffer at a flow rate of 24 ml/h. Fractions of 3.3 ml were collected and assayed for protein (see Fig. 26a). As shown, the protein appears as a relatively broad peak (tubes 55-80). A mixture of ribonuclease A and its glycosylated form (5.7 mg total ribonuclease; ~ 3,000 cpm) was dissolved in 1 ml buffer and applied to the column and eluted with the 0.2M phosphate buffer, pH 6.02. Fractions of 2 ml were collected and assayed for protein and radioactivity (Fig. 26b). The separation of the two components was not complete and the glycosylated ribonuclease appeared as a shoulder on the ribonuclease peak. The recovery of radioactivity was ~60%. Fractions 92-103 were combined, dialysed and freeze-dried. The freeze-dried sample

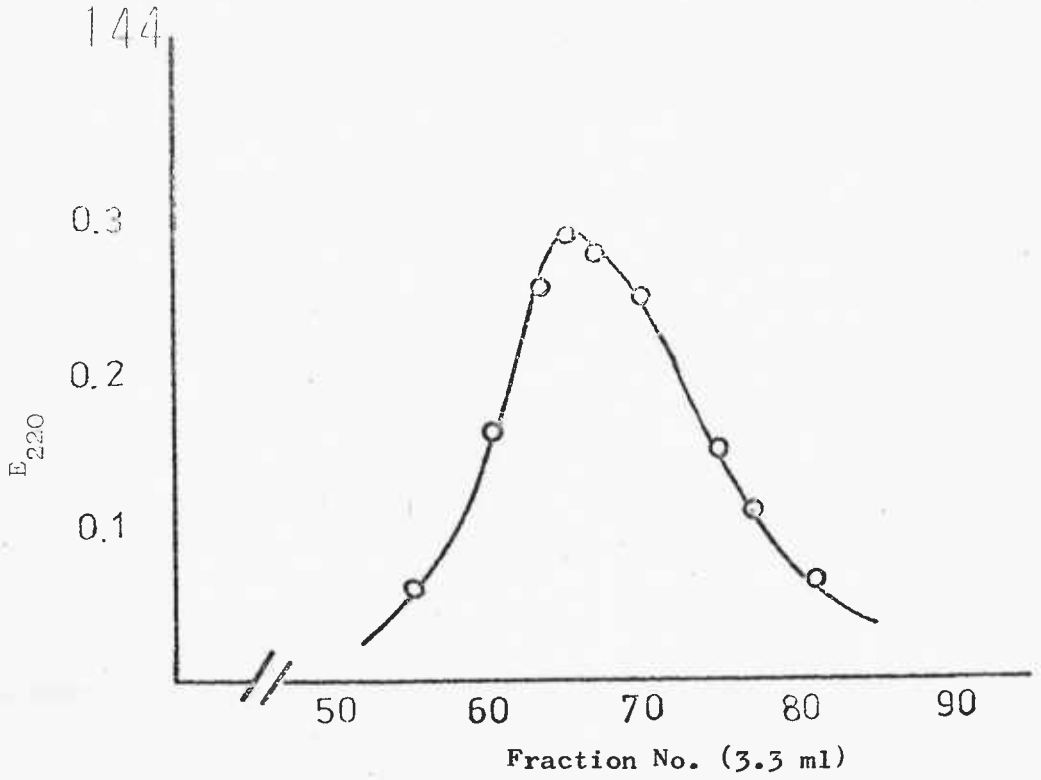


Fig. 26a. Elution profile of ribonuclease A (2.51 mg) on a CG-50 column (45 x 0.9 cm; 200-400 mesh). Elution was effected using a sodium phosphate buffer 0.2M, pH 6.02

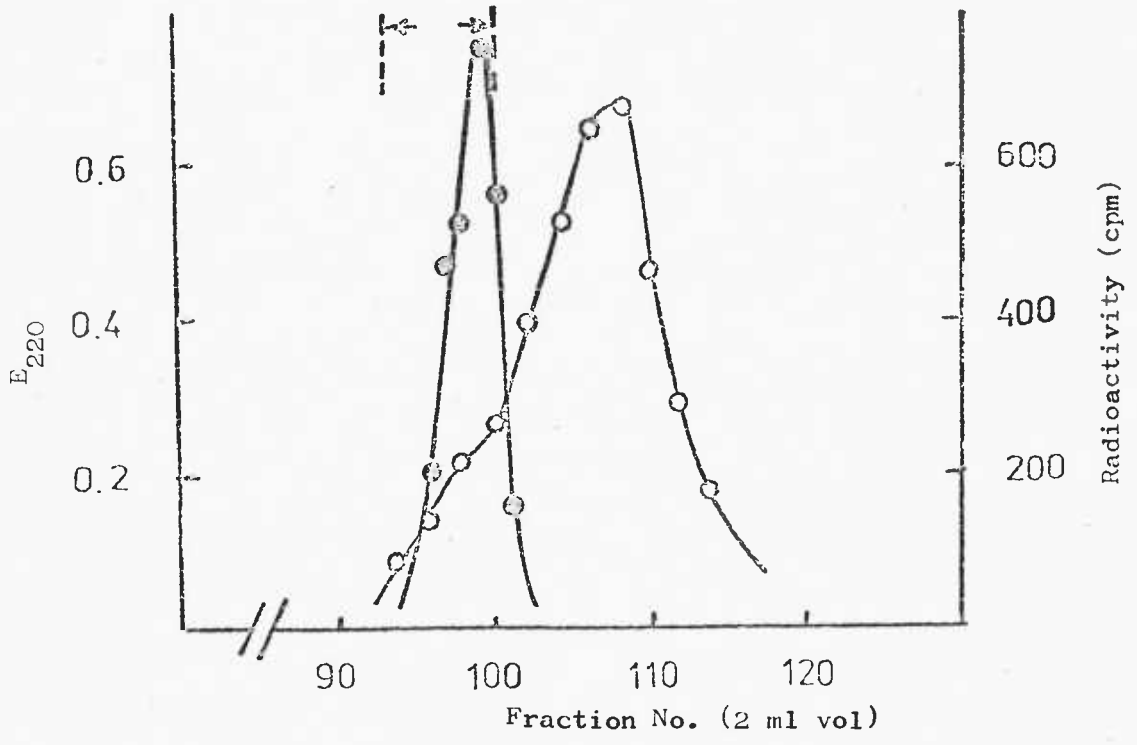


Fig. 26b. Chromatography of a mixture of ribonuclease A and its glycosylated form (5.7 mg total ribonuclease, ~ 3,000 cpm) on a column of CG-50 (45 x 0.9 cm)

o—o E_{220} ●—● radioactivity

was rechromatographed on the CG-50 column with the hope of obtaining a better separation, but no protein peak or radioactivity could be recovered from the column: It is possible that at the very low concentration of the protein used irreversible adsorption had occurred.

(iii) Isolation of radioactive glycopeptides from tryptic digest of a mixture of ribonuclease A and glycosylated ribonuclease

The mixture of ribonuclease A and its glycosylated product (containing 73.2nmoles glucosamine; 10626 cpm) was taken for tryptic digestion. The sample was first cyanoethylated according to the method of Anfinsen & Haber (1961) as described by Plummer & Hirs (1964).

Ribonuclease (7 mg) was dissolved in a freshly prepared solution of 8M urea (the urea solution was adjusted to pH 8.5 with 0.1% methylamine solution and subjected to a stream of nitrogen before use). The final concentration of protein in urea was $\sim$ 0.5mM. A 50-fold excess of the theoretical amount of mercaptoethanol was added with stirring and the pH was adjusted to 7.9 with NaOH. After $4\frac{1}{2}$ h, twice the quantity of acrylonitrile needed to react with the originally added mercaptoethanol was added to the mixture, the pH being maintained at a constant value of 7.9 with 0.02 N NaOH. The reaction was allowed to continue for a further 2 h and then the pH was lowered to 4 by glacial acetic acid and the solution immediately transferred to a G-25 column (30 x 2.5 cm) and eluted with 0.1M acetic acid. The protein peak, which appeared in the void volume was pooled, was freeze-dried and the modified protein taken up for tryptic digestion.

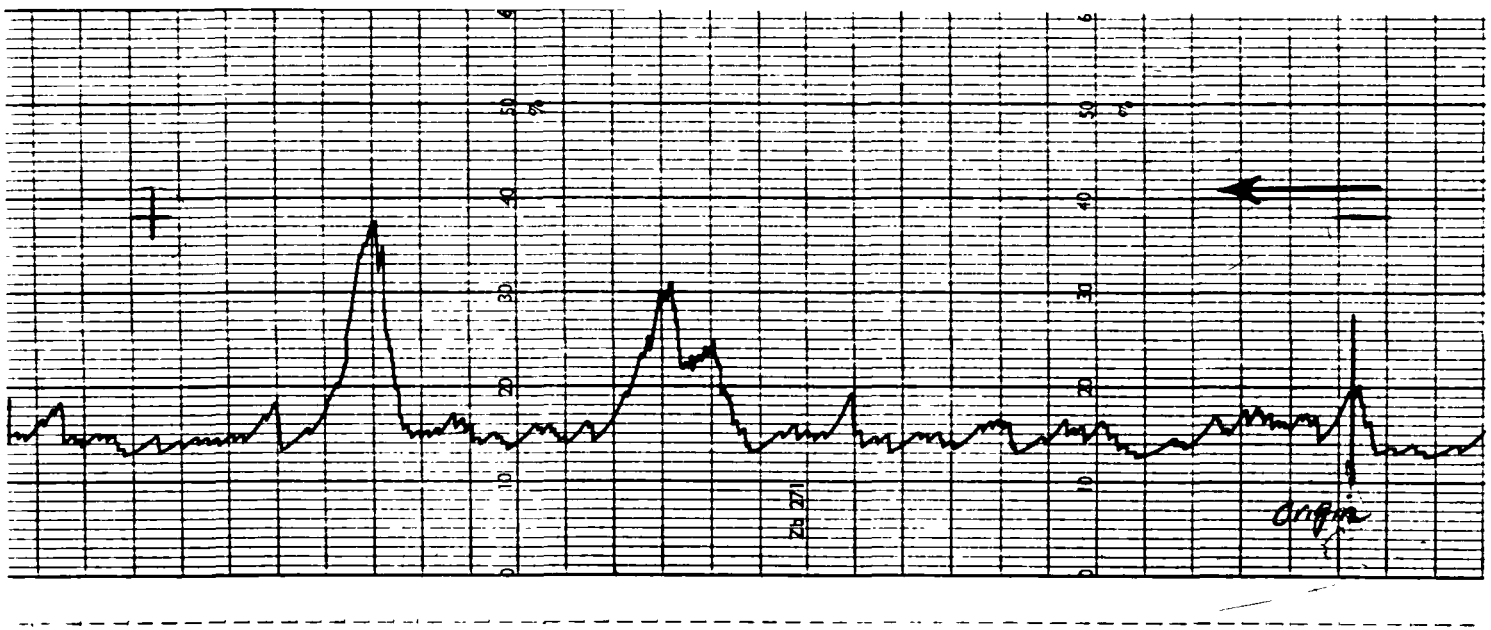


Fig. 27. High voltage electrophoresis of peptide fragments obtained by tryptic digestion of cyanoethylated ribonuclease-GlcNAc. The strips were scanned using a Panax gas-flow strip counter. The first radioactive peak had moved a distance of 14.1 cm and the second 20.2 cm towards the cathode.

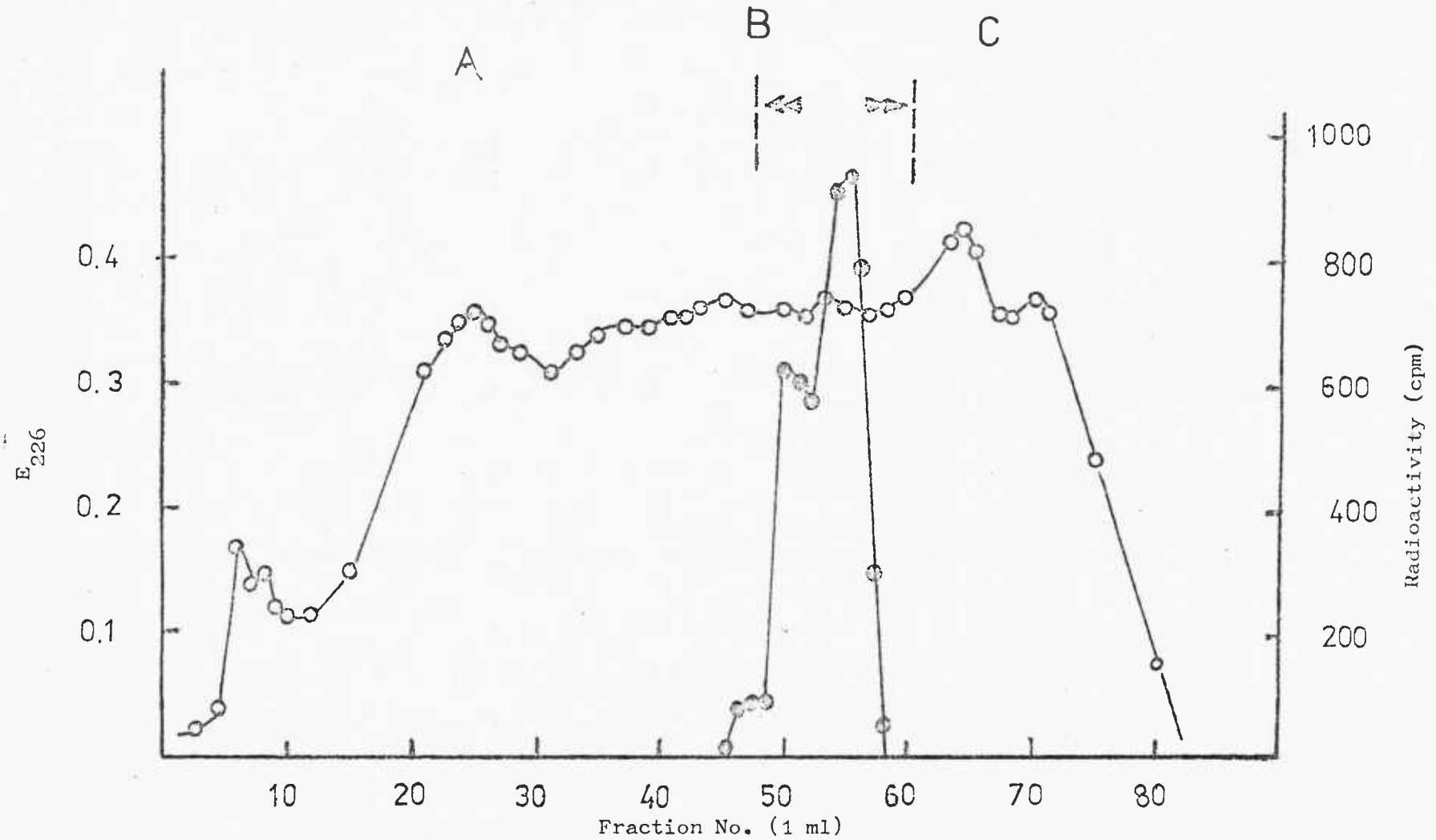


Fig. 28. Fractionation of tryptic digest of cyano-ethyl derivative of glycosylated ribonuclease (7 mg) on a Sephadex G-25 column (150 x 1.0 cm). Fractions 45-60 were combined, freeze-dried and subjected to further purification on another G-25 column (see Fig. 29).

o—o E_{226} ●—● Radioactivity

The cyanoethylated ribonuclease was rather insoluble in water. A suspension (1%) of cyanoethylated-protein was made up in water containing 0.02% purified trypsin (see p. 98). The pH was maintained at 8.0 by the addition of 0.02N NaOH. The digestion was continued for 3 h at room temperature. Then the pH was lowered to 3 using glacial acetic acid and the mixture was allowed to stand at 4°C overnight. The insoluble fraction was removed by centrifugation at 10,000 g and 4°C for 15 min. The pellet was suspended in 0.2M acetic acid and re-centrifuged. The supernatants were combined. A portion of the mixture was taken for high voltage electrophoresis (Atfield & Morris, 1961) on Whatman 3 MM paper, 120 V/cm, 40 min, in acetic acid-formic acid buffer, pH 1.8 (see p. 91). The strips were scanned using a Panax gas-flow strip counter. Two radioactive bands were detected (Fig. 27) which had moved a distance of 14.1 and 20.2 cm respectively towards the cathode. The faster moving peak contained ~55% of the radioactivity.

The remainder of the tryptic digest (two-thirds of the original; 7080 cpm) was subjected to column chromatography on Sephadex G-25, fine (150 x 0.9 cm) pre-equilibrated with 0.2M acetic acid. Elution was effected with 0.2M acetic acid and fractions of 2 ml were collected. The fractions were assayed for radioactivity and the absorbancy at 226 nm was also measured. The chromatogram was divided into three parts, A, B and C (Fig. 28). An appropriate prediction of the nature of the peptides in the peaks could be obtained from a consideration of the peptides expected in a tryptic hydrolysate of ribonuclease (Table 12). Part A corresponds to high molecular weight peptides,

Residue numbers in ribonuclease A	Amino acid sequence	Number of amino acid residues
1-7	Lys-Glu-Thr-Ala-Ala-Ala-Lys	7
8-10	Phe-Glu-Arg	3
11-31	Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-Ser-Ser-Ser-Asn-Tyr-Cys-Asn-Gln-Met-Met-Lys	21
32-33	Ser-Arg	2
34-37	Asn-Leu-Thr-Lys	4
38-39	Asp-Arg	2
40-41	Cys-Lys	2
42-61	Pro-Val-Asn-Thr-Phe-Val-His-Glu-Ser-Leu-Ala-Asp-Val-Gln-Ala-Val-Cys-Ser-Gln-Lys	20
62-66	Asn-Val-Ala-Cys-Lys	5
67-85	Asn-Gly-Gly-Thr-Asn-Cys-Tyr-Gln-Ser-Tyr-Ser-Thr-Met-Ser-Ile-Thr-Asp-Cys-Arg	19
86-91	Glu-Thr-Gly-Ser-Ser-Lys	6
92-98	Tyr-Pro-Asn-Cys-Ala-Tyr-Lys	7
99-104	Thr-Thr-Gln-Ala-Asn-Lys	6
105-124	His-Ile-Ile-Val-Val-Cys-Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val	20

TABLE 12. Peptides which might be expected to be present in tryptic hydrolysates of reduced cyanoethylated ribonuclease. Each assigned Cys residue has of course undergone conversion to an S-cyano-ethylcysteine.

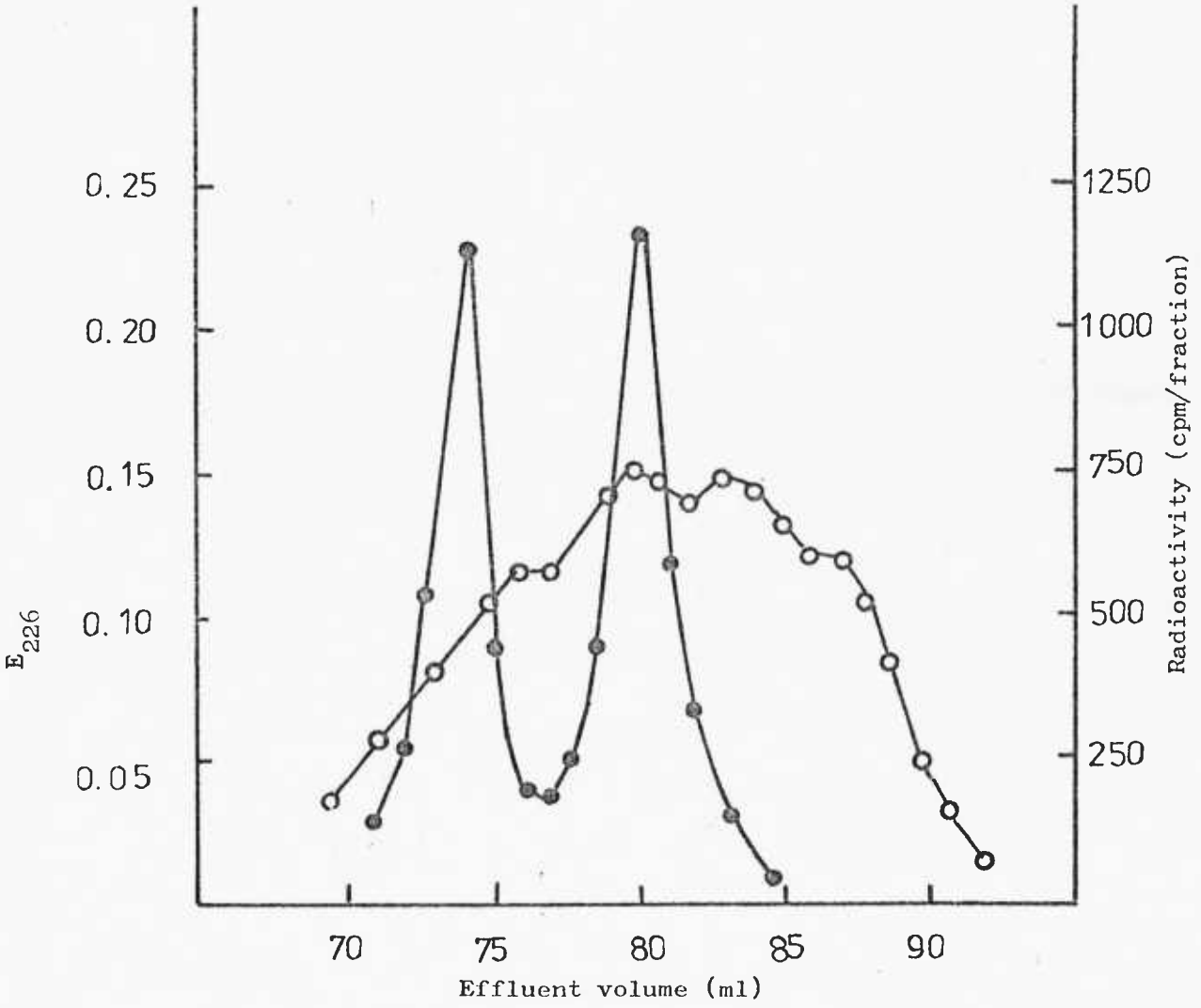


Fig. 29. Rechromatography in 0.2M acetic acid on Sephadex G-25 (200 x 0.6 cm) of a part of the effluent from Sephadex G-25 (Fig. 28) of a tryptic hydrolysate of glycosylated ribonuclease

●—● Radioactivity

○—○ E_{226}

Part B to medium size peptides and Part C to small peptides.

The small peak at the beginning, fractions 4-6, is probably unreacted trypsin. There were two radioactive peaks in the region of the medium peptide. They were not completely resolved from each other. They contained 5280 cpm corresponding to 36.4nmoles glucosamine. The total recovery was ~ 75%.

The radioactive fractions 45-50 were combined, freeze dried and rechromatographed in 0.2M acetic acid on a column of 0.6 x 200 cm Sephadex G-25 (fine; void volume = 32 ml as measured by Blue Dextran). Fractions of 1 ml were collected and assayed for peptide content (E_{226}) as well as for radioactivity (see Fig. 29). There was a clear separation of two radioactive peaks ((a) and (b)). Peak (a) corresponded to 2240 cpm and peak (b) to 2960. The residual amounts after assay of fractions 72-84 (Fig. 29) were combined (4550 cpm) and chromatographed on a column of cation exchange resin IR-120. The column was pre-equilibrated with pyridine formate (0.2M in pyridine, pH 3.25) and elution was effected using 50 ml of pyridine formate, pH 3.26, to which 75 ml 0.4M pyridine formate, pH 6.2, flowed. Fractions of 2 ml were collected and assayed for peptides by the ninhydrin reaction according to Hirs (1967). Measured volumes (0.5 ml) of each solution were transferred to polyethylene tubes and dried overnight. To these were added 0.15 ml volumes of 13.5N NaOH and the tubes were covered with aluminium foil and autoclaved for 20 min at 10 lbs (110°C). The tubes were removed and cooled,

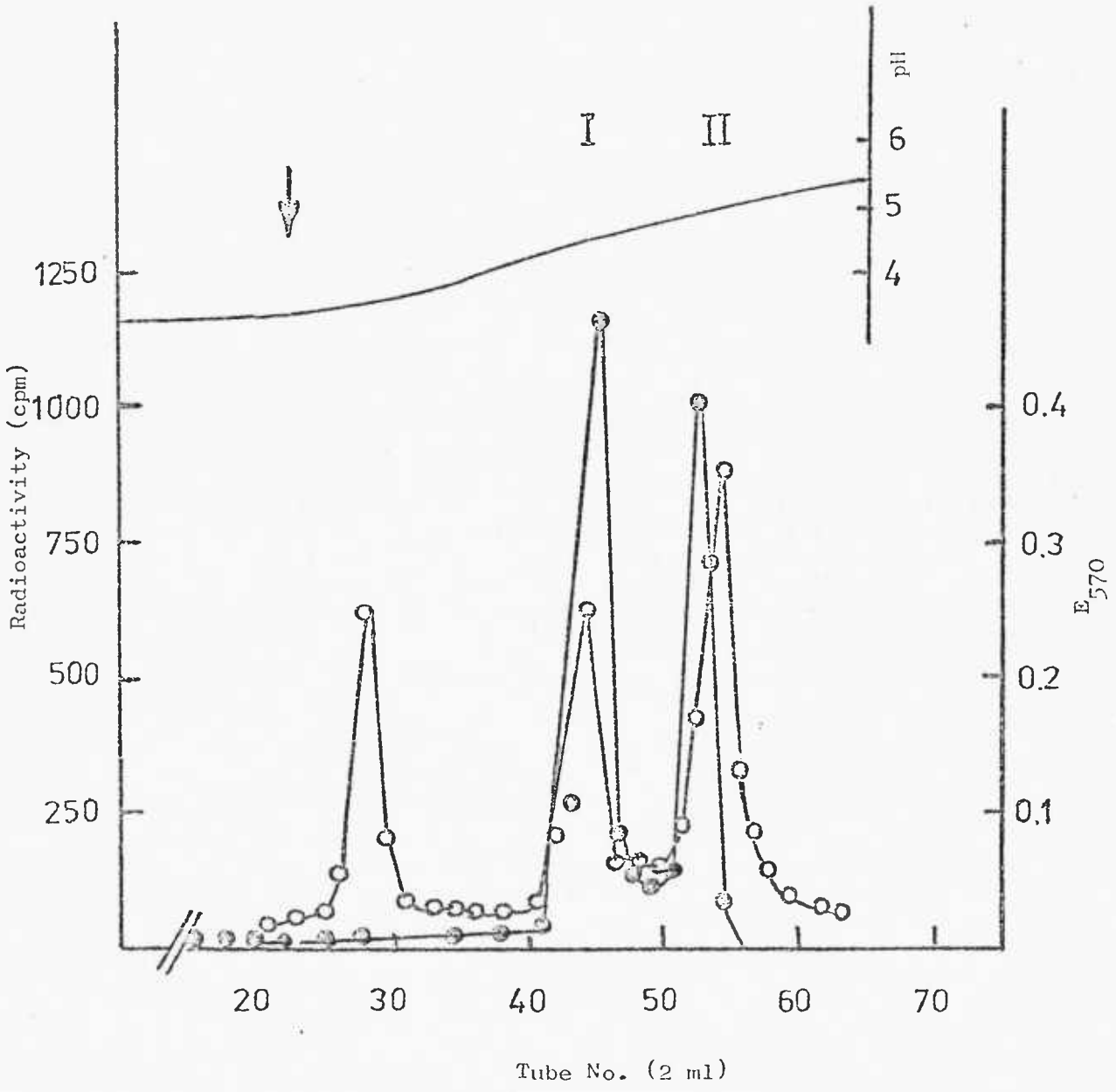


Fig. 30. Chromatography on Amberlite CG-120 of the combined fractions 72-84 from the Sephadex G-25 column (Fig. 29).

o —— o E_{570}

● —— ● Radioactivity

——— pH

and the contents were neutralized with glacial acetic acid (0.25 ml), followed by the addition of 0.5 ml of the ninhydrin reagent*. The tubes were then individually covered and heated in a boiling water bath for 15 min, cooled and 2.5 ml 50% ethanol were added. Absorbance at 570 was measured. The concentration of the peptide was obtained using a standard curve of leucine.

Three peptide peaks appeared, two of which were radioactive (Fig. 30), peaks I and II. The radioactivity measurements showed that peak I contained 2020 cpm and peak II 2100 cpm, corresponding to ~ 14 and 15 nmoles glucosamine respectively. Peak I corresponded to peak (b) of the Sephadex column. This was deduced from other similar experiments in which the peaks from the Sephadex G-25 column (Fig. 29) have been chromatographed separately on an Amberlite CG-120 column. The amino acid analysis of this peak after acid hydrolysis (6M HCl, 100°C, 3 h) showed the composition listed in Table 13, column 2.

This composition indicates that the peptide is almost certainly derived from the region 34-37 of the ribonuclease A (see Table 12). The glucosamine analysis of this peptide by the amino acid analyser (see Methods) revealed the presence of 0.12 moles glucosamine/mole of the peptide. It seemed probable

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* Ninhydrin reagent was prepared according to Moore & Stein (1954) and it contained 20 g ninhydrin, 3 g hydrindantin, 750 ml of 2-methoxy-ethanol and 250 ml 4M sodium acetate buffer, pH 5.

Component	Fraction I Column	Fraction II-2 Column
	2	3
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+
Glycine	0	0.12
Alanine	0	0.06

* Determined on acid hydrolysates with the use of amino acid analyser (Programme II, see p.

+ Determined by Elson-Morgan procedure

TABLE 13. Composition of the radioactively labelled glycopeptides obtained from glycosylated ribonuclease.

Component	Fraction II' -2' Column	Fraction II'' -2'' Column
	2	3
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

TABLE 14. Analysis of the peptides obtained by tryptic digestion of ribonuclease A and chromatographing in the same place as peptide II-2 derived from glycosylated ribonuclease.

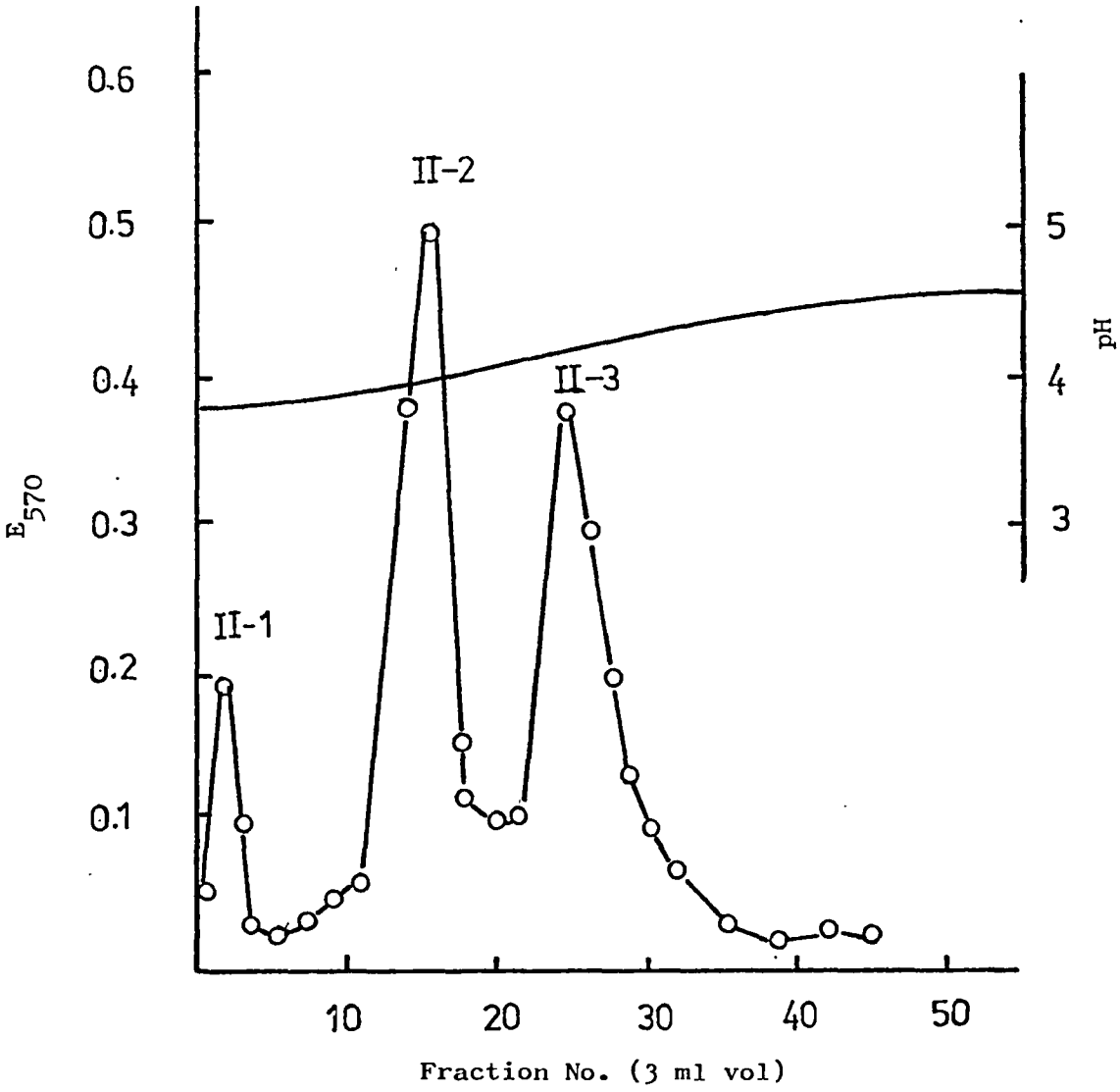


Fig. 31. Rechromatography of fraction II (Fig. 30) on a column (12 x 1.6 cm) of sulphopropyl-Sephadex C-25.

o — o E570

——— pH

at this stage of the work that the glycosylated tetrapeptide, (GlcNAc).Asn-Leu-Thr-Lys, co-chromatographs with the non-glycosylated tetrapeptide. This was indeed shown later (see Chapter 5).

The second radioactive peak (Fig. 30) from the Amberlite CG-120 column (fraction II) corresponds to the first radioactive peak on the Sephadex G-25 column (Fig. 29). The value of V_e/V_o 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. 30) 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. 31) 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 13, column 3. 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-Glu-Thr-Ala-Ala-Ala-Lys..).

Glucosamine analysis of fraction II-2 as performed by the Elson-Morgan procedure (Kraan & Muir, 1957) revealed 0.18 residues relative to one residue of aspartic acid. This peptide is probably a mixture of glycosylated and non-glycosylated peptides arising from residues 32-37 in the original ribonuclease A (see Table 12, p. 149) which are not separated from each other under chromatographic conditions used. The reasons for the low values of arginine were not wholly clear.

Arginine is an amino acid which is stable in hot aqueous acid and it seems rather unlikely that it had undergone partial destruction under the experimental conditions used.

The possibility that during the incubation period the arginine residue at position 33 (see Table 12) had undergone some change in its side groups was investigated as follows: Ribonuclease A was incubated with a preparation of rough endoplasmic reticulum in the absence of UDP-N-acetyl-glucosamine under the conditions described on p. 139. The incubation mixture was chromatographed on CM-cellulose column under conditions identical with those used in the previous experiments. The ribonuclease peak was collected, dialysed and freeze dried. It was then cyanoethylated and hydrolysed with trypsin as stated before (p. 145). The hexapeptide (residues 32-37, see Table 12) was separated, purified and hydrolysed in acid. The hydrolysate was analysed for amino acid content (Table 14, Column 2). An incomplete recovery of arginine relative to the other amino acids present

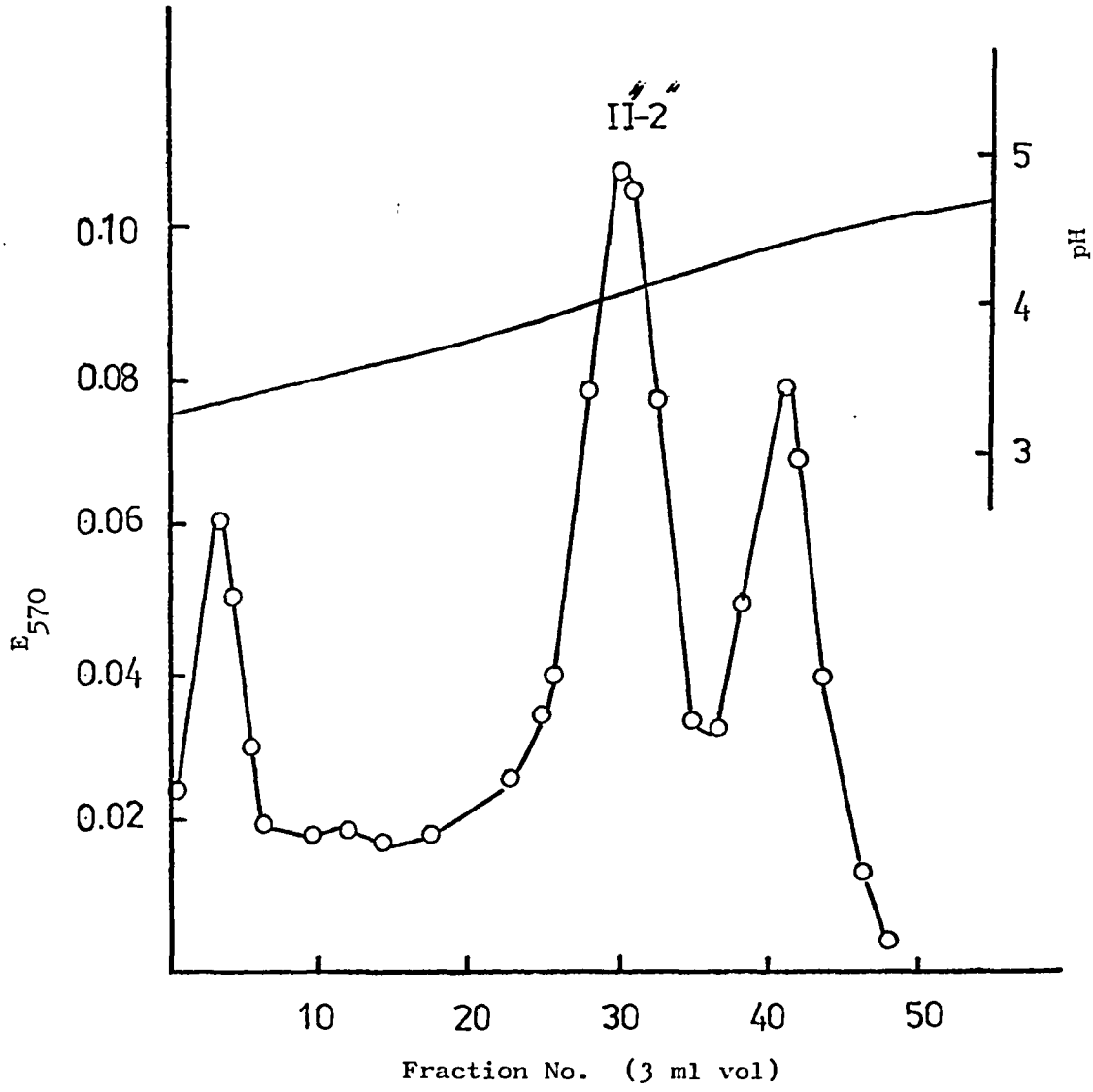


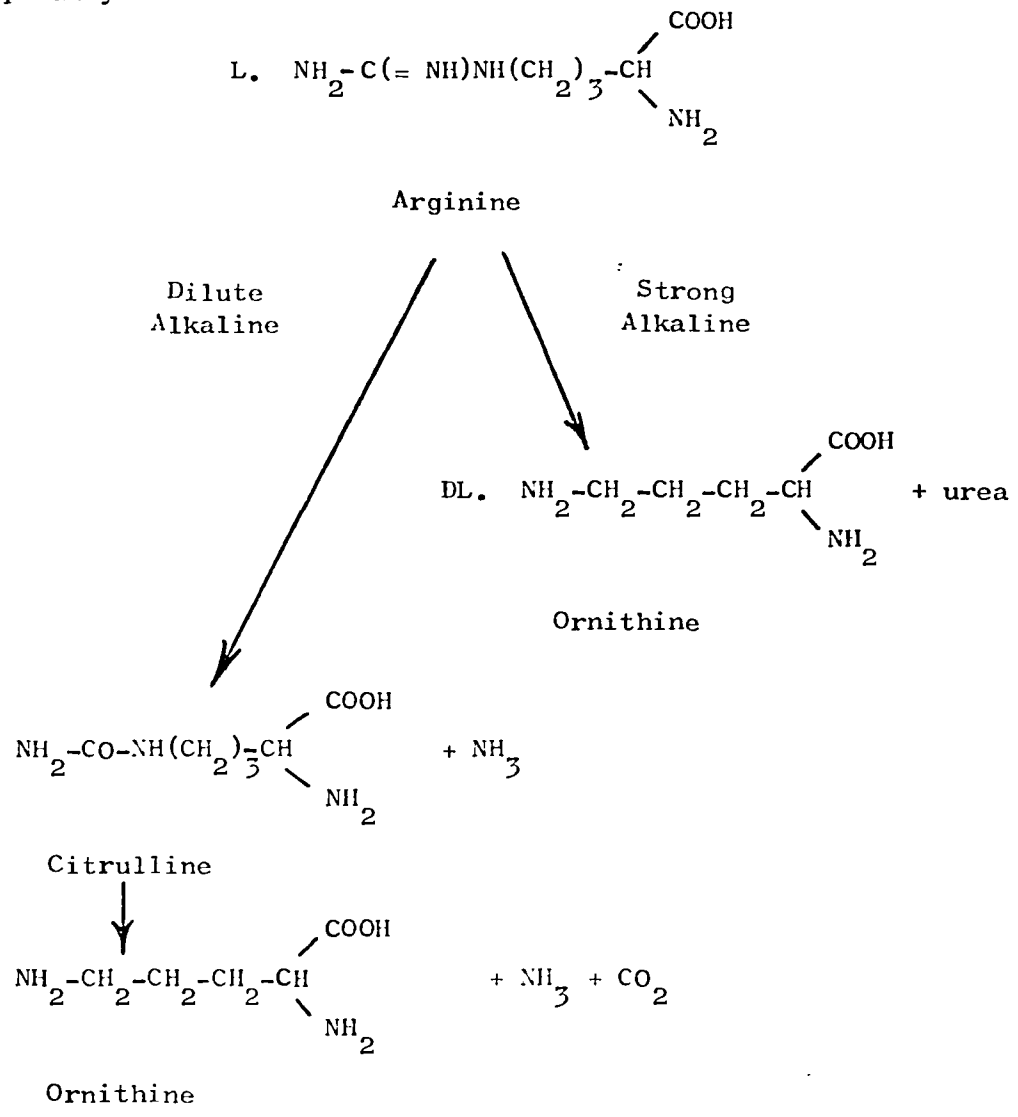
Fig. 32. Chromatography on sulphopropyl-Sephadex C-25 of a peptide from ribonuclease A corresponding in behaviour to the glycopeptide II obtained from monoglycosylated ribonuclease A.

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in the hexapeptide was observed. In a similar control experiment, ribonuclease not previously treated with preparations of rough endoplasmic reticulum was digested with trypsin and a corresponding peptide fraction (II'' - 2'') was taken and analysed (Fig.32) (Table 14, column 3). The results of this analysis also show low value for arginine, although these losses appear to be less than those observed in experiments with glycosylated ribonuclease.

It is known that arginine may be converted under alkaline conditions to either ornithine or citrulline through the following pathways:



Since the cyanoethylation of ribonuclease was performed at pH 8, as was the tryptic digestion, it seemed possible that at least some arginine could undergo changes in these alkaline conditions. But the results of the control experiment carried out above rules out this possibility because, although the arginine recovery is incomplete, there is neither ornithine nor citrulline observed in the chromatogram of the hydrolysate. The losses of arginine might result from the effects of pyridine formate and the hydrolysis of the peptide. Each was examined separately as follows: A mixture of the amino acids present in the peptide II^1-2^1 was made up with final concentrations of

Lys	5 mmoles/l
Asp	5 mmoles/l
Ser	5 mmoles/l
Arg	5 mmoles/l
Leu	5 mmoles/l
Thr	5 mmoles/l

1) A portion of this containing 50 nmoles of each was applied directly to the amino acid analyser (programme I, p. 92).

2) Another portion of this mixture (1 ml) was taken up in 5 ml of pyridine-formate buffer and left overnight. The solution was then evaporated to dryness, and the residue was taken up in water. A measured portion was applied to the analyser.

3) 50ml of the solution was evaporated to dryness and taken up in 6N HCl, 100°C, 6 h and the hydrolysate was dried *in vacuo* and the residue was taken up in water; a portion of it was run on the analyser.

4) Finally a portion of the mixture was treated with trypsin exactly as mentioned for the hydrolysis of ribonuclease but with the omission of Sephadex and Amberlite CG-120 column chromatographies. The residue after this treatment was hydrolysed with HCl and run on the analyser. The results clearly showed no losses of arginine in any of the cases.

4. Some aspects of the specificity of the enzyme

The results of the experiments described earlier in this Chapter reveal that the enzyme present in the rough endoplasmic reticulum of liver catalyses the incorporation of N-acetyl-glucosamine into ribonuclease A. It was of interest to examine how specific the enzyme is with respect to the size and structural arrangement of the acceptor substrate, and some limited studies of this nature were made.

(i) Does reduced cyanoethylated ribonuclease A act as an acceptor?

The four disulphide bridges present in ribonuclease A connect residues 59 to 72, 26 to 84, 40 to 95 and 58 to 110 and therefore giving the molecule its characteristic spatial arrangement. When the protein is reduced, the molecule unfolds. The reduced ribonuclease A could be used as a tool for investigating whether the secondary or tertiary structure of the molecule plays a regulatory role for the enzyme activity. The reduced ribonuclease A was prepared according to the method of Anfinsen & Haber (1961) as described by Plummer & Hirs (1964). The reduced ribonuclease A was cyanoethylated with acrylonitrile according to the procedure described in Chapter 2. The reduced,

Substrate	N-Acetyl-glucosamine (nmoles) / mg enzyme protein / h		
	endogenous	endogenous+ exogenous	difference
Ribonuclease A	3.97	4.85	0.88
RCN-ribonuclease*	3.42	3.25	--
Peptide mixture	2.92	3.18	0.26
Hen egg albumin	2.25	2.60	0.35

* RCN-Ribonuclease is reduced cyanoethylated ribonuclease

TABLE 15. Effect of size and structural arrangement of the acceptor substrate on the activity of the enzyme.

cyanoethylated ribonuclease A was purified on a column of Sephadex G-25 and freeze-dried. The eluting solvent was 0.2M acetic acid.

Incubations were carried out in 100 µl volume containing the following components: Mn^{+2} (50mM), UDP-N-acetyl-glucosamine (0.28mM containing 250 cpm/nmole), enzyme protein (rough endoplasmic reticulum 250 µg), Tris-HCl buffer, pH 7.4 (45mM) and reduced cyanoethylated ribonuclease A (0.035mM). Control experiments were performed simultaneously, one with ribonuclease A in place of reduced cyanoethylated ribonuclease, and the other without any exogenous acceptor added. Reduced cyanoethylated ribonuclease A was not completely soluble at the pH of the incubation mixture. The incubation was performed for one hour and terminated by the addition of 1% phospho-tungstic acid in 0.5M HCl. Radioactivity measurements revealed no incorporation into reduced cyanoethylated ribonuclease as judged by the difference in the count rate of the control and reduced cyanoethylated ribonuclease containing sample, while under the same experimental conditions incorporation was observed into ribonuclease A (Table 15).

(ii) Does a peptide containing the sequon act as a substrate for the enzyme?

The asparagine sequon occurs at position 34 in the ribonuclease molecule. When the ribonuclease molecule is allowed to react with CNBr, four peptide fragments may be expected to be produced (see Table 6 for the position of methionine residues), one of which, 31-79, contains the asparagine sequon. This peptide was used in preliminary studies for elucidation of

the specificity of the enzyme towards the size of the receptor molecule.

Cyanogen bromide cleavage of ribonuclease A was performed by the method of Steers et al. (1965) as described in the Method section (p. 98) . The peptides produced were not separated from each other and the peptide mixture was used for the analysis directly. As shown earlier, the mixture does not contain any undigested ribonuclease A. Incubations were performed under conditions similar to those described above for reduced cyanoethylated ribonuclease, except that 0.056 absorbance units (280 nm) of the peptide mixture was used instead of reduced cyanoethylated ribonuclease A. The results of this experiment showed negligible incorporation into peptide fragments (Table 15). It may be that the peptide in question is not a substrate, but a final answer must await studies carried out with the pure peptide; other peptides might act as inhibitors.

(iii) Does hen egg albumin act as acceptor?

It was of equal interest to see whether acceptors other than ribonuclease A are effective as substrate for enzymic incorporation of N-acetyl-glucosamine. Among the proteins suitable for this is hen egg albumin. This glycoprotein contains a sequence ..Asn-Leu-Ser.. (Wiseman et al., 1972) which is normally not glycosylated in addition to the sequence ..Asn-Leu-Thr.. which is. Egg albumin was prepared by ammonium sulphate precipitation (Warner, 1954). The conditions for the glycosylation were similar to those used for ribonuclease A. The preparation of rough endoplasmic reticulum was suspended in

Tris-malcate buffer, pH 7.4, to an equivalent concentration of 1 g liver/ml. An amount equal to 200 µg endoplasmic reticulum protein was used for each incubation. The incubation mixtures contained the following in the final volume of 50 µl, egg albumin 0.02 mM, UDP-N-acetyl-glucosamine 0.1 mM containing 247 cpm/nmole, Mn^{+2} 50 mM and buffer Tris-maleate 50 mM. Control experiments were also carried out with the same components except that a preparation of boiled enzyme was used, and in another control egg albumin was omitted. The incubations were performed for 2 h at 37°C and then terminated by the usual acid precipitation technique; radioactivity was measured by scintillation counting of the precipitates. There was incorporation into endogenous protein (Table 15) and in the mixture containing egg albumin it was higher. In the case of boiled enzyme, incorporation was minimal. The results obtained with egg albumin suggested that there was an incorporation of N-acetyl-glucosamine into the protein, but the position of incorporation is unknown. It is possible that further substitution of the carbohydrate moiety present had occurred.

5. Some general comments

The experimental results so far presented demonstrate the presence of an enzyme in preparations of rough endoplasmic reticulum of rabbit liver which can catalyse the incorporation of N-acetyl-glucosamine from UDP-N-acetyl-glucosamine into asparagine 34 of ribonuclease A. Experimental evidence shows only one of the ten asparagine residues in ribonuclease A underwent glycosylation. The most probable reason for this is

the presence of an appropriate sequon around asparagine 34 (..Asn-Leu-Thr...) which does not occur at any other position in bovine pancreatic ribonuclease. This probably implies that the enzyme involved in the formation of the GlcNAc-Asn linkage requires a special type of recognition site irrespective of the source of the enzyme. The involvement of Asn-(X)-Thr(Ser) in the formation of the linkage may in fact indicate the size of the binding site on the enzyme.

It is interesting that a peptide fragment obtained by CNBr cleavage of ribonuclease A may not act as acceptor although it contains the asparagine sequon (peptide 31-79). This might imply that a certain number of amino acids is necessary for recognition of the peptide as a substrate. It was also observed that reduction of ribonuclease A followed by blocking the SH group with cyanoethyl moieties resulted in products with no acceptor properties. This might be due to unfolding of the molecule and therefore changes in the spatial arrangements, but the cyanoethyl groups themselves might inhibit the enzyme. Hen egg albumin, which has one carbohydrate prosthetic group, contains another non-glycosylated asparagine sequon (the complete amino acid sequence of this glycoprotein is not yet known), but it is not clear from the present results whether this second position can accept a single residue, and this problem needs pursuing. Studies of Pless & Lennarz (1977) on the enzymic transfer of the oligosaccharide moieties from an oligosaccharide lipid to denatured forms of some secretory proteins show that ribonuclease A, when subjected to sulphitolysis acts as an acceptor in an <u>in vitro</u> system using hen

oviduct membrane preparations. Their studies on different acceptors such as bovine α-lactalbumin and bovine ribonuclease A show similar results. Bovine deoxyribonuclease, porcine pancreatic elastase, bovine pancreatic carboxypeptidase and horse liver alcohol dehydrogenase did not act as acceptors, although they all contained one or more asparagine sequons. In no case was the acceptor amino acid identified.

There is, however, no explanation as to how the degree of glycosylation *in vivo* is regulated. It was suggested that restriction is imposed by peptide folding. Compartmentalization, presence and specificity of the transferase may also account for the variations in the degree of glycosylation (Marshall & Neuberger, 1968; Hunt & Dayhoff, 1970; Sox & Hood, 1970).

The experimental results presented here show that only partial glycosylation of ribonuclease A occurs *in vitro* under the experimental conditions used. The underlying reason for this might be the same as those leading to *in vivo* production of only partially glycosylated ribonuclease A. It is possible that one or more of the products of the reaction acts as an inhibitor for the enzymic reaction. If the formation of glycosylated ribonuclease is simplified as follows:

$$\text{UDP-GlcNAc} + \text{ribonuclease A} \xrightarrow[\text{Enzyme}]{Mn^{+2}} \text{UDP} + \text{GlcNAc-ribonuclease}$$

the two major products, UDP and glycosylated ribonuclease are the possible inhibitors of the reaction. But it was shown that concentrations of UDP as high as 0.5mM are required to cause ~ 50% inhibition of the enzymic activity. The maximum concentration of UDP in the experiments done assuming that all

UDP-N-acetyl-glucosamine were cleaved either by transfer to the substrate or by hydrolysis to yield UDP would not be greater than about 0.35mM. It is unlikely, therefore, that UDP is the sole inhibitor which prevents complete glycosylation of ribonuclease A. The inhibitory effect of glycosylated ribonuclease could not be elucidated at the time many of the earlier experiments were done, because ribonuclease A and its glycosylated product exhibited very similar chromatographic behaviour which rendered their separation extremely difficult. This whole problem requires further study now that mono-glycosylated ribonuclease can be isolated fairly easily (see Chapter 5).

The possible involvement of lipid intermediates such as dolichyl monophosphate N-acetyl-glucosamine (Leloir, 1971; Mookerjea et al., 1972; Richards & Hemming, 1972) or dolichyl pyrophosphate N-acetyl-glucosamine (Behrens et al., 1973) is unknown, and is an important topic for further investigations. However, in general terms, the two mechanisms, one involving the transfer of sugar from nucleotide-sugar and the other from lipid-sugar, are not necessarily mutually exclusive in vivo. It is possible that the enzyme carries a binding site for the asparagine sequon, for the sugar nucleotide donor and also for dolichyl mono- or pyrophosphate N-acetyl-glucosamine (Marshall, 1974). It must also be borne in mind that in the in vitro system the enzyme is removed from its topographical environment and might not have an absolute need for the lipid intermediate, while in vivo there is a need to transfer the sugar from the sugar nucleotide mainly present in the

cytoplasm to the membrane via a lipid intermediate (Marshall, 1974). However, considerations of this nature can only be fully investigated if the enzyme can be obtained in a pure form and its mechanism of action thoroughly elucidated.

The enzyme in question appears to be located mainly in the rough endoplasmic reticulum. However, the relatively high levels of the enzyme in the mitochondrial pellet suggests the possibility that the enzyme might also be present in other subcellular fractions of liver, e.g. cell membranes, but this needs further investigation. The enzyme is membrane bound and probably can be partially released by treatment with non-ionic detergents such as Triton X-100.

The activities of several enzymes which are membrane bound depend on the structural integrity of the membrane concerned. The glucose-6-phosphatase (De Duve et al., 1949; Hers et al., 1951) activity of preparations of liver microsomes becomes less stable to heat when the membranes were treated with lecithinase, which had been isolated from cultures of Clostridium welchii and was probably a mixture of phospholipase A_1 and A_2. Moreover the substrate specificity requirement of the glucose-6-phosphatase became less rigid. In addition, low concentrations ($\sim$ 0.05%) of deoxycholate led to an activation of membrane bound glucose-6-phosphatase, but higher concentrations (greater than about 0.1%) inactivated it. Considering the results of these experiments it was suggested that certain phospholipids contribute to the substrate specificity of glucose-6-phosphate and that the enzyme is a lipoprotein (Beaufay & De Duve, 1954).

Membrane bound UDP-glucuronyl transferase (defined as that enzyme catalysing glucuronylation of 4-nitrophenol) also becomes less stable if the microsomal preparations from guinea-pig liver are treated with phospholipase A. Moreover, the specificity of the enzyme is altered by treatment with phospholipase A and, in particular, UDP-N-acetyl-glucosamine no longer functions as an allosteric activator (Zakim & Vessey, 1974). When preparations of guinea-pig liver microsomes were treated with relatively low levels of phospholipase A for short periods of time, initial increases in UDP-glucuronyl transferase activity were observed (Zakim et al., 1973). It was for this reason (see p. 138) that preparations of rough endoplasmic reticulum from rabbit liver were treated with phospholipase A under the conditions of Zakim et al. (1973) and the UDP-N-acetyl-D-glucosamine-asparagine sequon N-acetyl-β-D-glucosaminyl transferase activity assayed before and after treatment. However, no significant changes were observed when the enzyme preparations were incubated with phospholipase A for short periods of time, but there was extensive loss of activity when the treatment was prolonged (Fig. 23). Sonication appears to have an inhibitory effect if the treatment is prolonged (see Figs. 22 and 23).

The enzyme requires, at least under experimental conditions employed, a high concentration of metal ions such as Co^{+2}, Mn^{+2} or Ni^{+2} for its full activity. It is also stimulated by CDP-choline. It was shown earlier by Mookerjea (1970, 1971) that N-acetyl-glucosaminyl transferase activity in membrane fractions rich in rough and smooth endoplasmic reticulum was stimulated by CDP-choline. The point(s) of addition of the sugar in the

endogenous protein or glycoproteins in the preparation was (were) not identified. The effect of CDP-choline was shown to be primarily in a fraction rich in endoplasmic reticulum (Mookerjea, 1972). These findings might help to explain the in vivo effect of CDP-choline on glycoprotein synthesis.

CHAPTER 4

A SEARCH FOR THE UDP-N-ACETYL-GLUCOSAMINE-ASPARAGINE SEQUON-N ACETYL-β-D-GLUCOSAMINYL TRANSFERASE ACTIVITY IN MEMBRANES FROM SOURCES OTHER THAN LIVER

The liver is known to be producing glycoproteins, both for export to the serum (see Chapter 1, p. 20) and for renewal of structural glycoproteins of the hepatocyte (see Cuatrecasas et al., 1975 and Hughes, 1976). If transfer of the sugar which is covalently linked to an asparagine residue occurs directly as a monosaccharide, then the presence of the transferase under consideration is not so surprising.

Experiments, described below, were carried out to examine the distribution of the transferase in sources other than liver. Kidney and pancreas were of interest, especially the latter organ, because of the known ability in many species to produce ribonuclease in vivo. At the same time when these experiments were performed data were available on the ability of yeast cell membrane preparations to catalyse the incorporation of N-acetyl-glucosamine into endogenous acceptors. A search for the enzymic activity in the sources mentioned above led to the discovery of the enzyme in the yeast cell membrane preparation, while extracts of kidney and of pancreas appeared to be devoid of enzymic activity under the assay conditions used.

1. Pancreas

Pancreas from rabbits was used in these experiments. Although rabbit pancreas does not appear to produce ribonuclease

Assay conditions		2	3	4
Tris-HCl buffer	mM	50	50	50
$MnCl_2$	mM	50	50	50
UDP-GlcNAc	mM	0.22	0.22	0.22
	cpm/nmole	4270	4270	4270
Ribonuclease	mM	0.071	0.071	0.077
	mg	0.25	0.25	0.27
Triton X-100	%	0.4	0.4	0.4
CDP-choline	mM	2.4	2.4	2.4
Total volume	µl	250	250	250
Enzyme protein	mg	0.5	0.5	1.4

TABLE 16. Conditions of incubation used in the enzymic N-acetyl-glucosaminylation of bovine pancreatic ribonuclease A using rabbit pancreas and hamster kidney preparations.

(Beintema, 1966), it seems likely in general terms that rabbit pancreas is involved in the formation of some glycoproteins and sugar lipid compounds have been isolated from calf pancreas (see Table 7, p. 56).

(i) Examination of the microsomal fraction of rabbit pancreas for the presence of the transferase

Pancreas (1.5 g) was decapsulated and homogenised in 7.5 ml 0.3M sucrose containing 0.01% soybean trypsin inhibitor (Tkacz et al., 1974). The homogenisation was carried out by five strokes of a motor driven glass-teflon Potter-Elvehjem homogeniser. The homogenate was centrifuged for 10 min at 650 g and 4°C. The resulting supernatant was centrifuged at 9,700 g, 4°C for 15 min and the pellet was discarded. The 9,700 g supernatant was finally centrifuged at 105,000 g, 4°C for 1 h to obtain the microsomal pellet. The pellet was suspended in 5 ml 0.9% KCl and recentrifuged for 30 min at the same force. It was then lightly homogenised in 1 ml of the buffer used subsequently in the assay. The protein content of the microsomal fraction was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. The transferase activity of the microsomal fraction was assayed under the conditions given in Table 16, column 2 (p. 173). The experiment was performed a number of times and in no case was greater incorporation seen in incubation mixtures which contained added ribonuclease A, when compared with controls done in the absence of ribonuclease A: Even incorporation into endogenous proteins or glycoproteins was small.

The possibility was considered that there was an inhibitor

of the reaction in the total microsomal fraction or, less likely, that the latter contained degrading enzymes for the substrate. Therefore an examination was made of the ability of the rough endoplasmic reticulum to catalyse the reaction in question.

(ii) Subfractionation of microsomal pellet

The microsomal pellet was obtained by homogenising 4 g of pancreas as described above. The pellet was then lightly homogenised in 0.3M sucrose (5 ml) and layered on top of 1.35M sucrose (7 ml) and the preparation centrifuged for 8 h at 105,000 g, 4°C. The rough microsomes were sedimented at the bottom as a tight pellet, while the smooth microsomes were a fluffy material at the interface. The upper layer (8 ml) was carefully removed and centrifuged for 1 h at 105,000 g (4°C) using the swing out head to obtain a smooth microsomal pellet. Both pellets were washed with cold 0.9% KCl and recentrifuged. Suspension of these pellets in buffer was used for transferase assay, the conditions of which are given in Table 16, column 3. There was no incorporation into ribonuclease A under the conditions used. There was also very little incorporation into endogenous protein.

2. Kidney

Hamster kidneys were used in these experiments, the animals being killed by a blow on the head. Their kidneys were removed immediately, washed in saline and decapsulated. The microsomes and rough endoplasmic reticulum fractions were prepared by the method of Keller & Zamecnik (1956) and Dallner (1963) respectively. The preparations were washed with

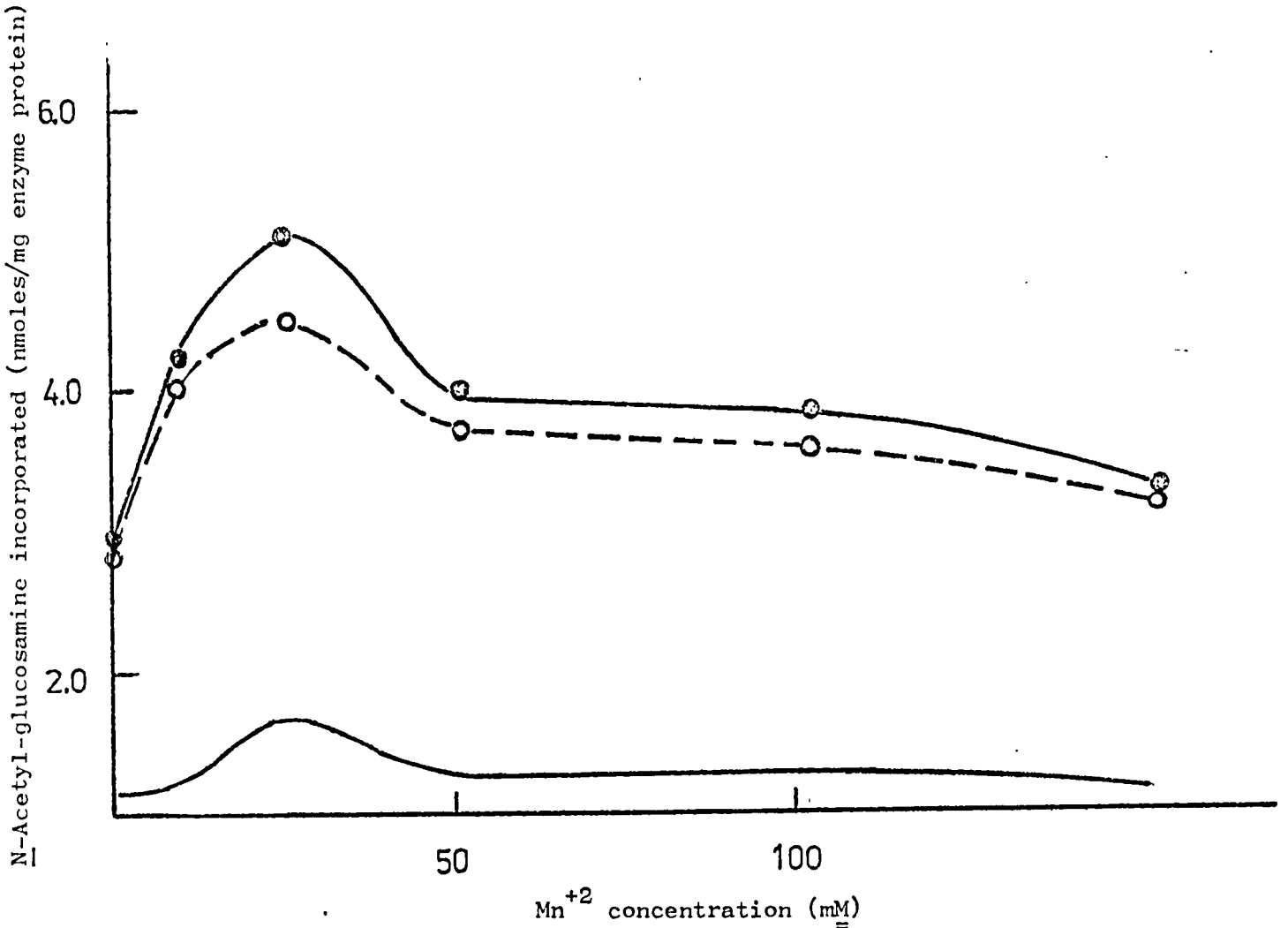


Fig. 33. Effect of Mn^{+2} concentration on the rate of incorporation of N-acetyl-glucosamine into ribonuclease A

●——●. ribonuclease was present inthe incubation mixture, O——O ribonuclease was omitted from the mixture, ——represents the difference curve.

0.9% KCl and suspended in buffer. Incubation of pancreatic ribonuclease A with both microsomal fractions and rough endoplasmic reticulum preparations under the conditions shown in Table 16, column 4 showed no transferase activity.

3. Yeast cell membrane preparations

A preparation of yeast cell membrane containing 21 mg protein/ml of 0.05M Tris-HCl buffer containing 1mM 2-mercaptoethanol, pH 7.0, was kindly supplied by Dr. Pieter Boer (Laboratory for Physiological Chemistry, University of Utrecht, The Netherlands). For details concerning the preparation of yeast cell membrane see Chapter 2, p.108.

(i) Incorporation of N-acetyl-glucosamine into ribonuclease A catalysed by yeast cell membrane preparation

Ribonuclease A was incubated with the yeast cell membrane preparation in the presence of Mn^{+2} using UDP-N-acetyl-glucosamine as the sugar nucleotide donor. The concentrations of the components in the final volume of 200 µl were: ribonuclease A, 71.4 µM; UDP-N-acetyl-glucosamine, 355 µM; Mn^{+2}, 50 mM; buffer Tris-HCl pH 7.1, 45 mM and the enzyme protein 420 µg. The incubation was performed at 37°C for 1 h both in the absence and presence of ribonuclease A. The incubation was terminated by the addition of 2 ml of 1% phosphotungstic acid in 0.5M HCl and the radioactivity incorporated into acid precipitable material measured by scintillation counting. It was shown that there was incorporation into endogenous protein (2166 pmoles GlcNAc/mg enzyme protein), but the incorporation is greater when ribonuclease A was present (2457 pmoles GlcNAc/mg enzyme protein).

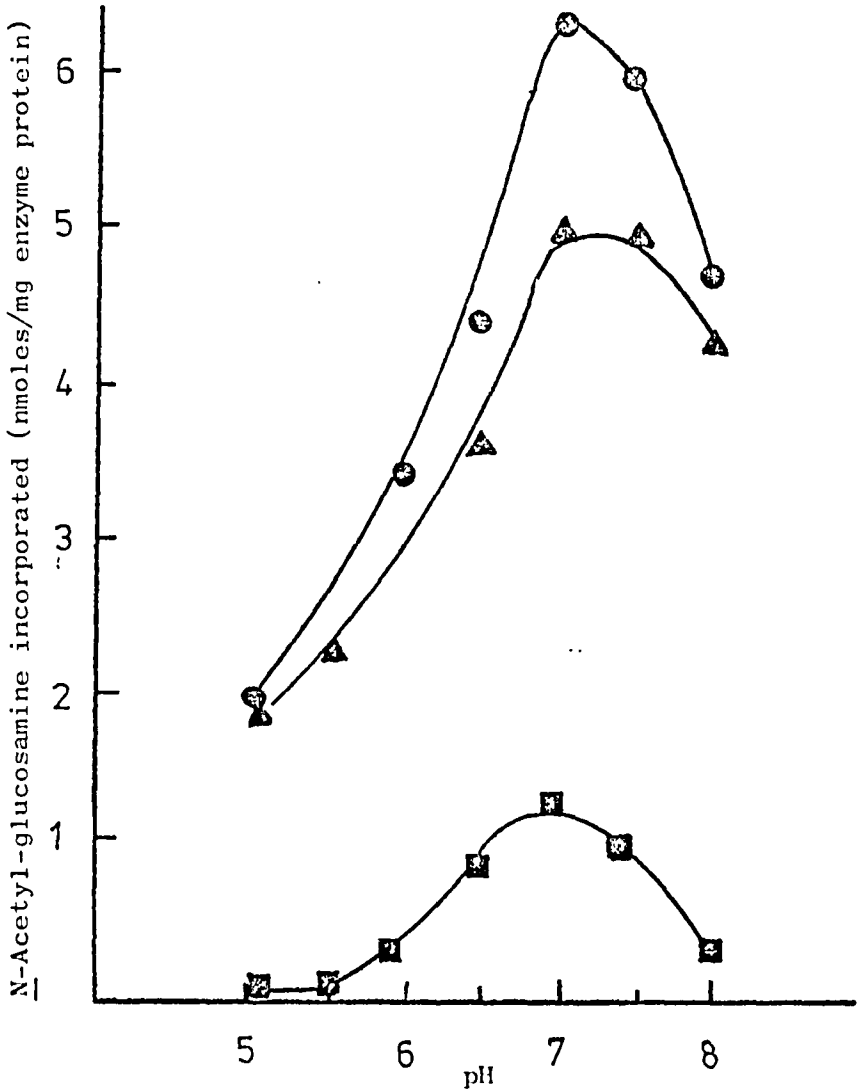


Fig. 34. Effect of pH on the rate of incorporation of N-acetyl-glucosamine into ribonuclease A

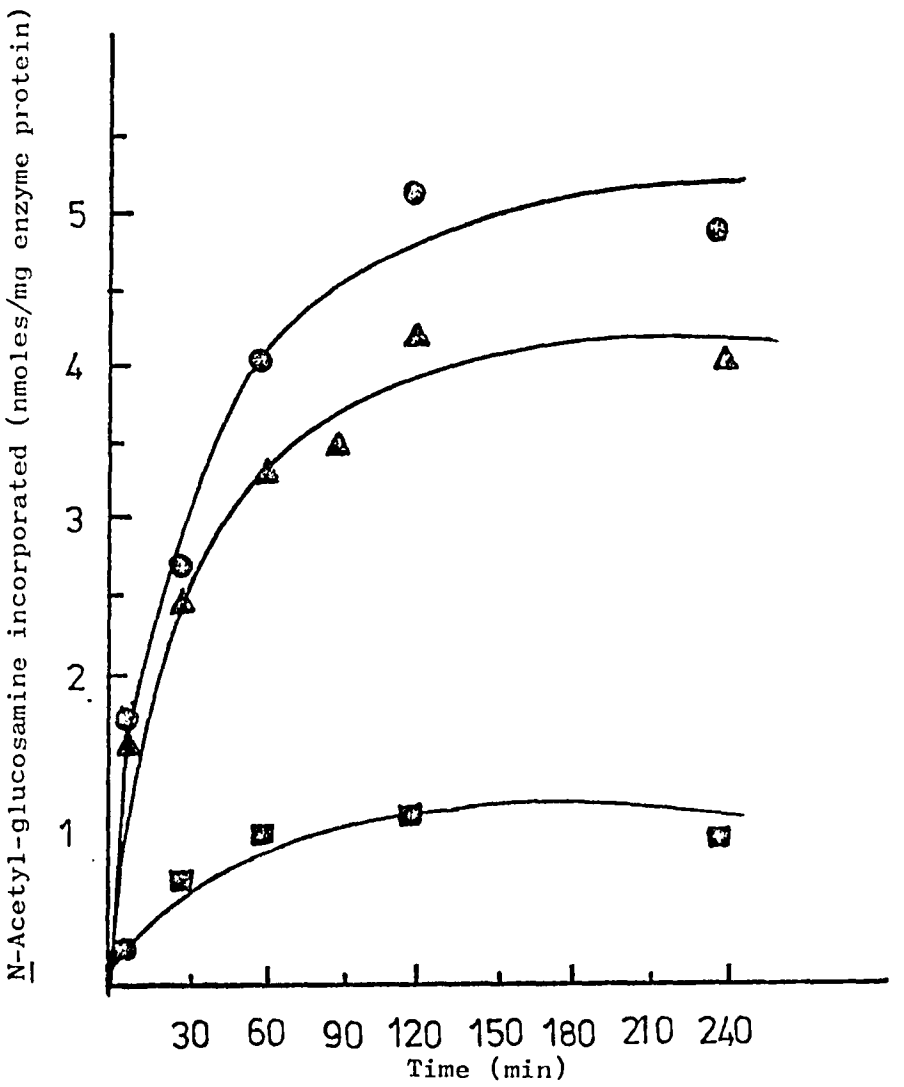


Fig. 35. Rate of incorporation of N-acetyl-glucosamine into ribonuclease A catalysed in vitro by yeast cell membranes

●—● Ribonuclease A present in the mixture: ▲—▲ Ribonuclease A omitted from the mixture: ■—■ Difference curve

(a) Effect of Mn^{+2} on the extent of incorporation

Experiments on liver showed that maximum incorporation was obtained when the concentration of Mn^{+2} ions was around 50m$\underline{M}$. To find the optimum concentration of Mn^{+2} for the incorporation of N-acetyl-glucosamine into ribonuclease A, the following experiment was performed:

Incubations were carried out both in the absence and presence of ribonuclease A. The conditions of incubation were closely similar to those mentioned above with the exception that the concentration of Mn^{+2} varied from 0-150m$\underline{M}$. The incubation was terminated by the addition of 1% phosphotungstic acid in 0.5$\underline{M}$ HCl and the radioactivity incorporated into acid precipitable material measured by scintillation counting. As shown in Fig. 33, the optimum concentration of Mn^{+2} for glycosylation of endogenous proteins and ribonuclease A appeared to be about 25m$\underline{M}$.

(b) Effect of pH on the activity of the yeast enzyme

The effect of pH on the extent of incorporation was investigated as follows:

Ribonuclease A was incubated with the UDP-N-acetyl-glucosamine in the presence of 25m$\underline{M}$ Mn^{+2} and the yeast cell membrane enzyme. The conditions were similar to those described above, the only variable being the pH of the incubating medium which ranged from 6-8.5. The results described in Fig. 34 show that with the increasing pH the rate of incorporation increases up to pH 7.0 and then gradually declines. The difference curve which exhibits the incorporation into ribonuclease A has an optimum around 7.0.

(c) Effect of time on the rate of incorporation

The incubations were carried out both in the absence and presence of pancreatic ribonuclease A under conditions similar to those described before. The incubation time varied from 10 min to 4 h, and the incubations were stopped by the addition of phosphotungstic acid (2 ml of 1% in 0.5M HCl) and the radioactivity in the precipitates measured. The results of the experiment are shown in Fig. 35 and, as will be seen, after 1 h the incorporation of N-acetyl-glucosamine into both ribonuclease A and endogenous protein was no longer occurring.

(ii) Separation of ribonuclease-GlcNAc from the incubation mixture

To demonstrate that the radioactivity was incorporated into ribonuclease A and was bound to ^{34}Asn of the molecule, the following incubations were performed:

Ribonuclease A (10 mg) was incubated with UDP-N-acetyl-glucosamine in the presence of the yeast membrane enzyme. The concentrations of the components in the final volume of 1 ml were: Ribonuclease A, 0.714mM; UDP-N-acetyl-glucosamine, 2.84mM containing 78.3 cpm/nmoles; Mn^{+2}, 25mM; yeast cell membranes enzyme protein 800 µl (~ 1.68 mg); and buffer, 45mM (Tris-HCl, pH 7.1). A control mixture containing all the components with the same molar concentration was also incubated, but ribonuclease A was omitted from this mixture. The incubations were performed at 37°C for 1 h and stopped by rapidly cooling the mixture to 2°C (ice-water mixture). The mixtures were centrifuged for 15 min at 2,000 g. That mixture in which ribonuclease A had been added was chromatographed on a column of CM-cellulose

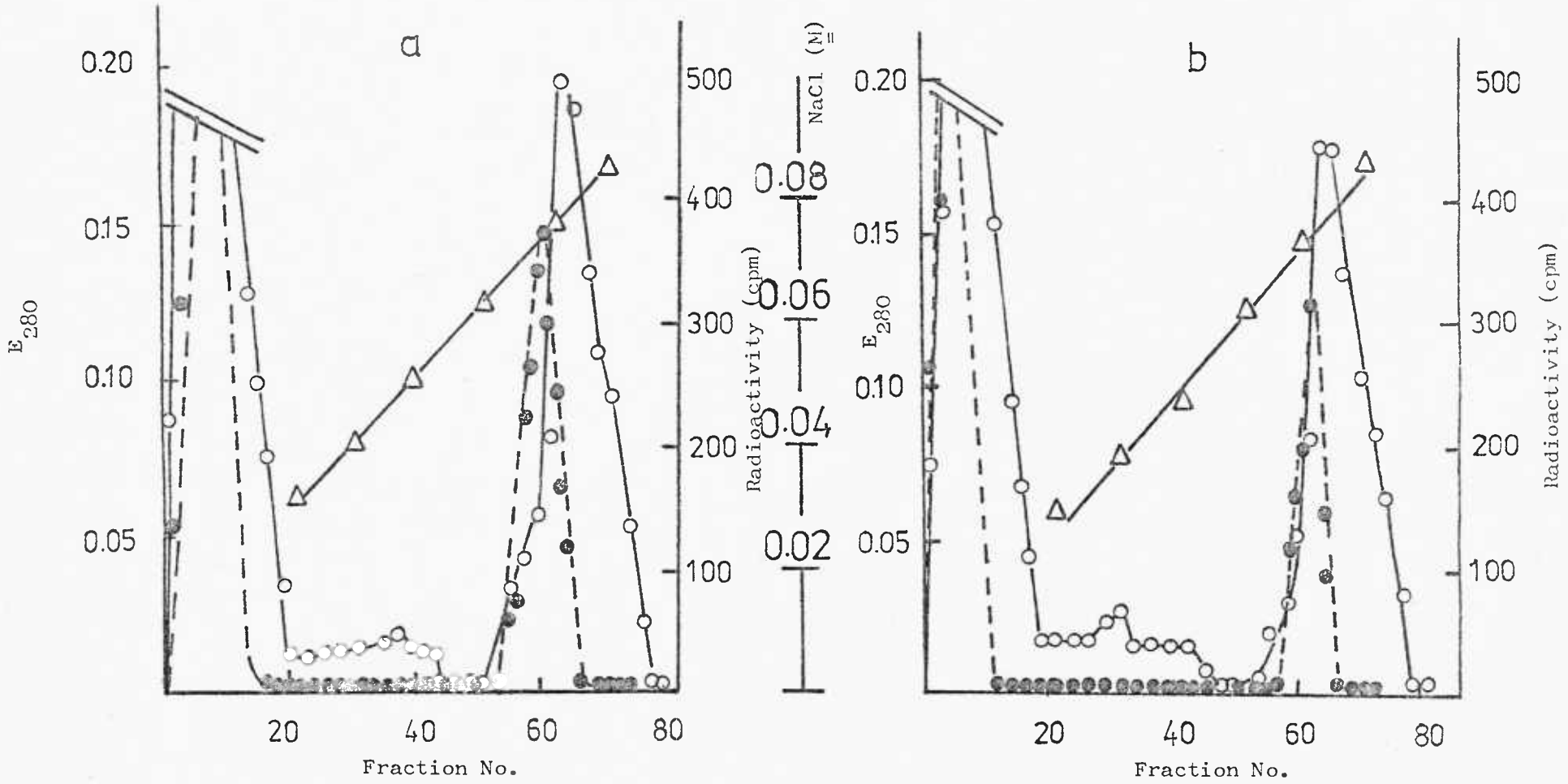


Fig. 36. Chromatography on CM-cellulose columns (0.9 x 9 cm) of (a) incubation mixture containing 10 mg ribonuclease A and a rabbit liver microsomal preparation: (b) ribonuclease A (10 mg) incubated with a preparation of yeast cell membranes. The one performed with rabbit liver microsomes was for comparative studies and contained identical components to those described for yeast (see text (p.177).

●—● radioactivity ○—○ E_{280} △—△ NaCl concentration (M)

(0.9 x 9 cm), pre-equilibrated with 0.01M Tris-HCl buffer pH 7.0 (Taborsky, 1959). The elution was effected using a gradient of NaCl in the above-mentioned buffer. Fractions of 3 ml were collected, assayed for protein at 280 nm and counted for radioactivity. Two ultraviolet light-absorbing peaks appeared, both being radioactive, the second of which was eluted at 0.07M concentration of the NaCl (Fig. 36b). This concentration of NaCl normally elutes the ribonuclease from the column. There were 0.029 residues of glucosamine (as measured by radioactivity counting) per mole of ribonuclease A. To establish the ultraviolet absorbing material at the position of ribonuclease did not arise from the yeast cell membranes preparation and was, in fact, the added pancreatic ribonuclease A, or its glycosylated derivative, the following experiment was performed. The control incubation mixture, having no ribonuclease A, was divided into two equal parts, one of which was chromatographed directly on the same CM-cellulose column and eluted in the same manner. The results (Fig. 37a) showed that at concentration of eluting NaCl of 0.07M there was no ultraviolet-absorbing material coming off the column, and when fractions were counted for radioactivity no measurable radioactivity could be detected in that region. To the other half of the mixture, which had already been incubated, was added 2.64 mg ribonuclease A and the preparation chromatographed on the regenerated CM-cellulose column. A peak of ultraviolet-absorbing material was eluted at 0.07M NaCl gradient (Fig. 37b), but there was no radioactivity in the peak. The results obtained confirm that the incorporation of radioactivity, as shown in the results described in Fig. 36, was into

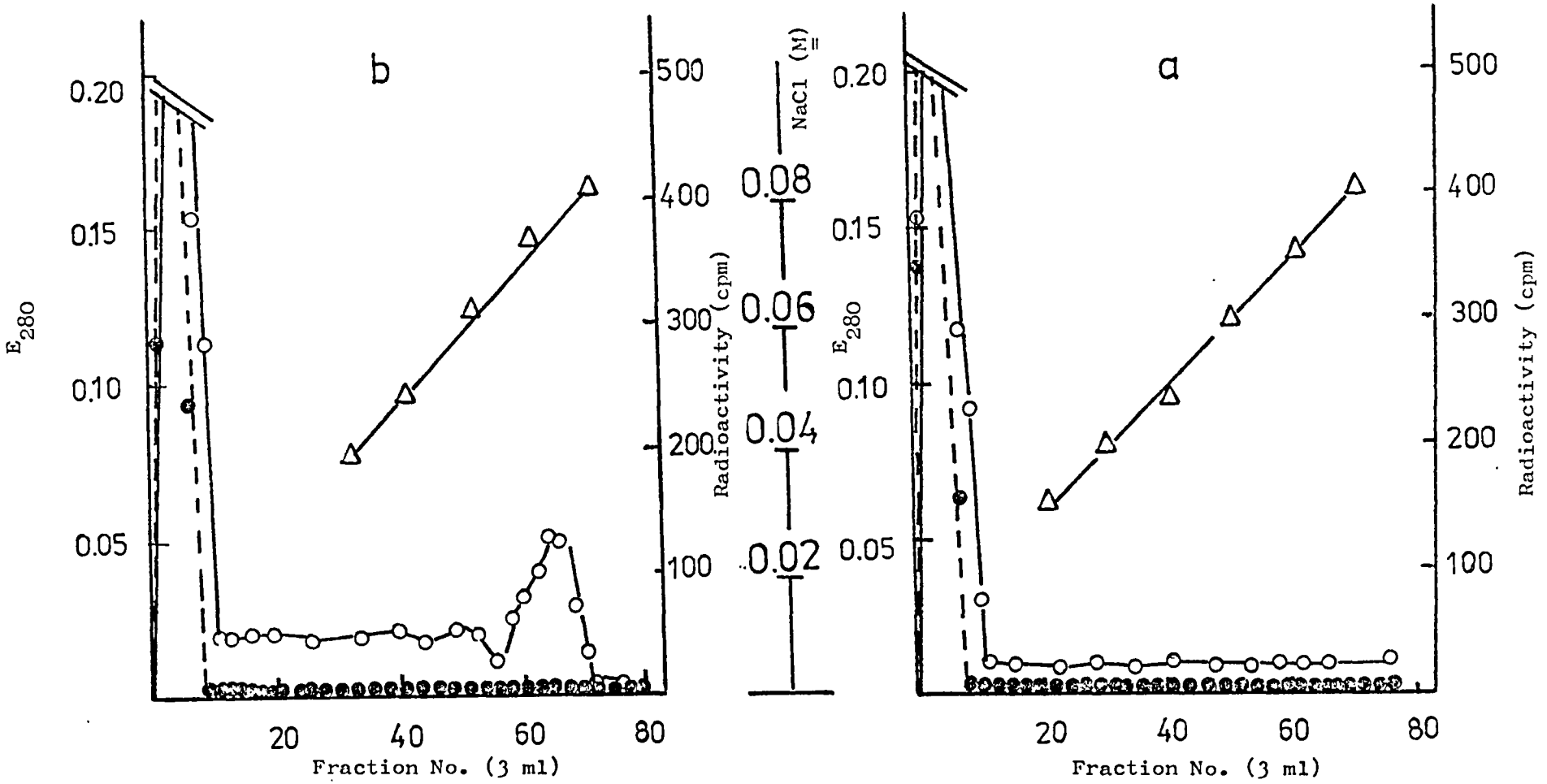


Fig. 37. Chromatography on CM-cellulose columns (0.9 x 9 cm) of incubation mixture prepared under the conditions described in the text (see p.177); (a) The incubation mixture contained no ribonuclease A: (b) Ribonuclease A (2.64 mg) was added, but only after incubation.

●—● Radioactivity o—o E_{280} Δ—Δ NaCl concentration

the pancreatic ribonuclease A and the preparation of yeast cell membrane, like liver, can catalyse the incorporation of N-acetyl-glucosamine into ribonuclease A. The point in the polypeptide chain at which the incorporation had occurred was determined as follows:

(iii) Isolation of the glycopeptide from tryptic hydrolysates of the glycosylated ribonuclease

Fractions 56-65, 1220 cpm (see Fig. 36) which corresponds to ~ 3.98 mg ribonuclease was subjected to digestion with trypsin under conditions described in the Methods section (p. 104). The tryptic digest was chromatographed on Sephadex G-25 (250 x 1 cm, two connected successive 125 x 1 cm) in 0.1M acetic acid. The fractions (1 ml) were measured at 226 nm (for protein) and counted for radioactivity. The peak of activity appeared in fractions 80-87; ~ 87% recovery. The active fractions were combined and freeze-dried, and this material was subjected to ion-exchange chromatography on Amberlite CG-120. The conditions of chromatography were identical with those in the experiments carried out with liver extracts (p. 151). Three ultraviolet-absorbing peaks were eluted from the column, the second of which was radioactive and contained 1061 cpm in total (96% recovery). The material in this peak was taken for acid hydrolysis in 4M HCl, 100°C, in vacuo for 4 h and the residue was evaporated to dryness. Amino acid analysis performed on a portion of the hydrolysate revealed the presence of aspartic acid, 1.0 (as basis for comparison); leucine, 0.99; threonine, 1.07 and lysine, 1.03 residues. There were 0.062 moles of glucosamine (Elson-Morgan reaction, Kraan & Muir, 1957) per mole of aspartic acid.

These results show that the peak consisted of a mixture about 94% of the peptide ..Asn-Leu-Thr-Lys.. and about 6% of the glycosylated derivative; it is known from earlier studies with liver (Chapter 3) that this peptide and the glycopeptide co-chromatograph under the conditions used.

4. Summary

The experimental work performed on extracts of hamster kidney and rabbit pancreas failed to demonstrate the source for transferase activity in either of the organs. However, there was some incorporation observed in the endogenous proteins of kidney preparations. The absence of the enzyme in the extracts of hamster kidney is not explicable, because kidney is known to synthesize glycoproteins. Definite evidence has accumulated that the bulk of urinary glycoproteins, however, has no counterpart in serum in any appreciable amounts and is apparently secreted by the urinary tract. Tamm-Horsfall glycoprotein is an example, the production of which is increased during tubular inflammation (see for example Bourrillon, 1972).

It seems possible that the enzyme is present in kidney at low activities and therefore cannot be detected under the experimental conditions, but no final conclusion can be drawn until we understand the mechanism of synthesis of glycoproteins in general. It has also been shown in the case of preparations of rough endoplasmic reticulum from the liver of the rat, in contrast to those from the rabbit (see Chapter 6), that the enzyme level is just at the limit of detectability.

The absence of the enzyme in pancreas could be due to the

possible effect of proteases or inhibitors present in the preparation. Addition of soybean trypsin inhibitor to the incubation mixture is, in fact, a drawback since it shows some inhibitory effect when added to the liver preparations, although the inhibitory effect in rabbit preparations is not particularly great; it is not known whether it exerts the same effect when added to the preparations of pancreas.

There is the appropriate transferase in particulate preparations from the yeast Saccharomyces cerevisiae, and this can catalyse the in vitro incorporation of N-acetyl-glucosamine into bovine pancreatic ribonuclease A. The incorporation is almost certainly at asparagine 34 of the polypeptide chain. The mechanism of enzymic reaction is not known because cell-free preparations of yeast are known to catalyse the transfer of N-acetyl-glucosamine into glycolipids. The latter have been identified tentatively as a mixture of dolichyl pyrophosphate-N-acetyl-glucosamine, dolichyl pyrophosphate di-N-acetyl-chitobiose and an unidentified compound (Lehle & Tanner, 1975; Reuvers et al., 1977a). This type of particulate preparation could also catalyse the transfer of mannose from GDP-mannose to dolichyl pyrophosphate di-N-acetyl-chitobiose. The oligosaccharide moiety produced contained two mannose residues attached to di-N-acetyl-chitobiose (Lehle & Tanner, 1975). Similar results have been obtained by Reuvers et al. (1977b) who have shown that incubation of particulate preparation of yeast with UDP-N-acetyl-glucosamine results in a mixture of two alkali stable polyprenol like glycolipids. The sugar moieties of the lipids

were N-acetyl-glucosamine and di-N-acetyl-chitobiose respectively. The lipids, after purification, could serve as donors of sugar to endogenous protein(s). The sugars were shown to be linked glycosidically to protein (Reuvers et al., 1977a), but whether the enzymic incorporation of N-acetyl-glucosamine into ribonuclease A in fact occurs by way of similar lipid intermediates is still open for investigation.

Experiments using an inhibitor such as tunicamycin led to very interesting results. At concentrations of 12μM there was an extensive inhibition (80%) of the formation of monoglycosylated ribonuclease A by the preparations of yeast cell membrane. Tunicamycin is a glucosamine-containing antibiotic (Takatsuki et al., 1971) which is known to interfere with glycoprotein biosynthesis in mammalian and microbial cells (Takatsuki & Tamura, 1971; Kuo & Lampen, 1974). Studies of Tkacz & Lampen (1975) on calf liver rough microsomes have shown that tunicamycin inhibits the enzymic formation of polyisoprenyl N-acetyl-glucosaminyl pyrophosphate. However, this reaction may not be the only N-acetyl-glucosamine transfer reaction inhibited by the antibiotic. Yet further experiments are needed to establish whether the inhibition of the glycosylation of ribonuclease by tunicamycin is due to the lower rate of synthesis of lipid intermediates.

CHAPTER 5

A SOLUBLE FORM OF UDP-N-ACETYL-GLUCOSAMINE-ASPARAGINE-SEQUON-N-ACETYL-β-D-GLUCOSAMINYL TRANSFERASE ACTIVITY PRESENT IN HUMAN SERUM

Recent studies on the mechanism of addition of sugars in the formation of glycoprotein molecules have led to the discovery of several soluble forms of glycosyl transferases in colostrum (McGuire et al., 1965), milk (Babad & Hassid, 1966; Kobata & Ginsburg, 1970) and serum (Whitehead et al., 1974a; Fraser & Mookerjea, 1976).

Occurrence of the enzymes in soluble forms has stimulated a great deal of attention to their sources, functions and characteristics. Although some of these enzymes have been purified, a great deal of knowledge is yet to be gained with respect to their origins and also to their intercellular or intracellular functions.

Considerations of this nature prompted a search for the transferase under study in human serum.

1. Experimental and results

Blood (8 ml), taken from a normal subject (Z.K.) was allowed to clot at room temperature and centrifuged at 4°C and 2,000 rpm for about 20 min to separate the serum. A portion of the serum was incubated with ribonuclease A under the conditions described in Table 17, column 2, p. 190. The incubation was carried out for 1 h and terminated by the addition of 1% phosphotungstic acid in 5% trichloracetic acid. The radioactivity incorporated into acid precipitable material

was estimated by scintillation counting. Corrections were made for the quenching caused by protein present in the serum. Control experiments in which ribonuclease A was omitted from the mixture were carried out simultaneously. The results shown in Table 18 reveal that there is incorporation into both endogenous protein and also into the added ribonuclease A.

(i) Formation of glycosylated-ribonuclease catalysed by fresh serum

In this experiment ribonuclease A (3 mg) was incubated with UDP-N-acetyl-[^{14}C]-glucosamine in the presence of Mn^{+2} with fresh human serum which was taken from Z.K. immediately before the experiment. The experimental conditions are given in Table 17, column 3. In control experiments the conditions were exactly the same, except that no ribonuclease was added. The reactions were stopped after 1 h by cooling the mixture to 2^{o}C; the incubation mixtures were centrifuged for 15 min at 3,000 g and the supernatants were chromatographed on a CM-cellulose column 0.9 x 9 cm as described below:

The control incubation mixture which did not contain ribonuclease A was divided into two equal parts; one part was directly chromatographed on CM-cellulose and elution was effected using a linear gradient of sodium chloride. Fractions of 3 ml were collected and absorbance at 220 nm of each fraction was measured. The radioactivity was estimated by counting a small portion of the eluate in scintillation liquid. Three radioactive peaks were detected (Fig. 38a), but none of them in the region where ribonuclease A is normally eluted

Assay conditions	2	3	4	5	6	7	8	9	10	11	12	13	14
Tris-HCl buffer (mM)	45	25	33	45	45	45	45	45	45	50	42	42	45
$MnCl_2$ (M)	50	50	50	50	50	50	50	50	50	50	0-100	0	50
CDP-choline (mM)	0	3.40	2.26	0	0	0	0	0	0	0-4.7	0	0	0
UDP-GlcNAc (mM)	0.086	4.0	2.6	0.086	0.086	0.625	0.75	0.75	0.75	0.57	0.24	0.24	12
cpm/nmole	258	50	40	258	258	310	310	310	310	420	5050	5050	75
Ribonuclease (μM)	20.4	428	285	20.4	20.4	178	178	178	178	143	159	159	2850
Amount mg	0.1	3.0	3.0	0.1	0.1	0.25	0.25	0.25	0.25	0.2	0.2	0.2	20
Enzyme vol (μl)	250[a]	250[a]	250[a]	50[b]	50[c]	25[d]	25[d]	25[d]	25[d]	30[e]	25[f]	25[d]	150[g]
pH	7.2	7.2	7.2	7.2	7.2	5-8.5	7.2	7.2	7.2	7.2	7.2	7.2	7.2
Time (h)	1.0	1.0	1.0	1.0	1.0	1.0	1-6.0	1.0	1.0	1.0	1.0	1.0	1.0
Triton X-100 %	0	0	0	0	0	0	0	0-1	0	0	0	0	0
UDP (mM)	0	0	0	0	0	0	0	0	0-0.2	0	0	0	0
Spermine or Spermidine (mM)	0	0	0	0	0	0	0	0	0	0	0	0-84	0
Total vol (μl)	350	500	750	350	350	100	100	100	100	100	90	100	500
Ribonuclease glycosylated %		23	14		12								9.5[h]

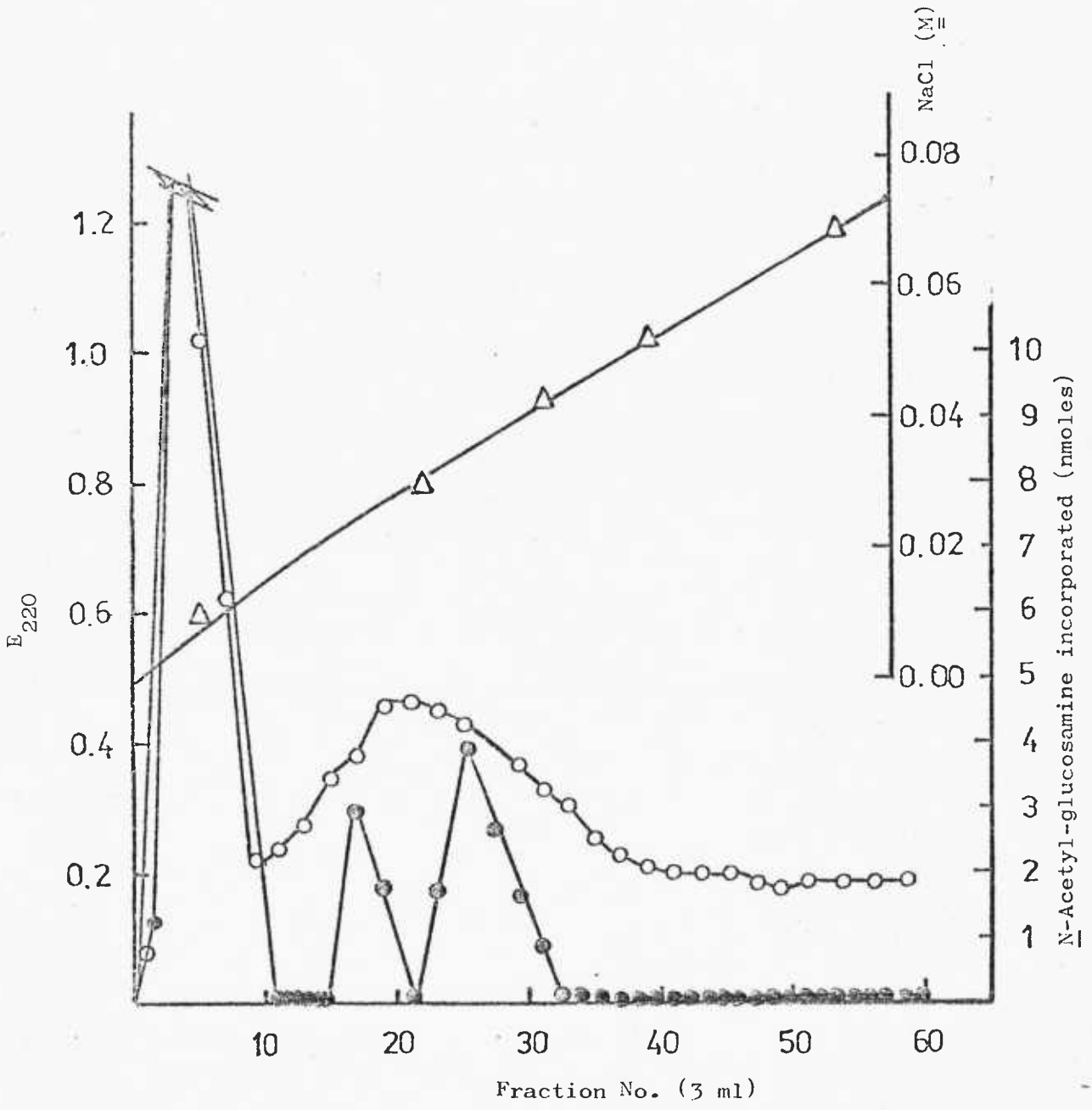
(a) Fresh human serum: (b) Out-dated human plasma: (c) The enzyme used in this series of experiments was one of the various ammonium sulphate-precipitated fractions (see Table 9): (d) Containing 0.5 mg of a fraction precipitated from human plasma between 35-45% ammonium sulphate saturation: (e) Containing 300 μg of 30-40% ammonium sulphate-fractionated material: (f) Containing 100 μg of 30-40% ammonium sulphate-fractionated material: (g) Containing 1.5 mg of a fraction precipitated between 30-40% ammonium sulphate saturation: (h) This is the mean value of several experiments (9.9, 10.9, 9.2, 8.6, 9.1, 9.6 and 9.0%).

TABLE 17. Conditions of incubation used in the enzymic incorporation of N-acetyl-glucosamine into bovine pancreatic ribonuclease A. The concentrations expressed are those present in the incubation mixture.

(0.07M NaCl concentration). There was, however, a residual level of ultraviolet-absorbing material in that region. To the other half of the control mixture was added 2.5 mg ribonuclease A and it was chromatographed on a CM-cellulose under conditions identical with the previous ones. An ultraviolet-absorbing peak appeared at that part of the chromatogram where the NaCl concentration was 0.07M. This was due to ribonuclease A, but still there was no radioactivity detected at that region (Fig. 38b). The incubation which originally contained ribonuclease A was also chromatographed on CM-cellulose and four radioactive peaks appeared (Fig. 38c), the first three in the same positions as in the control experiment. The fourth of these peaks (fractions 45-51), which was later shown to be glycosylated ribonuclease, occurred in a position slightly ahead but overlapping the ribonuclease A peak, which is eluted at 0.07M NaCl.

These results show that incubation under appropriate conditions of ribonuclease A with human serum and UDP-N-acetyl-glucosamine leads to the formation of glycosylated ribonuclease A. There were 49.2 nmoles of N-acetyl-glucosamine incorporated per 3 mg ribonuclease A, which is equivalent to 0.23 residues/mole of protein, taking the molecular weight of ribonuclease A as 14,000.

In a similar experiment performed under slightly different conditions (see Table 17, column 4) where the concentration of ribonuclease A was two-thirds of that described in the above-mentioned experiment, there were 30.2 nmoles of N-acetyl-glucosamine incorporated per 3 mg ribonuclease A which



o—o E_{220}

●—● N-acetyl-glucosamine incorporated

△—△ NaCl gradient

Fig. 38a. Chromatography on a CM-cellulose column (9 x 0.9 cm) of an incubation mixture without added ribonuclease A. The conditions of incubation are given in Table 17, column 3.

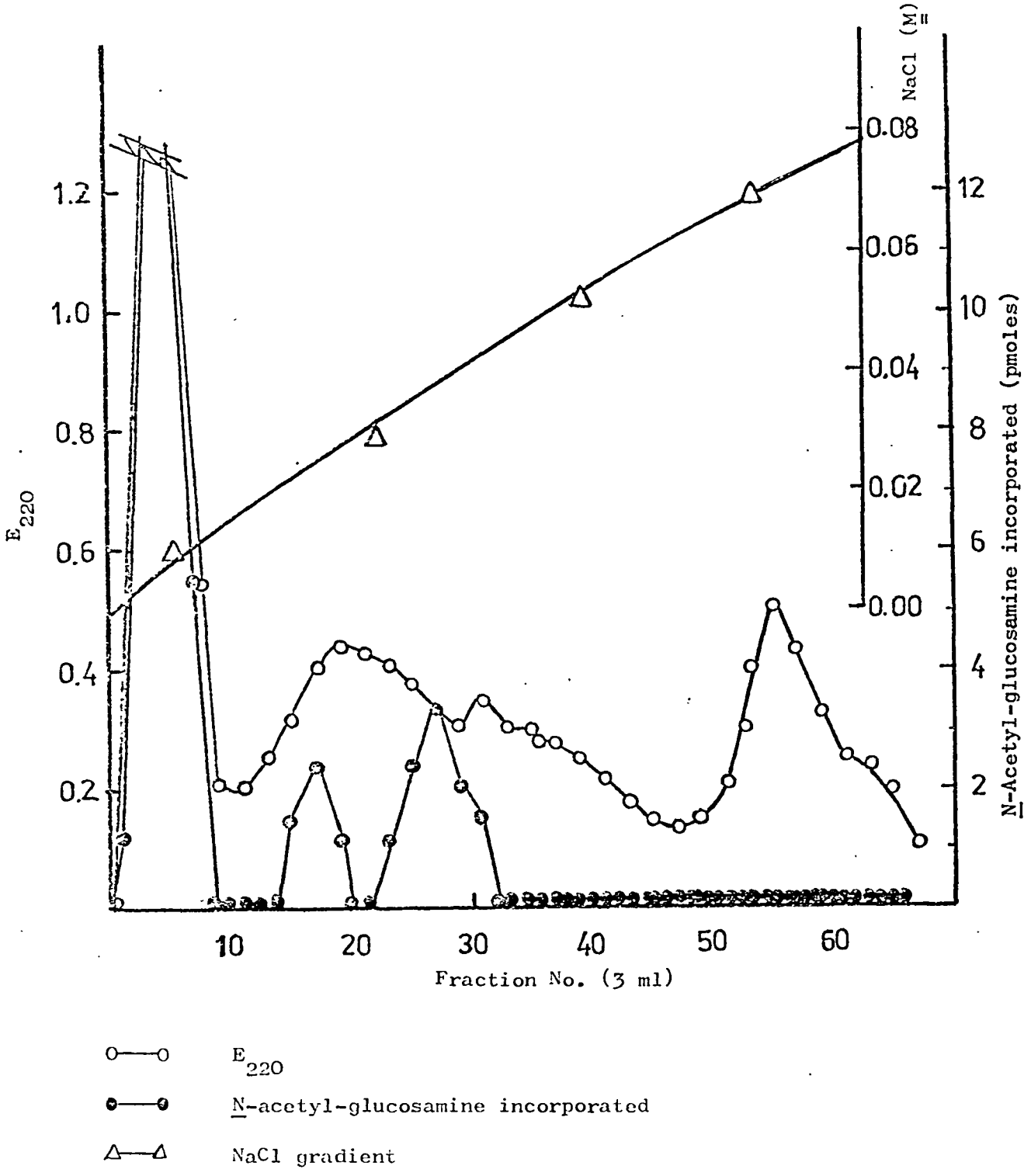
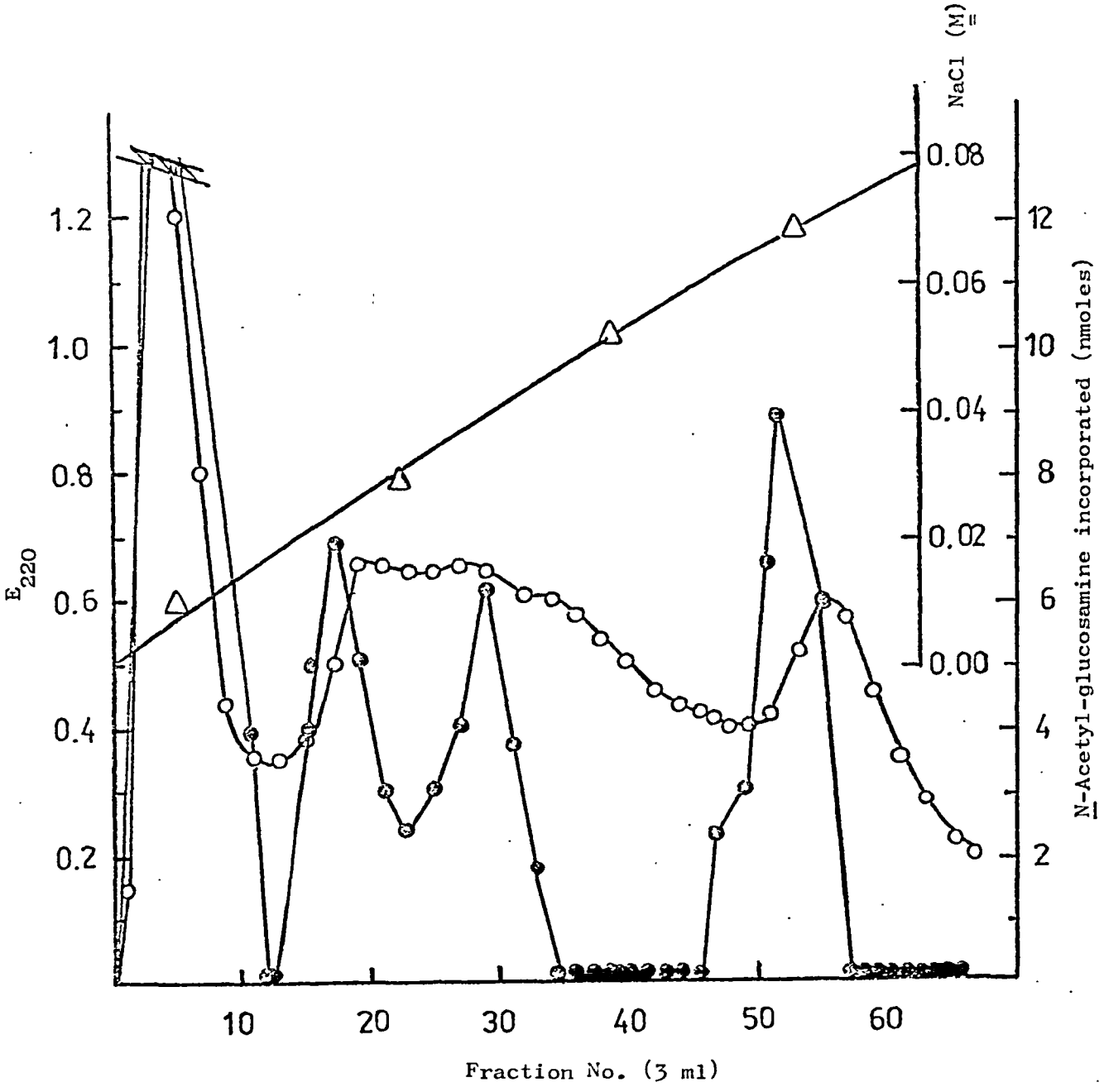


Fig. 38b. Chromatography on a CM-cellulose column of a control mixture incubated as described in Table 17, column 3. The ribonuclease was added to the mixture after incubation.



o—o E_{220}

Δ—Δ NaCl gradient

●—● N-acetyl-[^{14}C]-glucosamine incorporated

Fig. 38c. Chromatography on a CM-cellulose column of a mixture incubated as described in Table 17, column 3.

corresponds to 0.14 residues per mole of ribonuclease A.

(ii) Detection of the enzyme in outdated blood

Samples of outdated blood were centrifuged at 2,000 g for 4°C for 25 min to separate the plasma. The plasma was then transferred into small vials and frozen at -20°C; each sample was thawed immediately before use. The enzyme activity was measured in this plasma sample using the incubation conditions given in Table 17, column 5. The ribonuclease A was incubated with UDP-N-acetyl-[^{14}C]-glucosamine in the presence of the serum as the enzyme source. Incubations were terminated by the addition of 1% phosphotungstic acid in 5% trichloracetic acid solution and the precipitates were filtered on millipore filters. The radioactivity incorporated into acid precipitable material was measured by scintillation counting. The results of the experiment show that there is incorporation into ribonuclease and into endogenous protein, indicating the presence of the enzyme in the samples of outdated blood (Table 18).

(iii) Ammonium sulphate fractionation of plasma

The plasma sample obtained from outdated blood was dialysed in the cold against 2 x 40 vol of Tris phosphate buffer (0.04$\underline{M}$ in Tris; 0.005$\underline{M}$ NaH_2PO_4), pH 8.6). Ammonium sulphate (5.7 g) was dissolved in 50 ml of dialysed plasma, and the solution was kept in the cold room overnight. The precipitate was collected by centrifugation at 8,000 g at 4°C for 10 min and dissolved in 10 ml of 0.04$\underline{M}$ Tris phosphate buffer. The solution was dialysed overnight against the same buffer (2 x 2 l vol) at 4°C and freeze-dried. The supernatant was further fractionated by

Enzyme source	nmoles N-acctyl-glucosamine incorporated / 250µl*		
	Endogenous	Exogenous+endogenous	Difference
Out-dated human plasma	1.95	3.36	1.41
Fresh human serum	2.15	4.19	2.04

*This value refers to the volume of either serum or plasma.

The amount of ribonuclease A used in these experiments was 14.2nmoles.

TABLE 18. Incorporation of N-acetyl-glucosamine into acid precipitable material using out-dated human plasma as the source of the enzyme. The values for fresh human serum are also given.

successive additions of 1.45 g, 1.50 g, 1.50 g, 1.55 g, 1.6 g and 1.65 g ammonium sulphate to give respective precipitates which were removed in each case as above. The weights of ammonium sulphate are the ones needed to give the degrees of saturation at 25°C shown in Table 9 , in which are also shown the recoveries of protein assessed as total absorbance at 280 nm.

The freeze dried samples obtained as mentioned above were dissolved in the Tris phosphate buffer, pH 8.6, to give a final concentration of 10 mg/ml. They were then assessed for enzyme activity as described below:

Incubations were carried out under the conditions shown in Table 17, column 6. The reactions were stopped by the addition of 1% phosphotungstic acid in 5% trichloracetic acid, the precipitates were filtered and the radioactivity incorporated into acid precipitable material was determined from the results of scintillation counting. The results of the experiments show that fractions precipitated by ammonium sulphate concentrations of 25-30 and 30-35% contain most of the activity. Each of these fractions appeared to be about six times more active than whole serum.

(iv) Factors affecting the rate of incorporation of N-acetyl-glucosamine into ribonuclease A

(a) Effect of pH:

Incubations were set up containing the components described in Table 17, column 7. The pH was the only variable in the experiment, being from 5 to 8.5. Control experiments were performed omitting ribonuclease A from the incubation mixture.

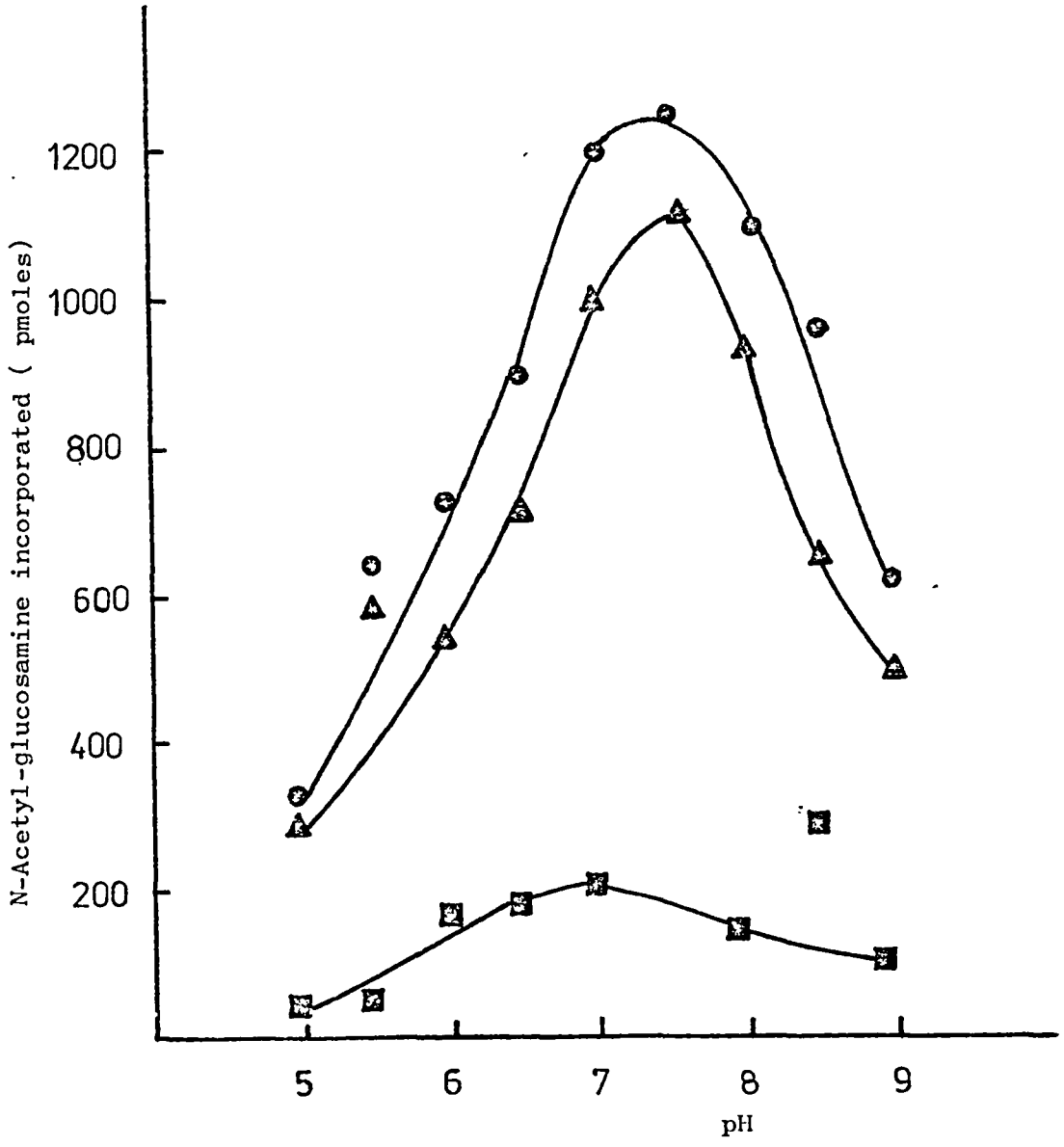


Fig. 39. Effect of pH on the incorporation of N-acetyl-glucosamine into ribonuclease A using human plasma as the source of the enzyme.

●—● Ribonuclease A was present in the mixture

△—△ Ribonuclease A was omitted from the mixture

▣—▣ The difference curve

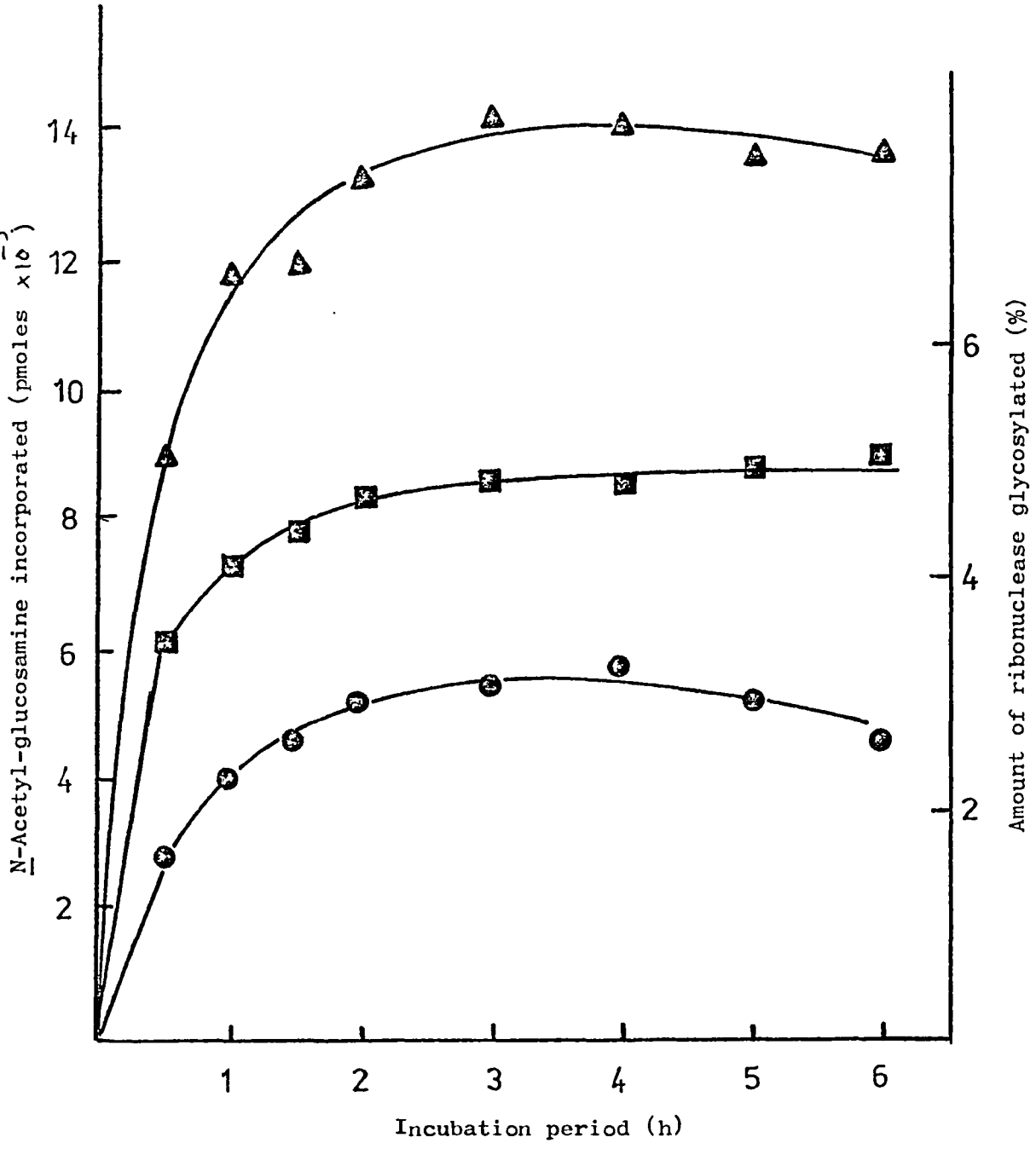


Fig. 40. Rate of incorporation of N-acetyl-glucosamine into ribonuclease A. For conditions of incubation see Table 17, column 8.

▲—▲ Incubation mixture containing ribonuclease A

■—■ Incubation mixture with no added ribonuclease A

●—● The difference curve showing incorporation into ribonuclease A

The reaction was stopped by addition of 1% phosphotungstic acid in 5% trichloracetic acid and the precipitates were filtered on millipore filters. The precipitates were washed, dried and counted in a scintillation counter. The results (Fig. 39) show that the incorporation into endogenous protein is maximum at pH 7.5, while glycosylation of ribonuclease A appears to have a pH optimum of about 7.

(b) Time:

The effect of time on the extent of incorporation was investigated by incubating ribonuclease A with UDP-N-acetyl-[^{14}C]-glucosamine in the presence of Mn^{+2} using plasma as the enzyme source (Table 17, column 8 gives the conditions of incubation). The results shown in Fig. 40 indicate that incorporation has ceased within about 2 h, giving rise to ~5% incorporation of radioactivity into ribonuclease A.

(c) Concentration of enzyme protein:

A portion of plasma (50 ml) was dialysed against 2 x 40 vol of Tris phosphate (0.04M Tris), pH 8.6. This dialysed plasma was used to investigate the effect of enzyme protein concentration on the rate of incorporation (Fig. 41). Fractions C and D (Table 9) were also assessed in the same way (for incubation conditions see Table 17, column 6). In the case of dialysed plasma, the extent of incorporation of N-acetyl-glucosamine is proportional to the amount of enzyme protein used. The original number of units of activity (for definition of units see Table 9) in the 50 ml of dialysed sample was 187×10^3. The rate of incorporation did not appear to

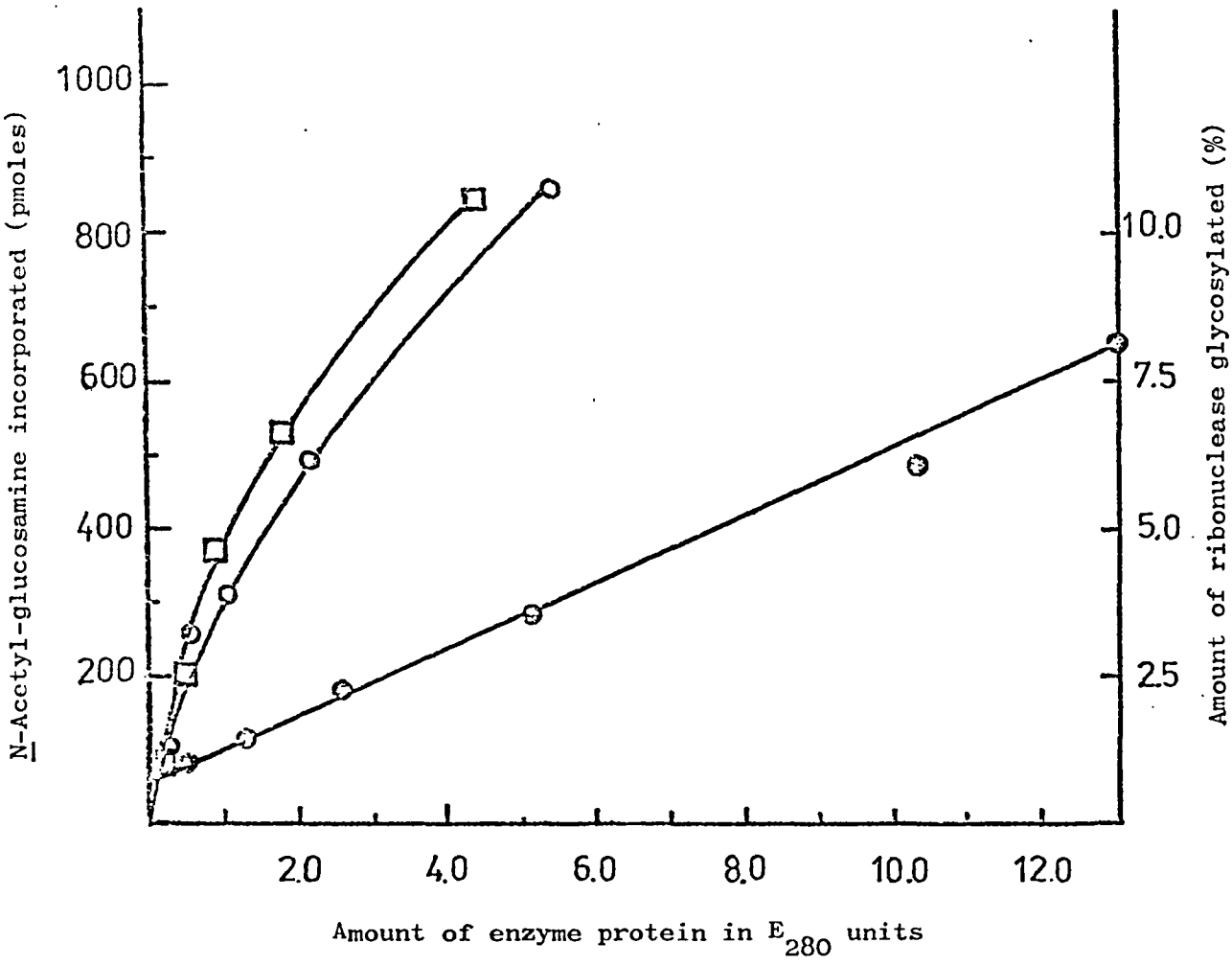


Fig. 41. Relationship between the amount of enzyme protein used and the extent of glycosylation of ribonuclease A.

●—● Dialysed whole serum

o—o Fraction C precipitated between 25-35% ammonium sulphate saturation

□—□ Fraction D precipitated between 30-35% ammonium sulphate saturation

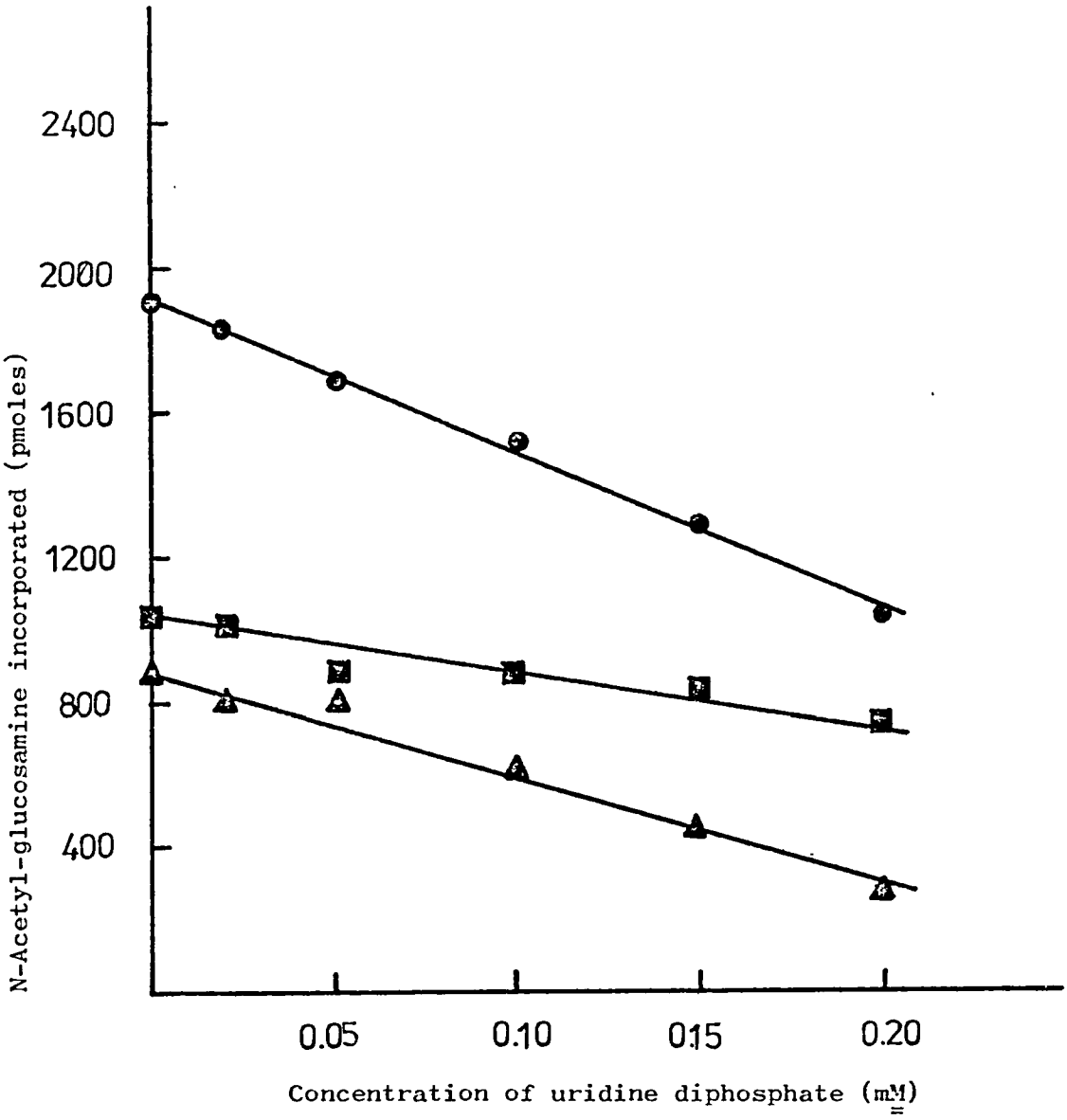


Fig. 42. Effect of uridine diphosphate on the activity of the enzyme.

●—● Incubations performed in presence of added ribonuclease

▲—▲ Ribonuclease was omitted from the mixture

■—■ The difference curve

be directly proportional to the amount of enzyme protein when partially purified fractions were used (Fig. 41). A total recovery of 579 x 10^3 units (309% recovery) of activity in the partially purified serum suggests that an inhibitor for the enzyme had been removed as a result of the ammonium sulphate fractionation.

(d) Effect of Triton X-100:

The effect of Triton X-100 on the activity of enzyme was examined by incubating ribonuclease with partially purified enzyme (35-45% ammonium sulphate fraction) under the conditions given in Table 17, column 9. The concentration of Triton X-100 was varied from 0-1%. It was shown that at concentrations of 0.1% and 0.2% Triton had no effect, while at greater concentrations it exhibited an inhibitory effect. At a 1% concentration there was 47% inhibition in the enzyme activity.

(e) Effect of uridine diphosphate:

The effect of uridine diphosphate on the enzyme activity was investigated by incubating ribonuclease A with partially purified serum at various concentrations of UDP ranging from 0-0.2m$\underline{M}$ (for conditions see Table 17, column 10). UDP appeared to inhibit the enzyme to the extent of 2% at 0.02m$\underline{M}$, 15% at 0.1m$\underline{M}$ and 27% at 0.2m$\underline{M}$ (see Fig. 42).

(f) Effect of CDP-choline:

The effects of adding CDP-choline on the extent of glycosylation of ribonuclease A were assessed. These experiments were carried out in the presence of Mn^{+2} ions at a concentration of

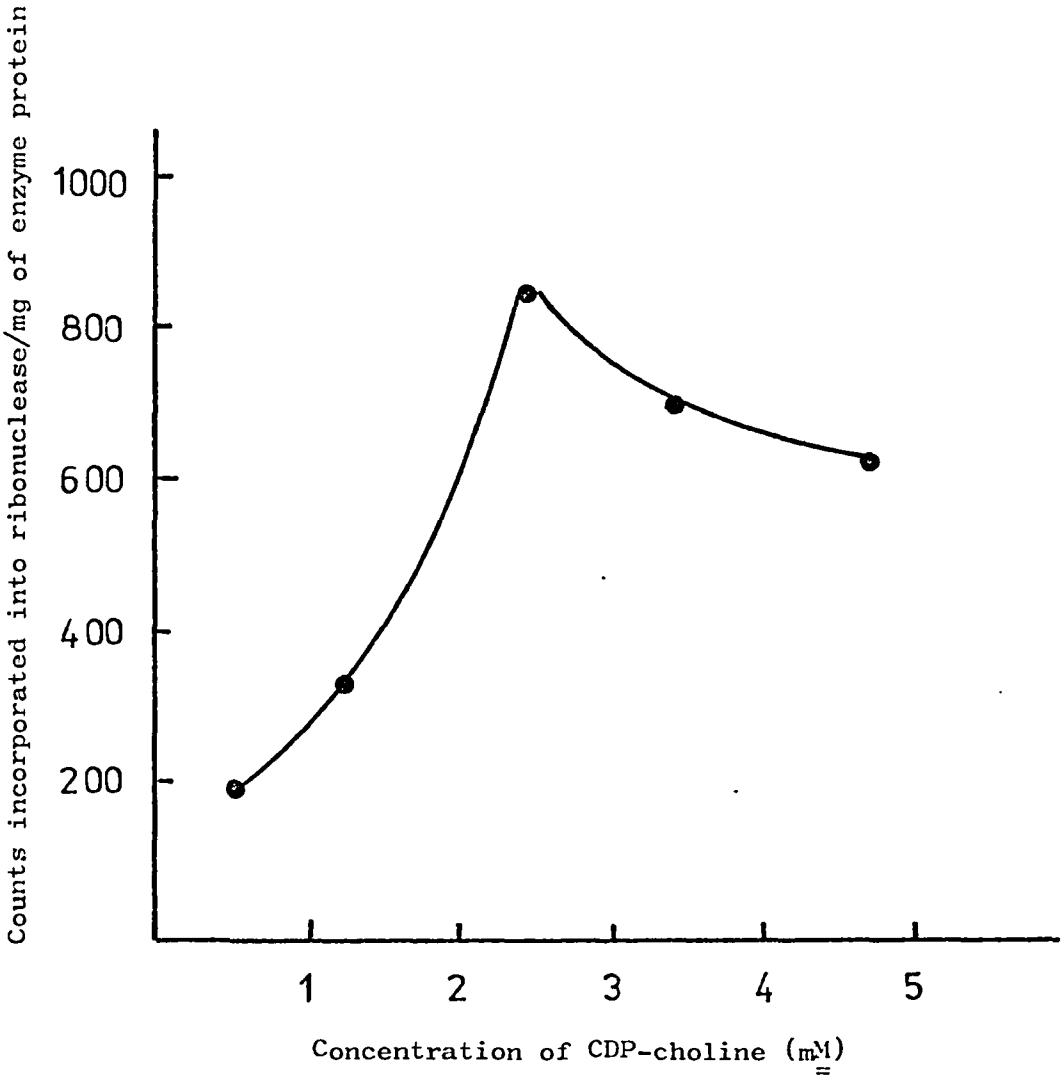


Fig. 43. Effect of CDP-choline on the incorporation of N-acetyl-glucosamine into ribonuclease A. The incubation conditions are described in Table 17, column 11.

50mM. The general conditions of incubation are described in Table 17, column 11. As shown in Fig. 43, the nucleotide enhances the ability of the enzyme to catalyse the transfer of N-acetyl-glucosamine from UDP-GlcNAc to ribonuclease A. The nucleotide also enhances the incorporation into the endogenous protein present in the enzyme preparation. The optimum concentration of CDP-choline appears to be 2.4mM.

(g) Effect of Mn^{+2} concentration on the activity of the enzyme:

Most of the previous experiments described were carried out using a concentration of 50mM Mn^{+2} ions. It was desirable to see if the serum enzyme needed a different optimum Mn^{+2} concentration from that of liver. Incubation mixtures were prepared with concentrations of components as described in Table 17, column 12. The final concentration of Mn^{+2} ranged from 0-100mM. The metal ion stimulated the incorporation both into endogenous protein and ribonuclease A, showing a similar optimum concentration of 50mM as compared with liver enzyme (Fig. 44).

(h) Effect of polyamines on the rate of incorporation:

Spermidine and spermine were used to study the effect of polyamines on the extent of incorporation of N-acetyl-glucosamine into ribonuclease A. At the time the presently described experiments were performed, the author was unaware of the studies carried out by Baker & Hillegass (1974b) on the activation caused by spermine of the galactosyl transferase in preparations of dog tracheal mucosal lining.

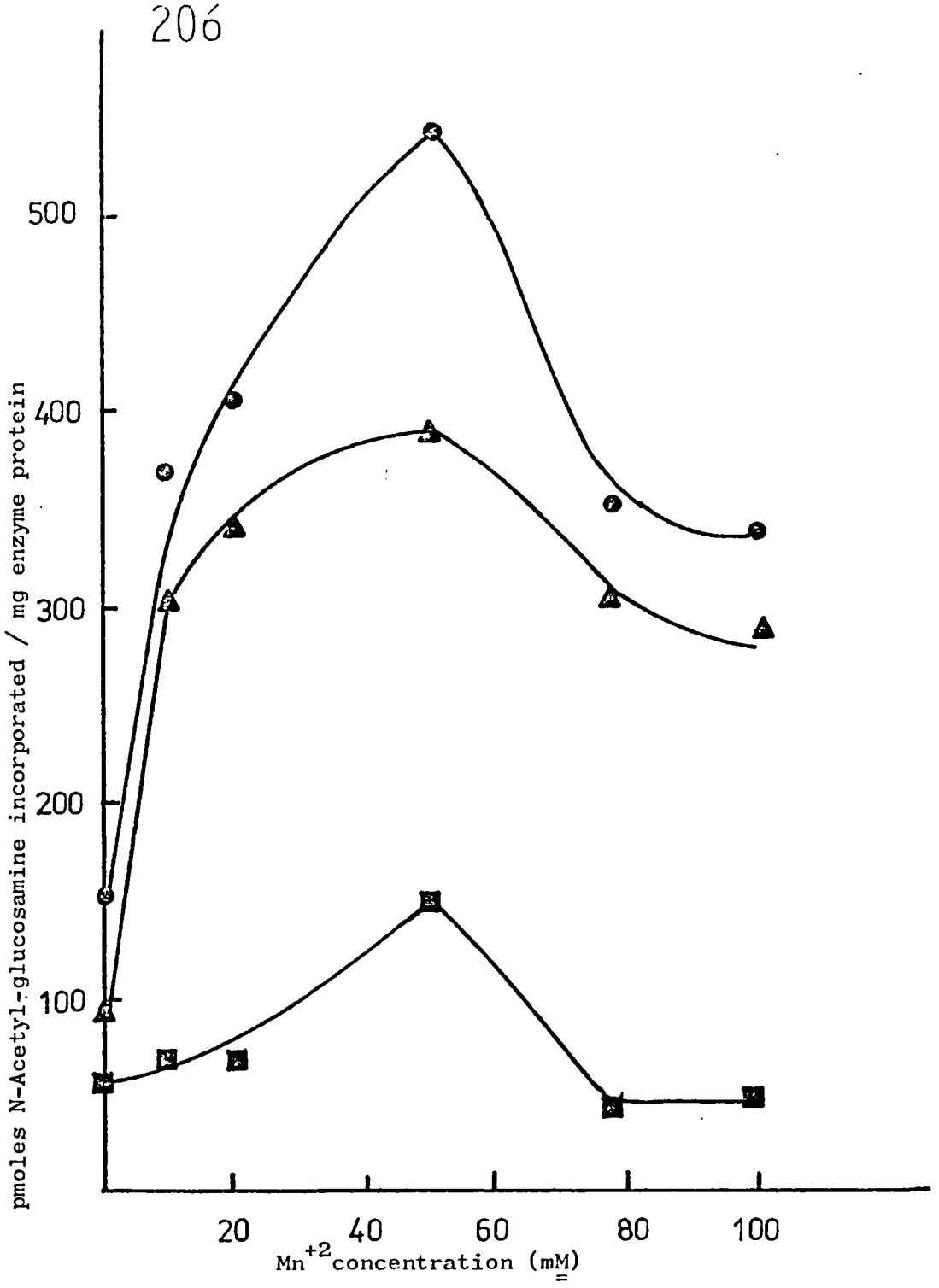


Fig. 44. Effect of Mn^{+2} concentration on the rate of incorporation of N-acetyl-glucosamine using human plasma as the source of the enzyme.

●—● Ribonuclease A was added to the mixture

▲—▲ Ribonuclease A was omitted from the mixture

■—■ The difference curve showing incorporation into ribonuclease A.

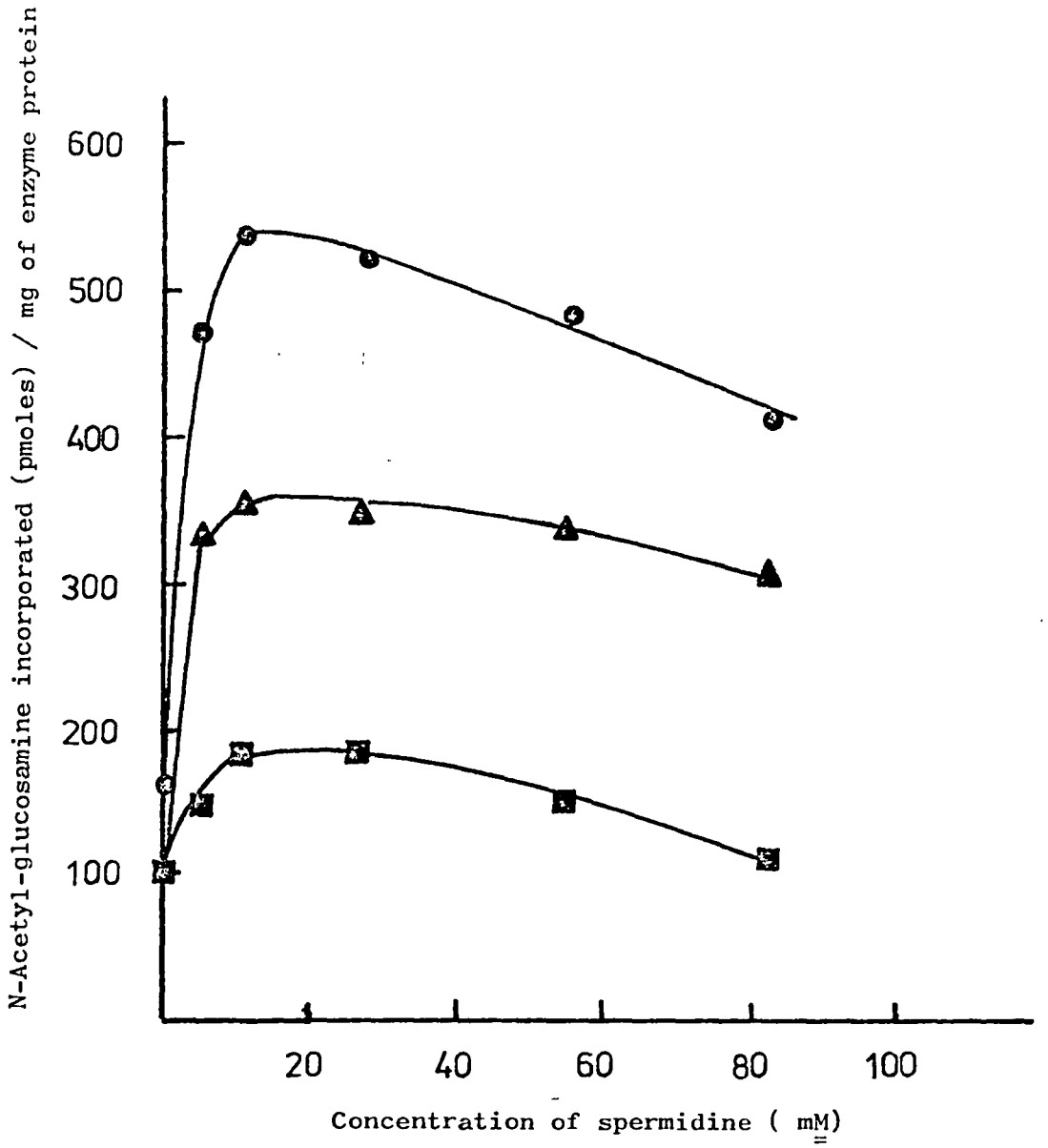


Fig. 45. Effect of spermidine on the extent of incorporation of N-acetyl-glucosamine into ribonuclease A.

●—● Incubations were carried out in presence of added pancreatic ribonuclease A.

▲—▲ Ribonuclease A was not added to the incubation mixture

■—■ The difference curve

The range of polyamine concentrations used was 0-8$\frac{4}{=}$mM. Both spermidine and spermine solutions were neutralized with dilute HCl before use. The conditions of incubation are given in Table 17, column 13. As Fig. 45 shows, both the polyamines activate the incorporation of N-acetyl-glucosamine into ribonuclease A and into endogenous proteins. Spermidine appears to be more effective and, at a concentration of 10-15$\frac{m}{=}$M, is a better activator than is Mn^{+2} at its optimum concentration.

(v) Separation of glycosylated ribonuclease from ribonuclease A

The chromatographic behaviour of ribonuclease-GlcNAc on CM-cellulose is known, from the experiments described in Chapter 3, to be only slightly different from that of ribonuclease A. Therefore a method was devised in which affinity chromatography involving the use of Sepharose-bound wheat-germ agglutinin was used. For this purpose a mixture of glycosylated and non-glycosylated ribonuclease was prepared as follows.

Ribonuclease A (20 mg) was incubated with UDP-N-acetyl-[^{14}C]-glucosamine under the conditions shown in Table 17, column 14. The enzyme was a portion of a fraction obtained by ammonium sulphate fractionation (25-35%) of plasma. The incubation was terminated by cooling the mixture to 2^{o}C. It was centrifuged at 2,000 g, 15 min, and the mixture chromatographed on CM-cellulose. Fractions of 3 ml were collected and absorbances at 220 nm were measured by spectrophotometer. Radioactivity was estimated by counting measured aliquots in a scintillation counter, and the extent of incorporation was 9.6%. The fractions containing

ribonuclease (Fig. 46) were pooled, dialysed against Tris-HCl buffer (0.01M, pH 7.0) and the retentate was freeze-dried. A column of 0.3 x 2 cm agarose-bound wheat-germ agglutinin was prepared and equilibrated with Tris-HCl buffer, 0.01M in Tris, pH 7.0. The freeze-dried sample from the CM-cellulose column was then dissolved in 1 ml of 0.01M Tris-HCl buffer, pH 7.0 (144 nmoles 10,860 cpm = GlcNAc) and the solution was put on the column. The column was eluted firstly with the 0.01M Tris-HCl buffer. Fractions of 1 ml were collected and absorbances were measured at 220 nm until no further ultraviolet-absorbing material was eluted. Ribonuclease A was not adsorbed on the column and therefore it passed straight through (Fig. 47). A gradient of N-acetyl-glucosamine in the buffer was then applied to the column (25 ml of 0.5mM GlcNAc in buffer was allowed to flow into 25 ml 0.01M Tris-HCl buffer and the latter chamber was connected to the column). Fractions of 1 ml were collected and measured at 220 nm and counted for radioactivity. The substance emerging from the column at a concentration of the eluting sugar of 0.175mM contained 9,100 cpm which is equal to 123 nmoles of GlcNAc (85% recovery) and was shown to be monoglycosylated ribonuclease as discussed later. The fractions containing the ribonuclease-GlcNAc ($\sim$ 1.7 mg) were combined and kept frozen. The peak of unreacted ribonuclease A eluted from the column was also pooled, dialysed against distilled water and freeze-dried. The freeze-dried ribonuclease was used for further glycosylation studies, the conditions for which were identical with those described above (Table 17, column 14). The incubation mixture, after centrifugation, was again chromatographed

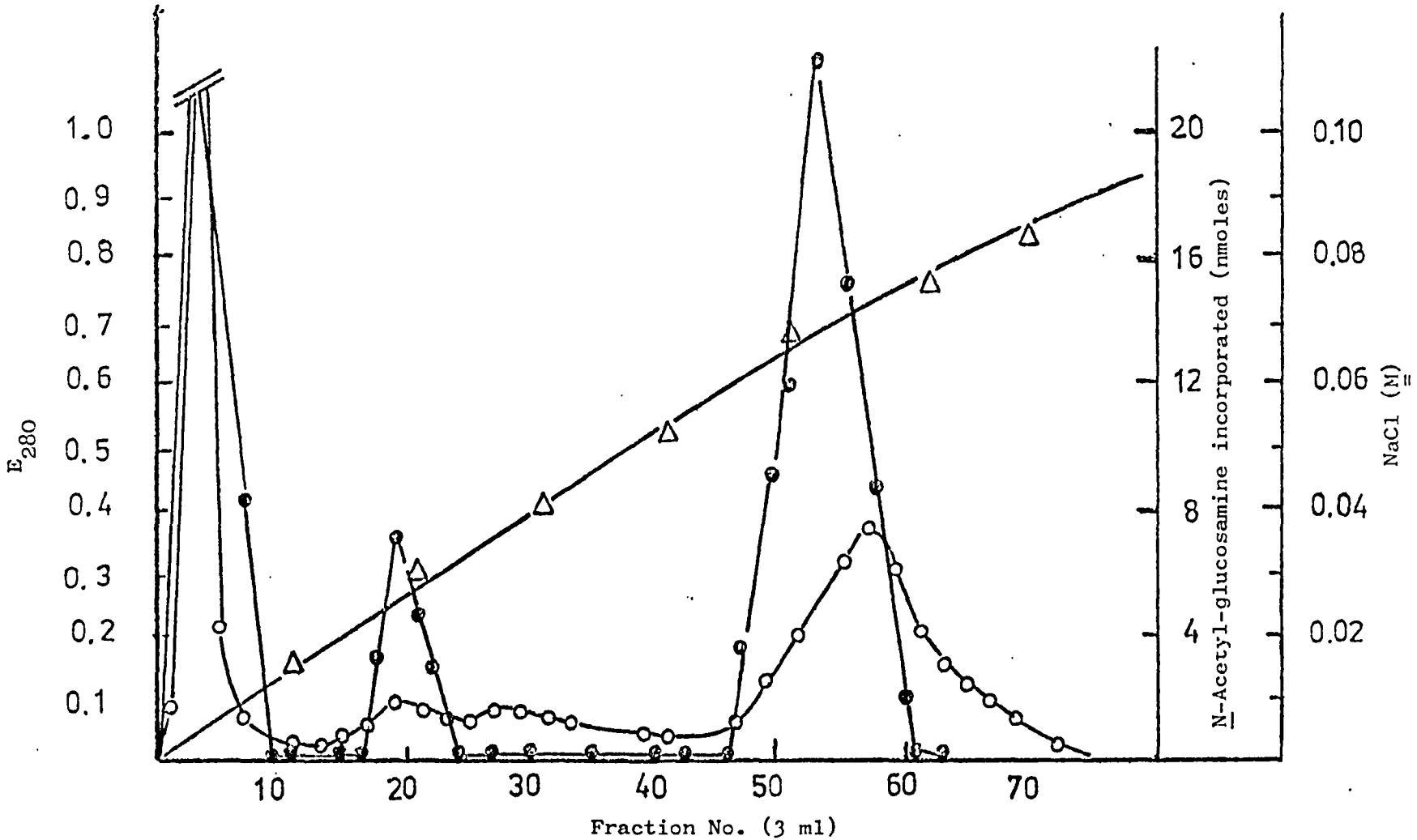


Fig. 46. Chromatography on a CM-cellulose column (0.9 x 9 cm) of a mixture incubated as described in Table 17, column 14. Fractions 48-65 were combined and used for further purification on lectin column (Fig. 47)

●—● N-acetyl-[^{14}C]-glucosamine incorporated o—o E_{280} Δ—Δ NaCl concentration (M)

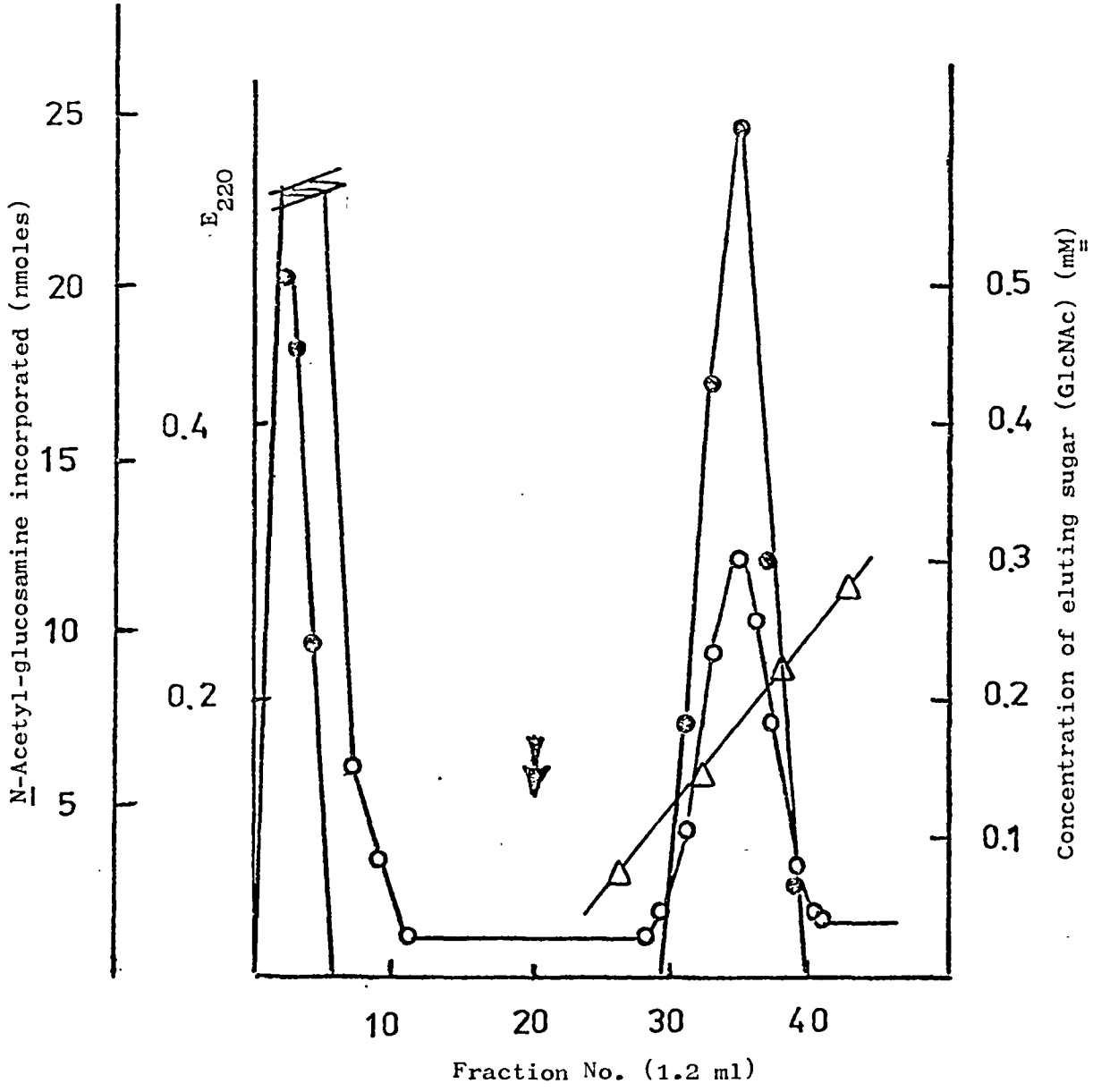


Fig. 47. Separation by chromatography at 4°C on a column (2 x 0.3 cm) of wheat-germ agglutinin Sepharose of ribonuclease A and glycosylated ribonuclease

●—● N-acetyl-[^{14}C]-glucosamine

o—o E_{220}

△—△ N-acetyl-glucosamine assayed by Morgan-Elson procedure

The arrow indicates the change of buffer.

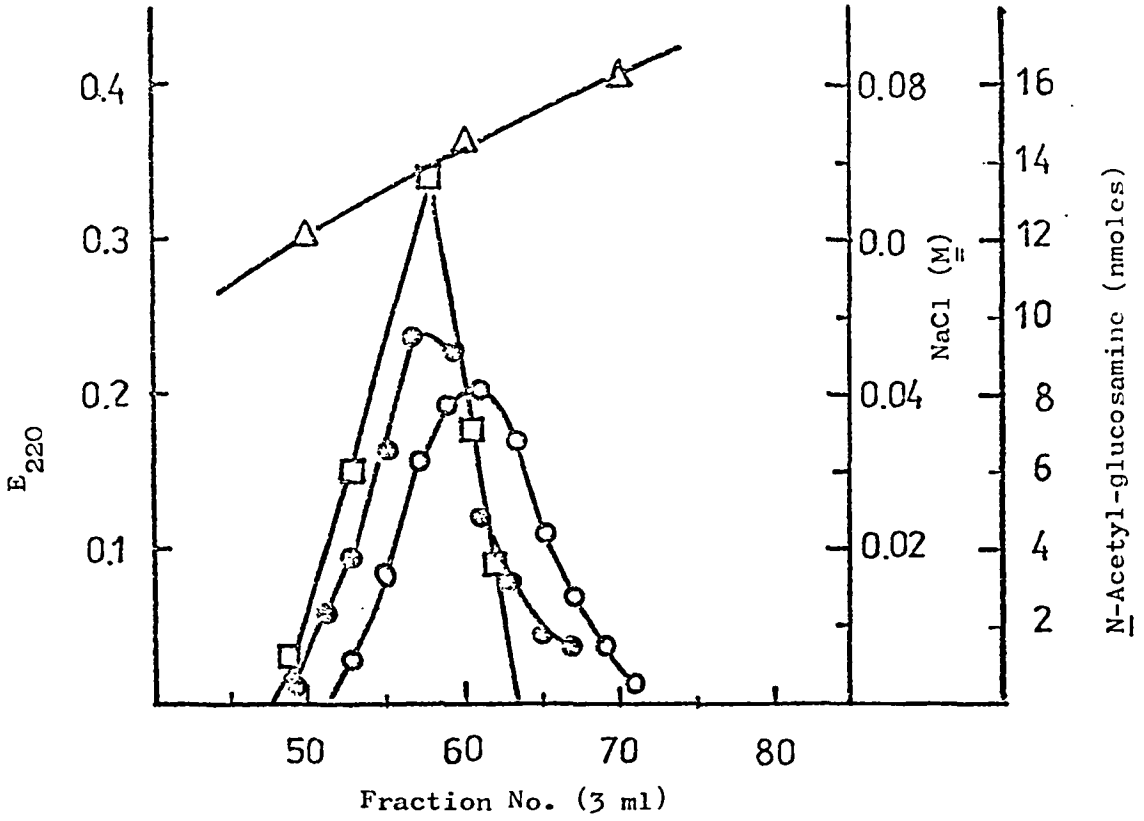


Fig. 48a. Chromatography on a CM-cellulose (9 x 0.9 cm) of either 1.5 mg ribonuclease GlcNAc (●—●, E_{220}; □—□ amount of N-acetyl-glucosamine bound to ribonuclease; △—△ NaCl gradient), or 1.0 mg ribonuclease A (○—○, E_{220}; △—△ , NaCl gradient)

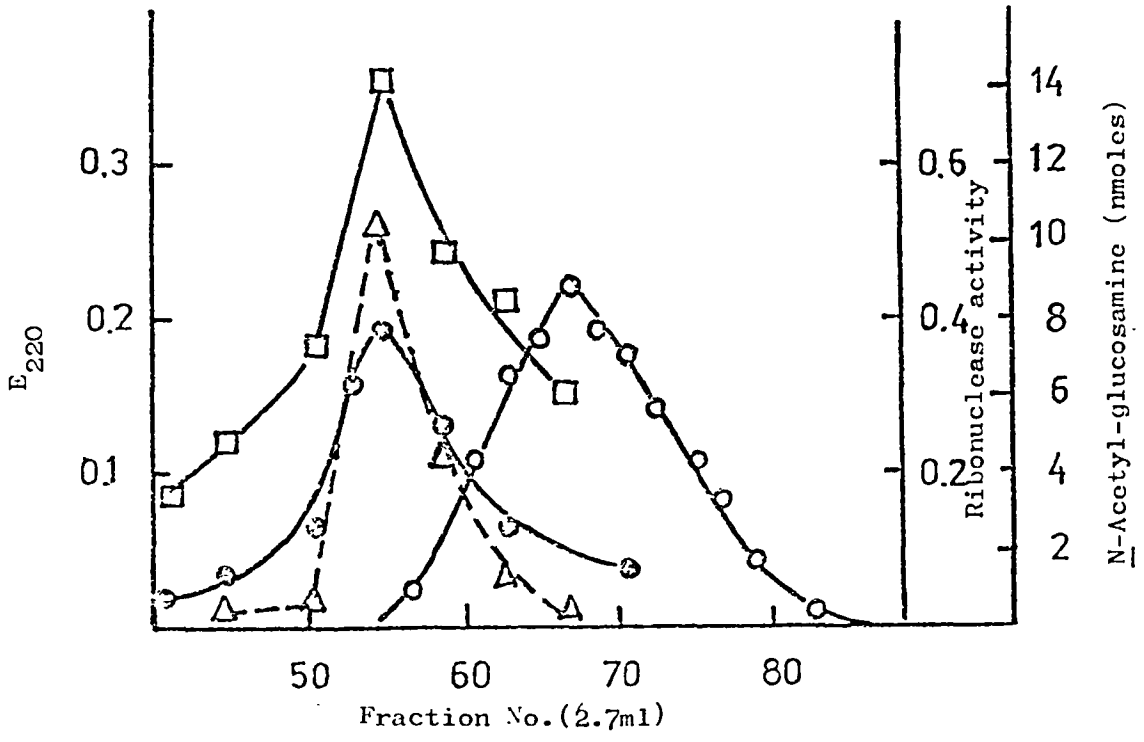


Fig. 48b. Chromatography on an Amberlite CG-50 column (45x0.9 cm) of either 0.5 mg ribonuclease-GlcNAc (●—●, E_{220}; □—□ , N-acetyl-glucosamine bound to ribonuclease; △—△ , enzyme activity of ribonuclease-GlcNAc), or of 0.8 mg of ribonuclease A (○—○, E_{220})

on a CM-cellulose column and the mixture of ribonuclease-GlcNAc and ribonuclease A was fractionated again on the wheat-germ agglutinin column. The peak of ribonuclease-GlcNAc was pooled and added to the previous fraction. The unreacted ribonuclease A was again used for further glycosylation. This procedure was repeated until about 7 mg ribonuclease-GlcNAc was prepared (five successive incubations). The extent of incorporation was very similar in each case, close to 10% (9.0%, 9.9%, 10.9%, 9.2%, 8.6%, 9.1% and 9.6%). The recoveries from the lectin column varied from 77 to 88%.

(vi) Some properties of ribonuclease-GlcNAc

(a) Chromatographic behaviour on CM-cellulose and Amberlite CG-50:

Ribonuclease-GlcNAc was found to be eluted from CM-cellulose slightly more readily than is ribonuclease A. Either ribonuclease-GlcNAc (1.5 mg) or ribonuclease A (1.0 mg) was chromatographed separately on a column (0.9 x 9 cm) of CM-cellulose in 0.01M Tris-HCl buffer, pH 7.0. The elution was effected using a linear gradient of NaCl. As shown in Fig. 48a, the two compounds have very similar chromatographic behaviour on CM-cellulose. This explains why in earlier experiments chromatography on CM-cellulose was not effective in separating the two components.

Ribonuclease-GlcNAc is also eluted more readily from Amberlite CG-50 than is ribonuclease A. Amounts of 0.5 mg ribonuclease-GlcNAc or 0.8 mg ribonuclease A were chromatographed in separate experiments on a CG-50 column (45 x 0.9 cm) in 0.2M

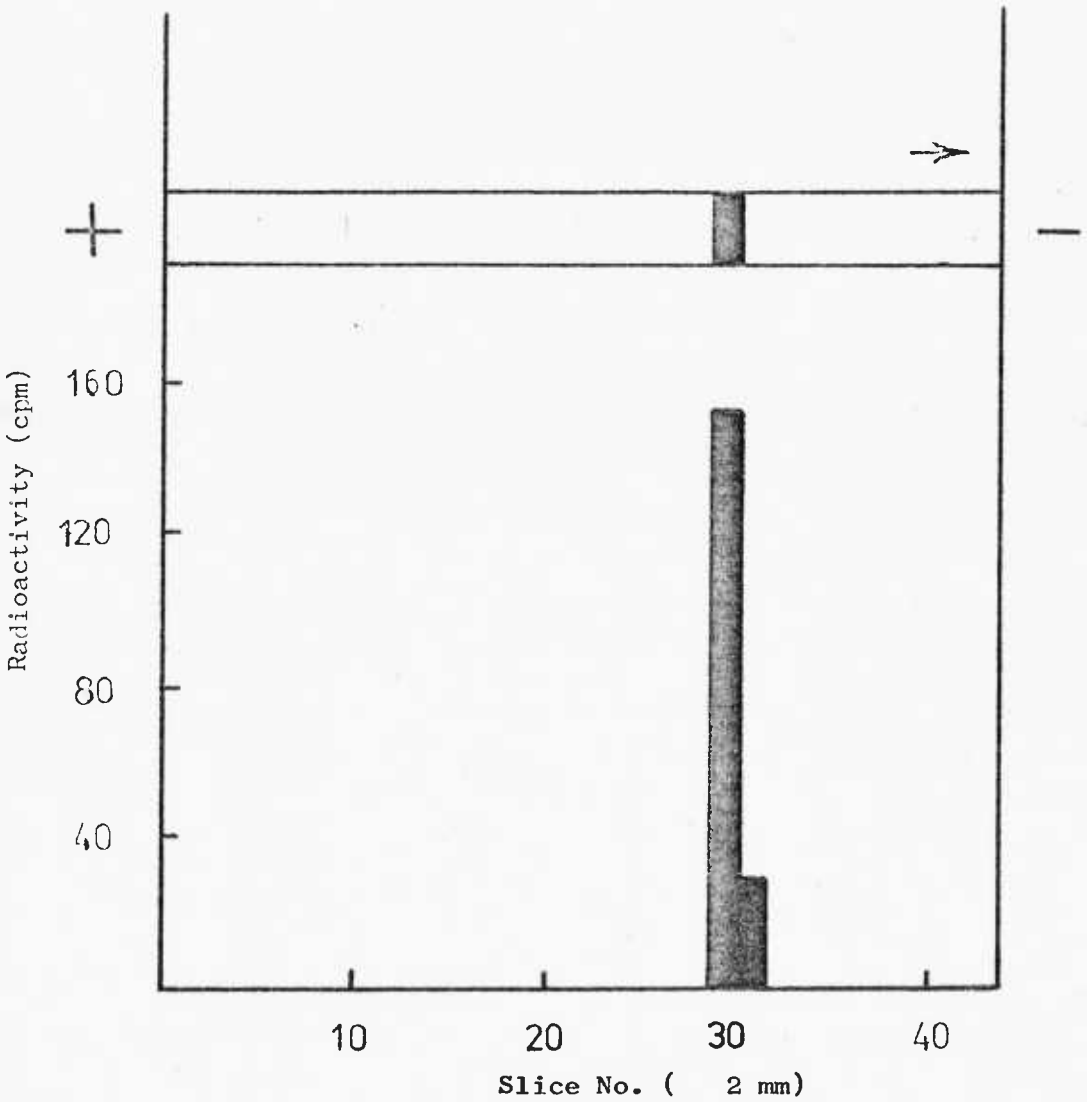


Fig. 49. Polyacrylamide gel electrophoresis of glycosylated ribonuclease. The gels were either stained with Coomassie blue for protein or cut in 2 mm slices and counted for radioactivity. The radioactivity was detectable only in slices corresponding to the position of ribonuclease-GlcNAc on gels which were stained with Coomassie blue.

Component	Residues/mole in Ribonuclease-GlcNAc	Ribonuclease 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 (Kraan & Muir, 1957) after hydrolysis in 4M HCl at 100°C 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.

TABLE 19. Amino acid and hexosamine content of glycosylated ribonuclease.

phosphate buffer (for composition see p. 143), pH 6.02 (Fig. 48b). The relative mobility of ribonuclease A to that of ribonuclease-GlcNAc was 1.22 which is close to the value found by Tarentino et al. (1974). Ribonuclease-GlcNAc also had enzymic activity, catalysing the digestion of ribonucleic acid, as assessed by the procedure of Kalnitsky et al. (1959) (Fig. 48b).

(b) Gel electrophoresis in the presence of sodium dodecyl sulphate:

Ribonuclease-GlcNAc was subjected to gel electrophoresis in 9.6% polyacrylamide gel containing sodium dodecyl sulphate (Marshall & Zamecnik, 1969). The gels were either stained with Coomassie blue for protein or counted for radioactivity. The gels were cut in 2 mm slices and the pieces were put in scintillation vials. The slices were dissolved in aqueous 30% H_2O_2 (v/v) at 40°C and the solution evaporated to dryness. Scintillation fluid (10 ml) was added to each and the samples were counted. The results of these experiments (Fig. 49) showed that the radioactivity associated with ribonuclease-GlcNAc was not released in a dissociating medium.

(c) Amino acid analysis:

A sample of ribonuclease-GlcNAc (500 µg) was hydrolysed in 6M HCl for 16 h at 100°C. The solution was evaporated to dryness and the residue dissolved in water. A portion of this was subjected to amino acid analysis using programme I (see p. 92). The results are given in Table 19. Amino sugar (Elson-Morgan) analysis was performed on a sample which was

hydrolysed in 4M HCl at 100°C for 4 h. This analysis revealed the presence of glucosamine; 0.78 residue/mole of protein (uncorrected for moisture and ash content), a result which is also in fair agreement with the radioactivity measurements.

(vii) Point of substitution of ribonuclease by the N-acetyl-glucosamine residue

Ribonuclease-GlcNAc (4.5 mg, 321 nmoles) was reduced and cyanoethylated (Anfinsen & Haber, 1961; Plummer & Hirs, 1964) (see Methods section). The cyanoethylated derivative was subjected to tryptic digestion with purified trypsin. The digest was put on a column of Sephadex G-25. Elution was effected with 0.2M CH_3COOH and fractions of 1 ml were collected. The fractions were assayed for protein and radioactivity. A single but asymmetric radioactive peak was eluted from the column (fractions 139-146, Fig. 50). This peak contained 304 nmoles glucosamine based on radioactivity measurements ($\sim$ 95% recovery). The fractions containing radioactivity were combined and freeze-dried. Of this material, an amount equivalent to 200 nmoles of glucosamine was put on an Amberlite CG-120 column, which was eluted with a gradient of increasing concentration and pH of pyridine formate buffer. Two radioactive peaks appeared, the first of which (peak I, Fig. 51) contained 104 nmoles of glucosamine. A portion of this was hydrolysed in 4M HCl, 4 h *in vacuo*, 100°C. The hydrolysate was subjected to analysis on the amino acid analyser using programmes I and II (see p. 92). The composition of the glycopeptide is given in Table 20 and it may be deduced that it had arisen from residues 34-37 of the original ribonuclease A.

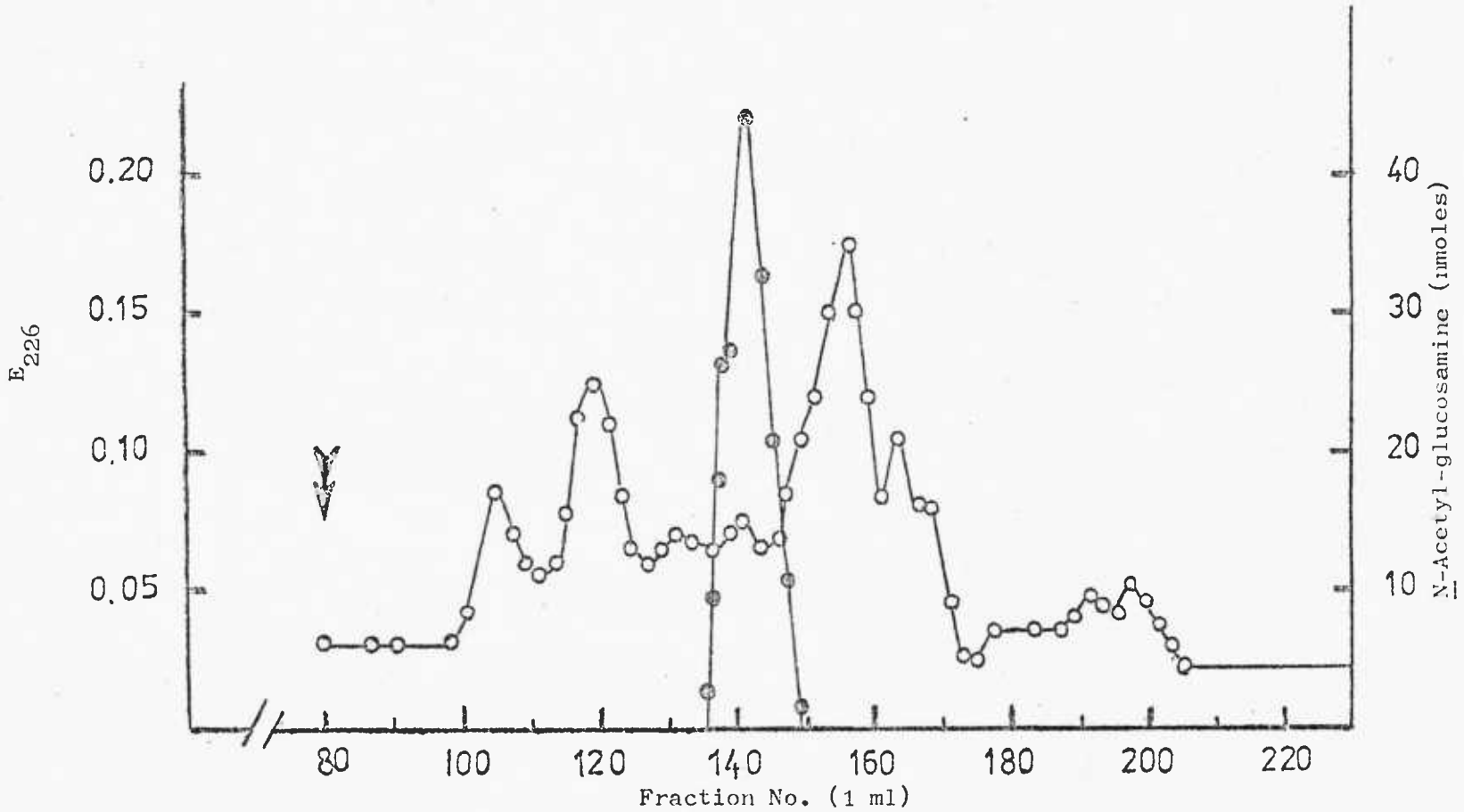


Fig. 50. Fractionation of tryptic digest of cyano- ethyl derivative of glycosylated ribonuclease on a column (250 x 1.0) of Sephadex G-25. ●—● radioactivity; O—O E_{226}

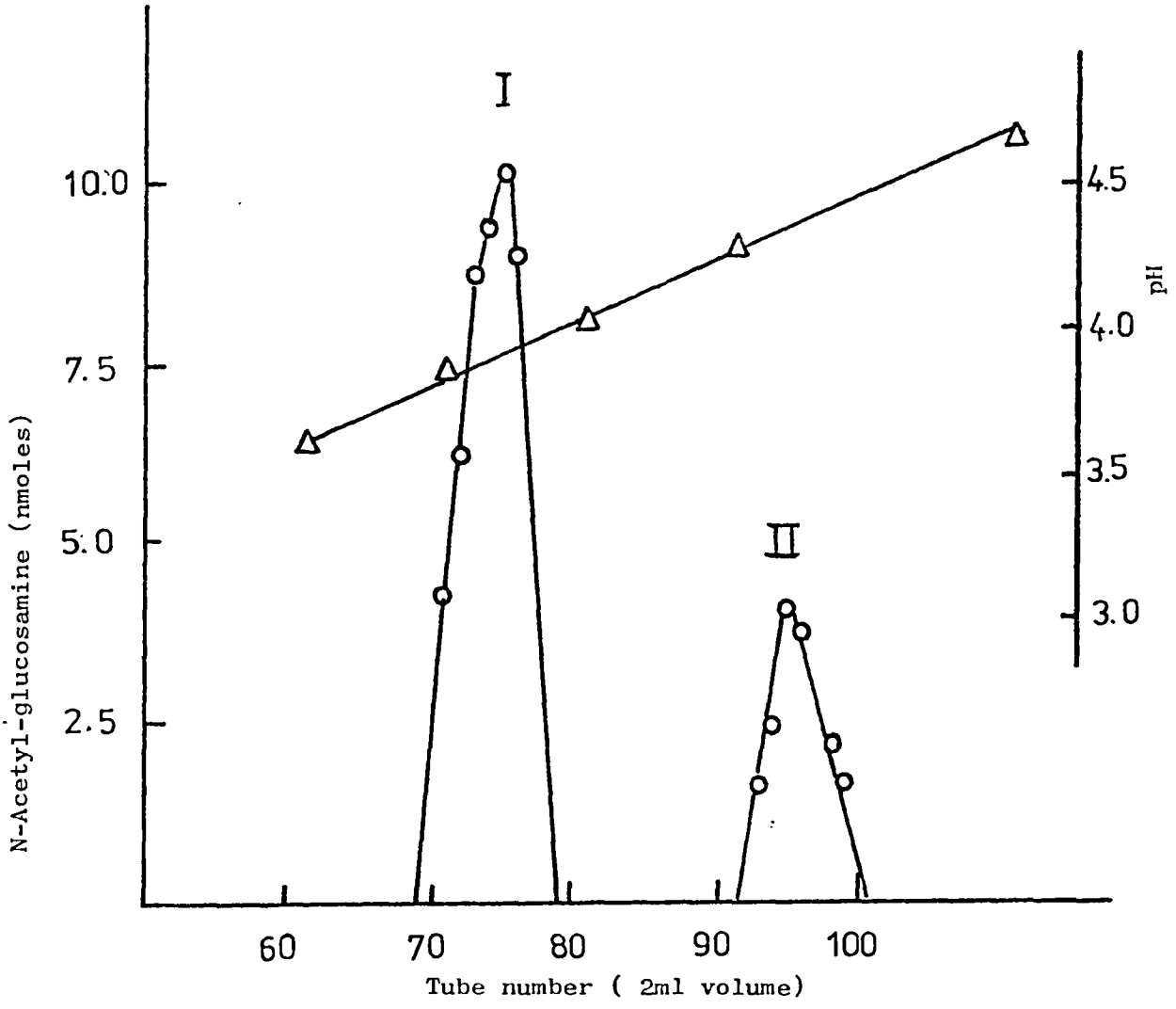


Fig. 51. Chromatography on Amberlite CG-120 column (7x0.9 cm) of a measured aliquot (≡ 200 nmoles of glucosamine-C^{14}) of combined fractions 139-146 from Sephadex G-25 column(Fig.27)

O—O N-Acetyl-glucosamine

△—△ pH

Substance	Glycopeptide I			Glycopeptide II'	
	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	0.97	13*	0.50
Aspartic acid	7.0	14.3	1.07	24	0.92
Threonine	7.4	16.3	1.02	36	1.38
Leucine	6.7	16.4	1.02	25	1.04
Lysine	8.3	13.1	1.01	18.5*	0.77
Glucosamine			1.01[b]		

*These values have been quoted wrongly in a paper (Khalkhali & Marshall, 1976)

(a) The amount of glycopeptide was calculated from the specific activity of UDP-N-acetyl-[^{14}C]-glucosamine 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.

TABLE 20. Amino acid and glucosamine contents of glycopeptides I and II' isolated from tryptic digests of cyanoethylated ribonuclease-GlcNAc-[^{14}C].

The second radioactive fraction (peak II, Fig. 51) contained radioactivity equivalent to 39 nmoles of GlcNAc and was further purified by chromatography on a column of sulphopropyl Sephadex C-25 (for conditions see p. 155). The radioactive peak was then subjected to acid hydrolysis in the usual way and analysed for the amino acid composition (Table 20). The results of the amino acid analysis suggest that it is the peptide from residues 32-37 of the original ribonuclease although the amount of arginine was low.

2. Conclusion

The results of the experiments described in this Chapter show the presence of an enzyme in human serum which can catalyse the *in vitro* incorporation of N-acetyl-glucosamine into ribonuclease A. The bulk of the enzyme was separated from albumin by precipitation at 25-35% saturation of ammonium sulphate. The enzyme was not stimulated by Triton-X-100 which, in fact, acted as an inhibitor at the higher concentrations used.

The transferase activity in serum had similar metal ion requirements to that of liver. However, it showed a sharper optimum at 50m$\underline{M}$ concentration of Mn^{+2}. The enzyme appeared to be stimulated better by spermidine, and other naturally occurring polyamines also stimulated the enzyme. The stimulation promoted by polyamines is perhaps of considerable importance *in vivo*. The polyamines are polycationic substances present in almost all living cells. It has been shown that they act as regulatory agents (Tabor & Tabor, 1964; Baker & Hillegass, 1974a; Bachrach, 1973). It was primarily suggested that polyamines affect the synthesis of ribonucleic

acid and deoxyribonucleic acid-dependent ribonucleic acid polymerase and deoxyribonucleic acid-dependent deoxyribonucleic polymerase respectively, and protein synthesis is also affected. Spermine and spermidine also stimulate *in vitro* the enzymic transfer of the methyl group from S-adenosyl methionine to tRNA. Extracts of rat liver and brain were used as sources of enzyme. The polyamines were considered to be potential regulatory factors *in vivo* for these reactions (Moller *et al.*, 1977).

Studies by Baker & Hillegass (1974b) on the effect of polyamines on the enzymic transfer of galactose from its nucleotide derivative to asialo-ovine submaxillary mucine showed a stimulatory effect caused by spermine. They, by using a microsomal preparation of mucosal lining of dog trachea, observed that the enzyme needs a high concentration of Mn^{+2} ($\sim$30mM) for its optimum activity and that spermine could not replace completely the metal ion requirement but could enhance the incorporation when suboptimal concentrations of Mn^{+2} were used. The activation observed by Baker & Hillegass (1974b) in their experiments was suggested to be due to a decrease in K_m of the enzyme for Mn^{+2} and the large molecular weight acceptor. However, in the present work the polyamine was shown to be able to replace the metal ion requirement of the enzyme. It seems likely therefore that there are two distinctly different mechanisms of activation in the two cases. The mechanism of binding of spermidine with the enzyme is unknown. It is possible that there is an electrostatic interaction which may induce conformational changes in the transferases, thereby facilitating the binding of the enzyme to the acceptor.

One can only speculate at this stage on the significance of the effect of polyamines. They are found to be associated mainly with microsomes (Giorgi, 1970) and this subcellular fraction is probably the site of their action. It is also known, as discussed earlier, that incorporation of sugars occurs partly at the polysomal level (Molnar et al., 1965; Molnar & Sy, 1967), where the enzyme presently being discussed is also found. It is possible, therefore, that spermidine or polyamines in general could play a regulatory effect on the rate of glycoprotein production.

There was also an increase in the activity of the enzyme when CDP-choline was added to the incubation mixtures, the stimulatory effect being maximum at a concentration of ~ 2.4mM. It is not at present known, however, whether this has any significance with respect to the probable requirement of choline for glycoprotein formation by the animal. In early stages of choline deficiency in rats there is an impairment of glucosamine incorporation into plasma glycoproteins (Mookerjea, 1966).

Studies on N-acetyl-glucosaminyl transferase, with unexamined acceptor requirements, present in rat liver showed an increase in the enzymic activity caused by CDP-choline in rough microsomal fraction of rat liver. The stimulatory effect, however, was absent in Golgi-rich membranes and serum (Mookerjea, 1971). The work described in this thesis shows that the N-acetyl-glucosaminyl — asparagine sequon transferase, irrespective of its source, be it serum or liver, is activated by CDP-choline.

The product of the enzymic reaction, monoglycosylated ribonuclease, was separated from the unreacted ribonuclease A by affinity chromatography on a column of wheat-germ agglutinin. It was found that ribonuclease-GlcNAc binds to the lectin affinity column rather weakly. This is due possibly to the fact that ribonuclease-GlcNAc contains only one N-acetyl-glucosamine. It has been shown that N-acetyl-glucosamine binds rather weakly to the lectin compared with larger oligomers (Burger & Goldberg, 1967; Privat et al., 1974; Goldstein et al., 1974). It is also worth noting that at the pH of the eluting buffer (pH 7.0), the monoglycosylated ribonuclease and lectin are positively charged. The isoelectric point of ribonuclease A is pI 7.8 and it is likely that the glycosylated ribonuclease has a similar isoelectric point. Wheat-germ agglutinin is also likely to have a high pI because it does not bind to DEAE-cellulose at either pH 7 or 8.6 (Nagata & Burger, 1974; Allen et al., 1973).

The product of the reaction after purification by affinity chromatography was characterized by its chromatographic behaviour on CM-cellulose, Amberlite CG-50, its relative movement with respect to bromophenol blue in sodium dodecyl sulphate gel electrophoresis and finally its amino acid content after acid hydrolysis. Glycopeptides were also obtained from tryptic digestion and glucosamine was estimated by the Elson-Morgan reaction or on the amino acid analyser. It was shown that in some cases the extent of incorporation was as high as 23% when preparations of fresh human serum were used. The enzyme seemed to be relatively stable because the extent of incorporation

seen with preparations of outdated blood were about 15% under closely similar conditions of incubation.

The occurrence in serum of UDP-N-acetyl-glucosamine — asparagine sequon N-acetyl-β-D-glucosaminyl transferase raises a number of questions, one of which is the source of the enzyme and its relation, if any, to the one in the liver. It has been suggested that some of the serum glycosyltransyl transferases originate from the liver. This assumption is based on the observation that activities seen in serum (N-acetyl-glucosaminyl-, galactosyl- and sialyl transferases) of patients with hepatic disorders are increased (Mookerjea et al., 1972; Kim et al., 1972) while they remain normal in patients with non-hepatic diseases. It is also possible that an enzyme similar to the one in the liver is present on the outer surface of the plasma membrane of a number of types of cells where several other types of glycosyl transferase are believed to occur (Shur & Roth, 1975; Porter & Bernacki, 1975). If this is so its function could be recognition of proteins that contain an unsubstituted asparagine sequon by a mechanism similar to that suggested by Roseman (1970) whereby a cell surface might recognize 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 (Whitehead et al, 1974a). However, a clear understanding of the relationship of this enzyme to the one from liver has been limited by the lack of a purified enzyme preparation from either source. If the enzyme is purified and antibodies are raised against it, the location of the enzyme might be elucidated by immunofluorescence methods.

CHAPTER 6

AN EXAMINATION OF THE UDP-N-ACETYL-GLUCOSAMINE-ASPARAGINE SEQUON N-ACETYL-β-D-GLUCOSAMINYL TRANSFERASE ACTIVITY IN PREPARATIONS OF ROUGH ENDOPLASMIC RETICULUM FROM REGENERATING RAT LIVER

Although preparations of rough endoplasmic reticulum from rabbit liver clearly contained UDP-N-acetyl-glucosaminyl transferase activity, as discussed in Chapter 3, attempts to demonstrate the activity in preparations from rat liver led to equivocal results. When similar quantities of rough endoplasmic reticulum from rat and from rabbit liver were used in the assay for incorporation of N-acetyl-glucosamine into ribonuclease A, the difference in the extent of incorporation seen when ribonuclease A was added to that found in its absence was in the order of 1% with preparations of rat liver (these experiments are discussed in more detail below).

The significance of this was not clear and therefore it was decided to carry out a series of experiments with the intention of examining whether the enzyme activity could be increased by the procedure of partial hepatectomy (for definition of partial hepatectomy see p. 109.

There is an increased rate of synthesis per unit wet weight of liver of some serum glycoproteins by regenerating rat liver (Mutschler & Gordon, 1966; Akamatsu et al., 1976). It seemed possible that the higher rate of production of glycoproteins might involve increased levels of activity compared with normal of the sugar transferases required for the formation of the types of linkage under consideration. There is probably a control exerted in the production of glycoproteins by glucosamine-

phosphate isomerase (glutamine-forming) (E.C.5.3.1.19, Akamatsu & Maeda, 1971), the activity of which is greater in regenerating liver than in normal. In the first experiments liver from normal rats was used.

1. Elucidation of possible occurrence of the enzyme in rat liver

Female Wistar rats (200-250 g) were killed by a blow on the head and the livers were removed, rinsed in ice-cold 0.25M sucrose and weighed. Liver (4 g) was homogenized in two volumes of 0.25M sucrose, and rough endoplasmic reticulum (see Fig. 10) was prepared by Dallner's method and finally washed in 0.9% KCl. It was then suspended in Tris-maleate buffer (0.05M in both, adjusted to pH 7.1 with NaOH) to equivalent concentration of 1 g liver/0.2 ml.

Incubations were performed both in the absence and presence of added ribonuclease A. The incubation mixtures contained in a volume of 0.1 ml the following: Ribonuclease A, 100 µg; Mn^{+2}, 50mM; buffer 50mM (Tris-maleate, pH 7.1); Triton X-100, 0.4%; UDP-N-acetyl-glucosamine, 0.506mM (1096 dpm/nmole) and rough endoplasmic reticulum corresponding to 200 µg protein (the amounts of rough endoplasmic reticulum from 1 g wet weight liver are shown in Table 10). The incubations were carried out for 1 h at 37°C. They were terminated by the addition of 1% phosphotungstic acid in 0.5M HCl and filtered on millipore filters. The radioactivity incorporated into acid precipitable material was measured by scintillation counting. There was incorporation into endogenous protein, and a small increase when ribonuclease A had been added to the incubation mixture. Values found in a number of experiments were (a) 123 pmoles and 105 pmoles in the presence and absence of ribonuclease A

respectively, (b) 108 pmoles and 84 pmoles respectively in the two cases, (c) 114 pmoles and 90 pmoles and (d) 113 pmoles and 89 pmoles. The differences observed are equivalent to about 0.003 moles glucosamine per mole of ribonuclease A (0.3% conversion of ribonuclease A to the glycosylated form). The meaning of the results obtained, with relatively small differences, is not fully clear. A preparation of rough endoplasmic reticulum from rabbit liver gave rise to conversion of about 5.7% of the ribonuclease A to its glycosylated form.

The results of these experiments suggested that rat liver contained much lower enzymic activity than rabbit liver.

2. The enzymic activity during liver regeneration

Partial hepatectomy was performed under ether anaesthetic according to Higgins & Anderson (1931) (see p. 109). Sham operation was also performed by laparotomy and palpation of the livers. The animals were killed by decapitation within 24, 48 and 72 h after partial hepatectomy or sham operation (the weights of the livers are given in Table 10). The preparations of rough endoplasmic reticulum were obtained from 4 g of liver according to Dallner's method and suspended in Tris-maleate buffer 0.05M, pH 7.1 to a concentration equivalent to 1 g liver/0.2 ml. The protein was estimated by Lowry's method (Lowry et al., 1951).

Incubations were performed using the preparations of the enzyme under the assay conditions mentioned above. The incubations were carried out at 37°C for 1 h and terminated by the addition of 1% phosphotungstic acid in 0.5M HCl. The precipitates were counted in a scintillation counter. Corrections

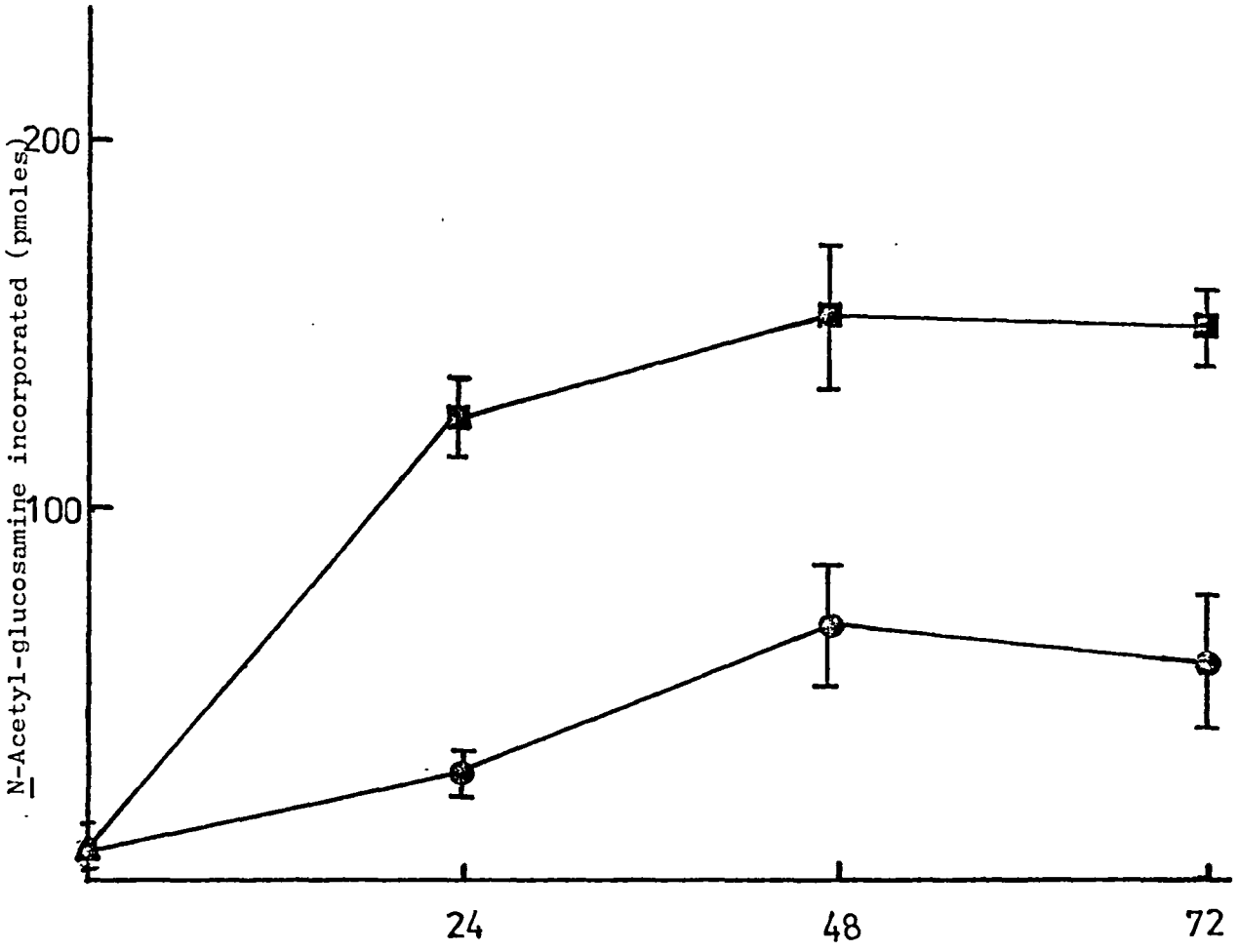


Fig. 52. N-acetyl-glucosaminyl transferase activity of preparations of rough endoplasmic reticulum made from regenerating rat liver (■—■) or from livers of sham operated animals (●—●). Results with control animals are represented as ▲ . Incubation conditions are given in the text (see p.227)

were made for protein content and the efficiency of the counter. The results are all given in Fig. 52. As is shown the specific activity of the enzyme is greater during liver regeneration than in preparations from sham operated rats. The maximum extent of incorporation obtained under the above-mentioned conditions, apart from using only 100 µg of rough endoplasmic reticulum protein, was obtained from preparations of rough endoplasmic reticulum made from rat liver 48 h after partial hepatectomy. There was $\sim$ 3% conversion of ribonuclease A to its glycosylated form under the conditions used.

3. Factors affecting enzymic activity in preparations of rough endoplasmic reticulum from regenerating rat liver

In these experiments preparations were made from rat liver 48 h post-hepatectomy.

(i) Effect of pH on the incorporation rate

The effect of pH on the incorporation rate was investigated by carrying out incubations as described above with 250 µg rough endoplasmic reticulum protein. The pH of the incubation mixtures varied from 5 to 8.5. As may be seen in Fig. 53, the peak of activity appears at around pH 6.

(ii) Effect of enzyme concentration on the extent and rate of incorporation

The amount of rough endoplasmic reticulum protein used in this experiment ranged from about 10 to 500 µg. The conditions of incubation were as before and the pH of the mixture was 7.1. Incubations were performed both in the presence and absence of ribonuclease A. The results are described in Fig. 54 and show

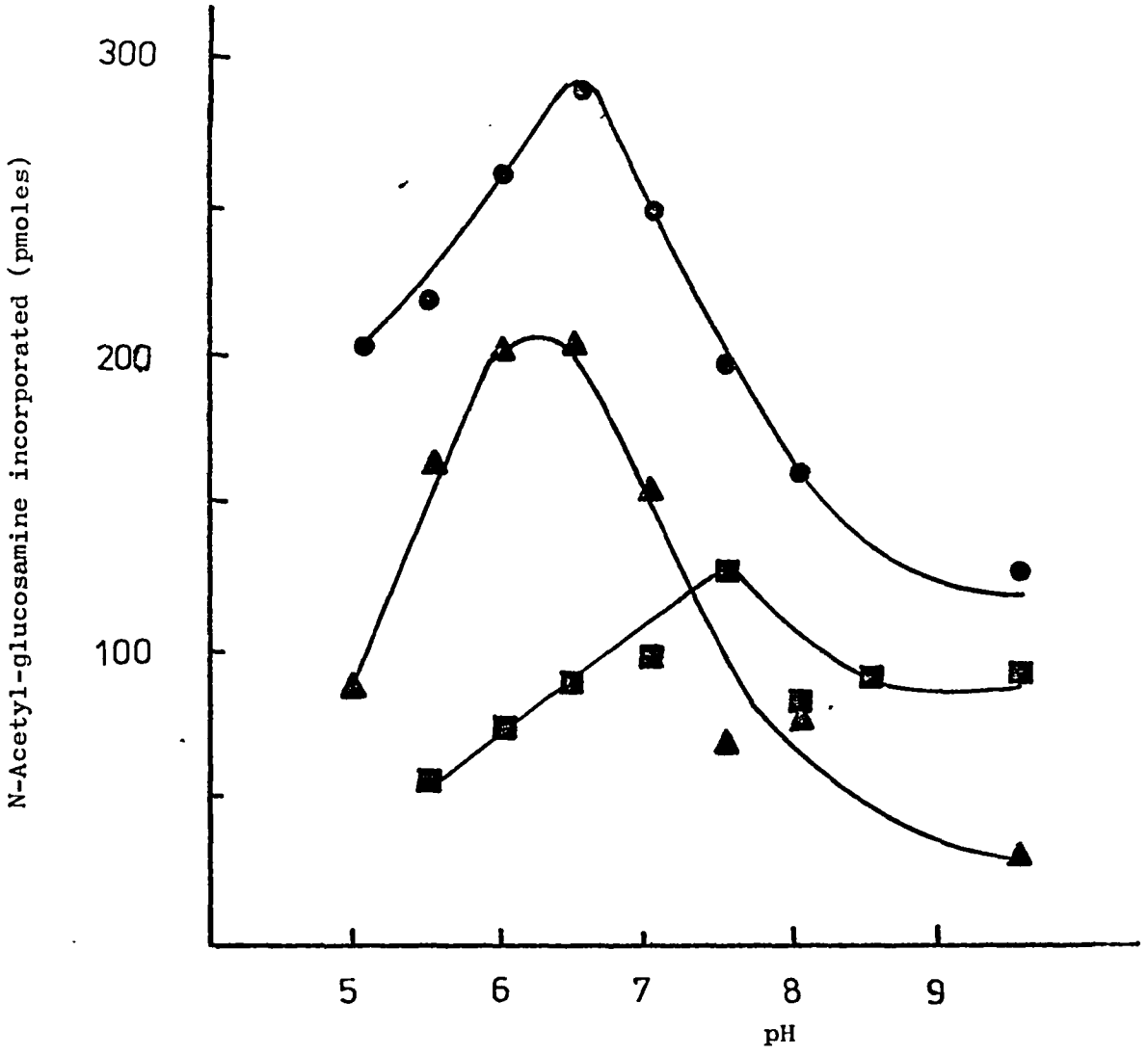


Fig. 53. Effect of pH on the extent of incorporation of N-acetyl-glucosamine into ribonuclease A using preparations of rough endoplasmic reticulum from regenerating rat liver 48 h after partial hepatectomy.

●—● Ribonuclease present in the mixture

■—■ Incubations performed in the absence of ribonuclease

▲—▲ The difference curve

that the incorporation rate increased as the enzyme protein concentration rose. This was observed in both cases, where added ribonuclease A was present or absent in the incubation medium. The difference curve, which is assumed to measure the incorporation into added ribonuclease A, also shows an increase relative to the protein concentration. The increase in the incorporation rate is not directly proportional to the amount of enzyme protein. Results for preparations from rabbit liver are included in Fig. 54b for comparative purposes. It is possible to compare the transferase activity of preparations of rough endoplasmic reticulum from normal rabbit liver, from rat liver 48 h after partial hepatectomy and from sham operated animals by considering the relative extents of conversion of ribonuclease A to its glycosylated form in the presence of relatively small amounts of enzyme protein (for example 20 μg), and it is suggested that the rough endoplasmic reticulum from the hepatectomized animal was about 4- to 5-fold as active as that from the laparatomized rat. There was even higher enzymic activity in preparations of rough endoplasmic reticulum from rabbit liver (Fig. 54b) which was tested at lower concentrations (0.28mM) of UDP-N-acetyl-glucosamine than was used in the experiments with preparations from rat liver. Activities of rabbit liver preparations in the presence of 100 μg of rough endoplasmic reticulum protein at concentrations of UDP-N-acetyl-glucosamine of 0.1, 0.2, 0.28, 0.4 and 0.5mM were in the proportions 0.54, 0.84, 1.0, 1.16 and 1.26 respectively.

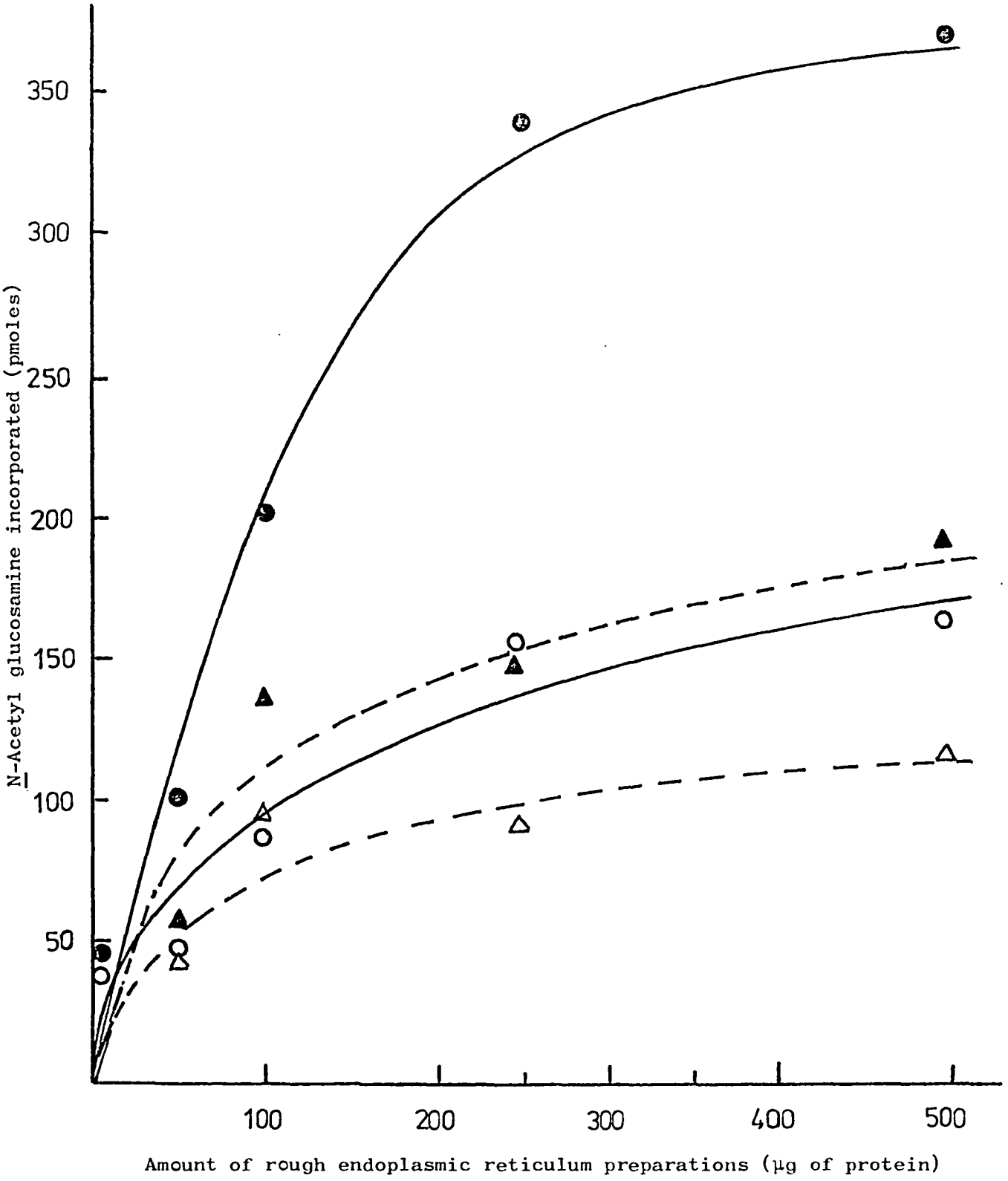


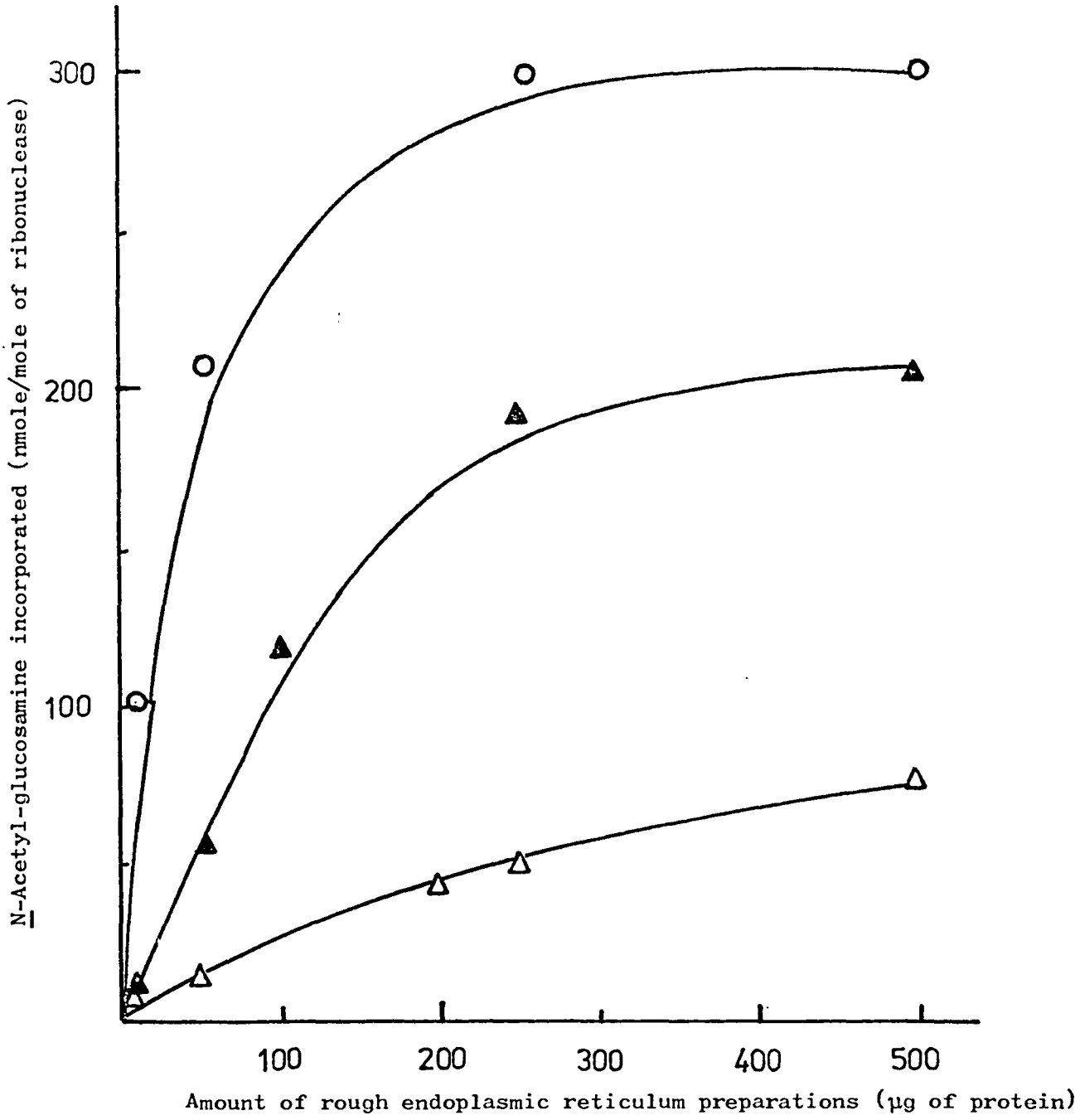
Fig. 54a. Effect of enzyme protein concentration on the incorporation of N-acetyl-glucosamine into acid precipitable material, using preparations of rough endoplasmic reticulum from rat liver.

●—● Incubations performed using preparations of regenerating rat liver 48 h after partial hepatectomy, with added ribonuclease A.

○—○ Without addition of ribonuclease A

▲—▲ Incubations performed using preparations of sham operated rat liver 48 h after operation, with added ribonuclease A.

△—△ Without added ribonuclease A.



▲—▲ 48 h after partial hepatectomy

△—△ Sham operated rat liver 48 h after operation

O—O rabbit liver, shown as a comparison

Fig. 54b. Incorporation of N-acetyl-glucosamine into ribonuclease A catalysed by preparations of rough endoplasmic reticulum from regenerating rat liver. The concentration of UDP-N-acetyl-glucosamine in the experiments with rabbit liver preparations was 0.28mM.

(iii) Effect of substrate concentration on the activity of the enzyme

In this experiment the variable factor was the concentration of ribonuclease A which varied from 20 to 1,000 µg per assay tube, but apart from that the conditions of incubation were as before but with 200 µg enzyme protein. The results obtained (Fig. 55) suggest that, at 37°C, K_m for ribonuclease is of the order of 2.2×10^{-5} M.

(iv) Effect of temperature on the rate of incorporation

The effect of temperature on the incorporation rate was investigated by incubating ribonuclease A with UDP-N-acetyl-glucosamine in the presence of Mn^{+2} and the enzyme protein at various temperatures. The molar concentration of the components was the same as stated (p.227). Control incubations were also performed having no added ribonuclease A in the mixture. As may be seen (Fig. 56) there is an increase in the rate of incorporation with increase in temperature, reaching a maximum around 37°C for both endogenous and exogenous acceptors.

4. Identification of the product of glycosylation of ribonuclease A

In the experiments described so far, it has been assumed that the differences obtained in incorporation of N-acetyl-glucosamine into acid precipitable protein in the presence or absence of the added ribonuclease A could be interpreted as an indication that the latter was indeed the acceptor of the sugar. This reasonable assumption was confirmed by isolation and identification of the glycosylated ribonuclease.

Ribonuclease A (1 mg) was incubated with UDP-N-acetyl-

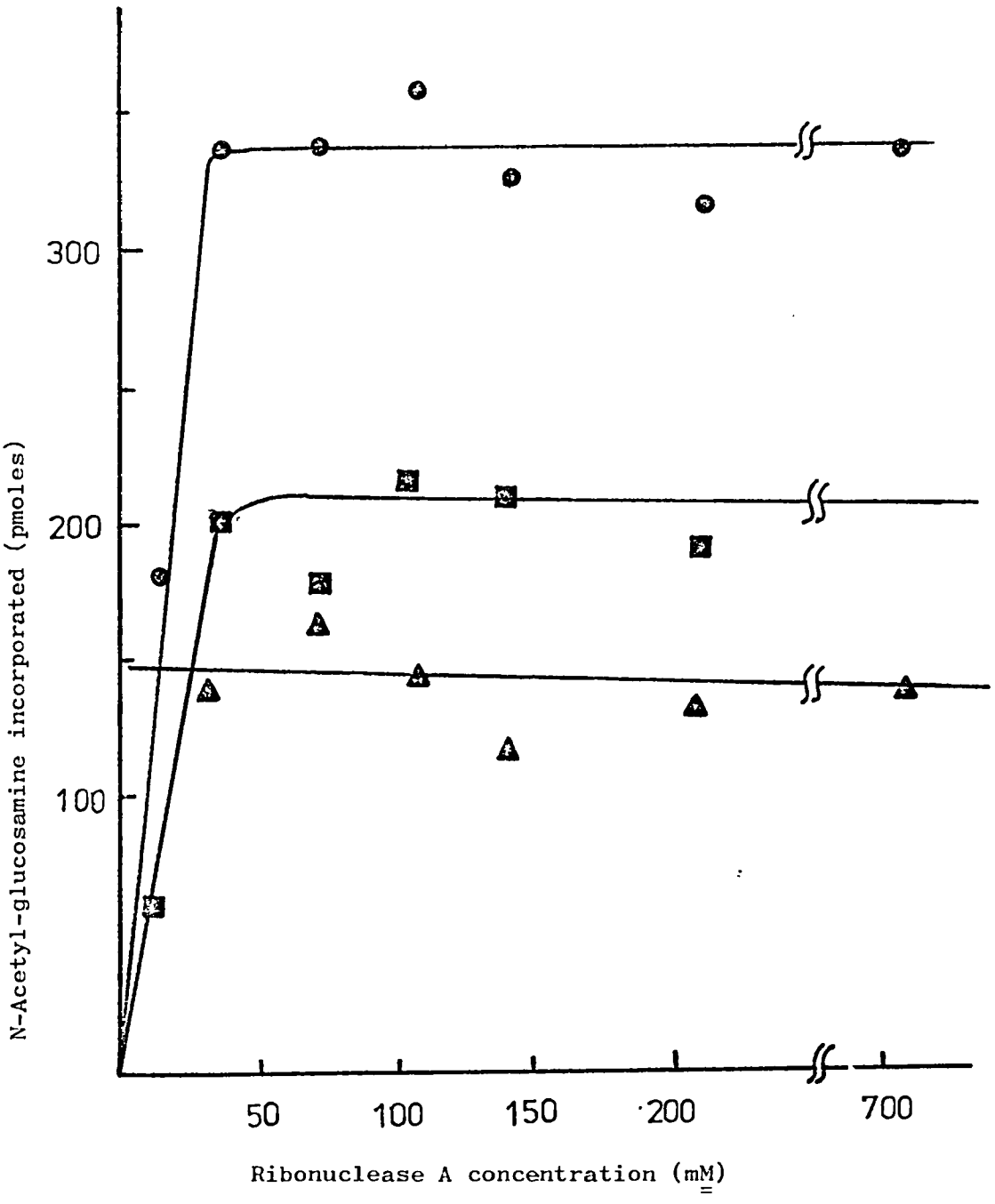


Fig. 55. Effect of ribonuclease A concncentration on the rate of incorporation of N-acetyl-glucosamine.

- Ribonuclease A was added to the mixture
- Ribonuclease A was omitted from the mixture
- The difference curve

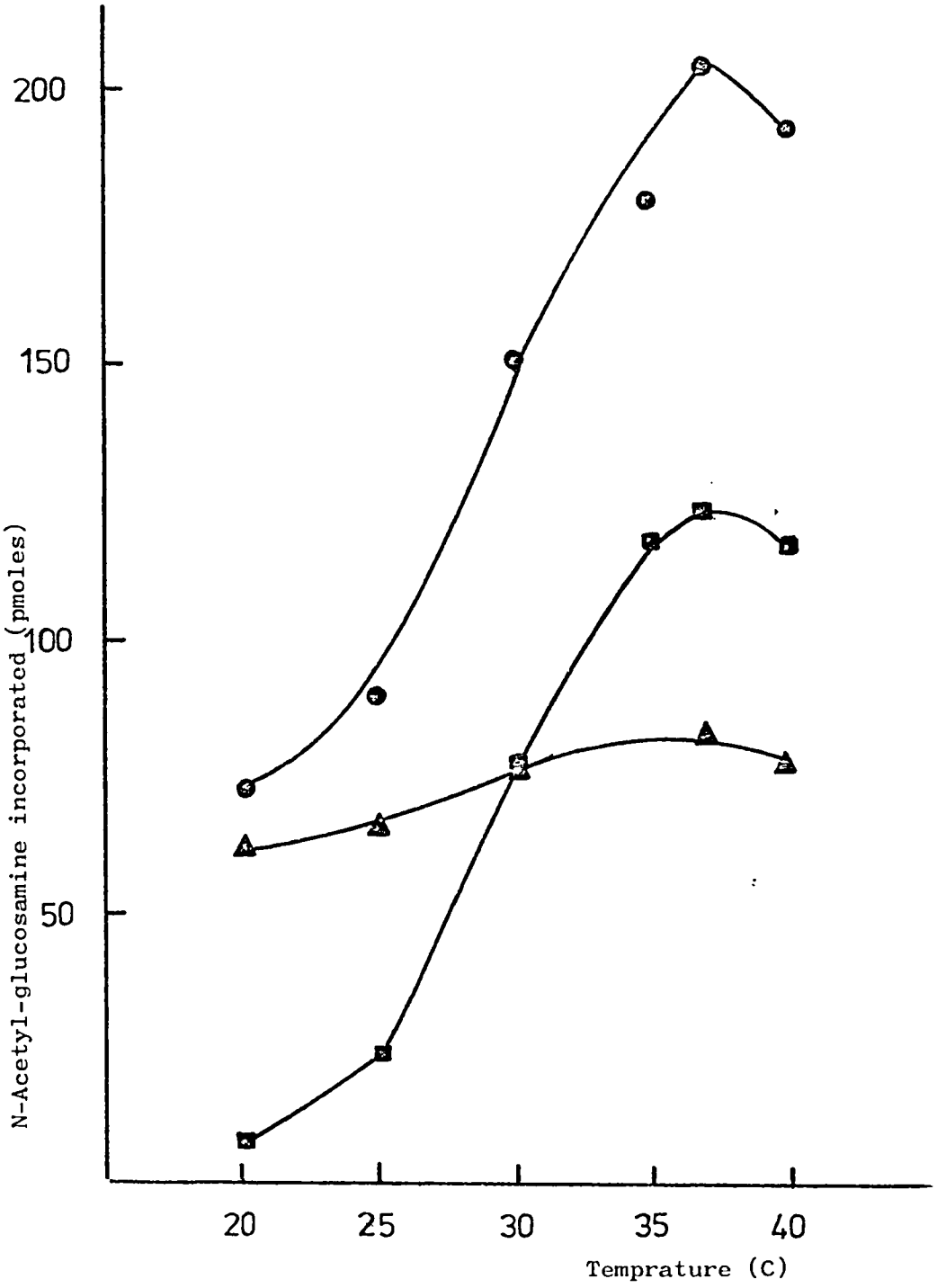


Fig.56. Effect of temprature on the incorporation of N-acetyl-glucosamine into ribonuclease A.

●——● Ribonuclease was added to the mixture
▲——▲ Incubations were carried out in absence of ribonuclease A
■——■ The difference curve

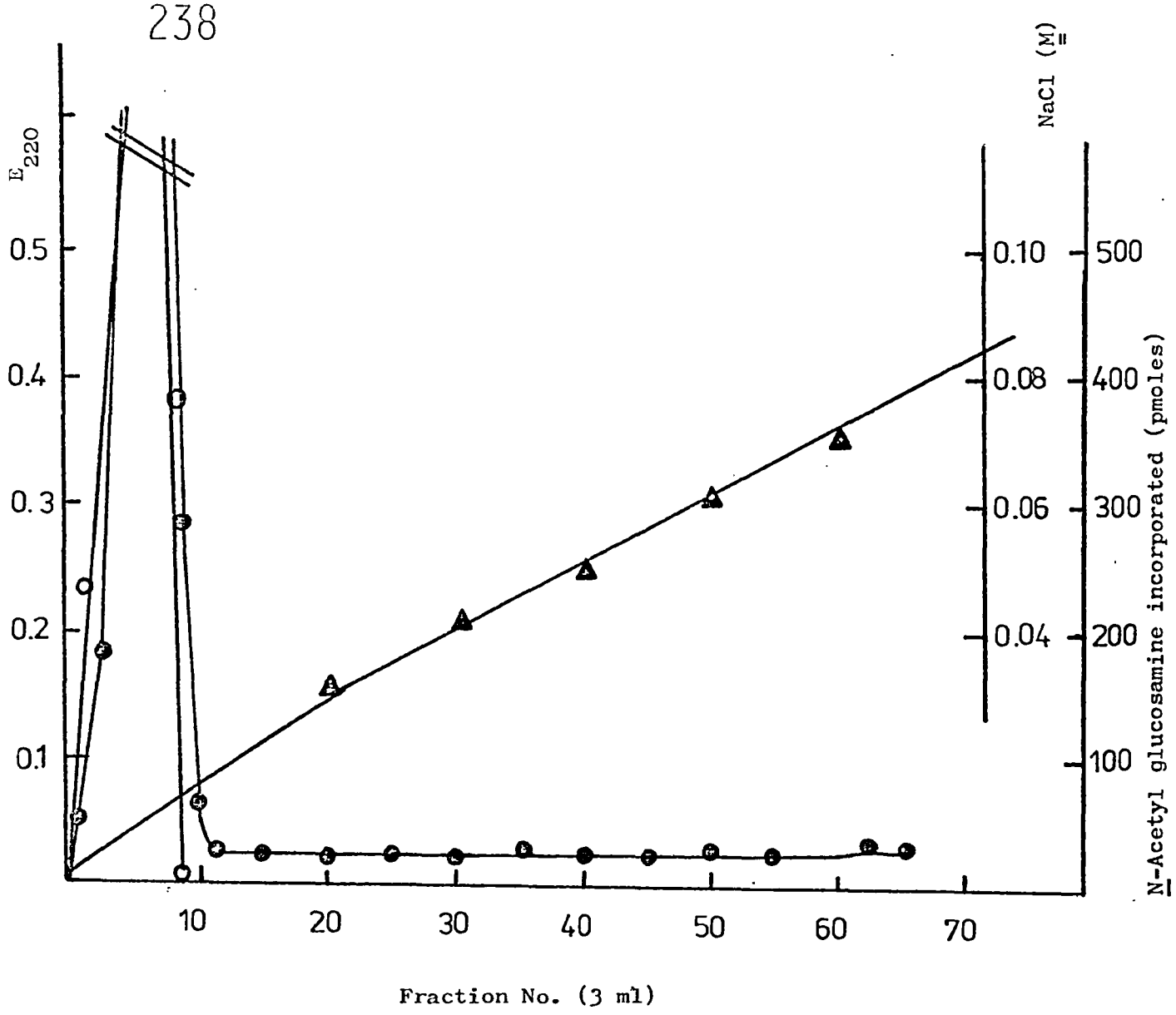


Fig. 57a. Chromatography on CM-cellulose (0.9 x 9 cm) of an incubation mixture containing a preparation of rough endoplasmic reticulum from regenerating rat liver 48 h after hepatectomy and no added ribonuclease A (for conditions of assay see text)

O—O N-acetyl-glucosamine incorporated (pmoles)

●—● E_{220}

▲—▲ NaCl gradient

glucosamine under the following conditions. The incubation mixture contained in a final volume of 1 ml: UDP-N-acetyl-glucosamine, 0.506mM containing 1096 dpm/nmole; Mn^{+2}, 50mM; Triton X-100, 0.4%; buffer (Tris-maleate pH 7.1), 50mM. The enzyme protein added was 1 mg of the rough endoplasmic reticulum prepared from the rat 48 h after hepatectomy. A control experiment was also performed simultaneously which had identical components with the one mentioned above, except that ribonuclease A was omitted from the mixture. Incubation was for 1 h at 37°C, the mixtures were frozen at -80°C and centrifuged at 2,000 g for 15 min after being thawed. The supernatants were chromatographed separately on a CM-cellulose column (see Fig. 57a,b). The chromatography of the control incubation mixture with no added ribonuclease A revealed no ultraviolet absorbing material in the region where ribonuclease A is normally eluted (Fig. 57a). Radioactivity measurements also showed no incorporation in that region of the chromatogram. When the incubation mixture containing ribonuclease was chromatographed (see Fig. 57b), it was found that there was radioactivity present in fractions 50-78 and the peak of activity was again eluted immediately before and overlapping ribonuclease A. Radioactivity measurements revealed 2.5% conversion of ribonuclease A to its glycosylated product (1752 pmoles GlcNAc). Fractions 50-70 (see Fig. 57b) were combined, dialysed and the retentate in the dialysis bag was freeze dried. The residue was subjected to affinity chromatography (see p.241) on wheat-germ agglutinin column. The peak of radioactivity was eluted at a concentration of N-acetyl-

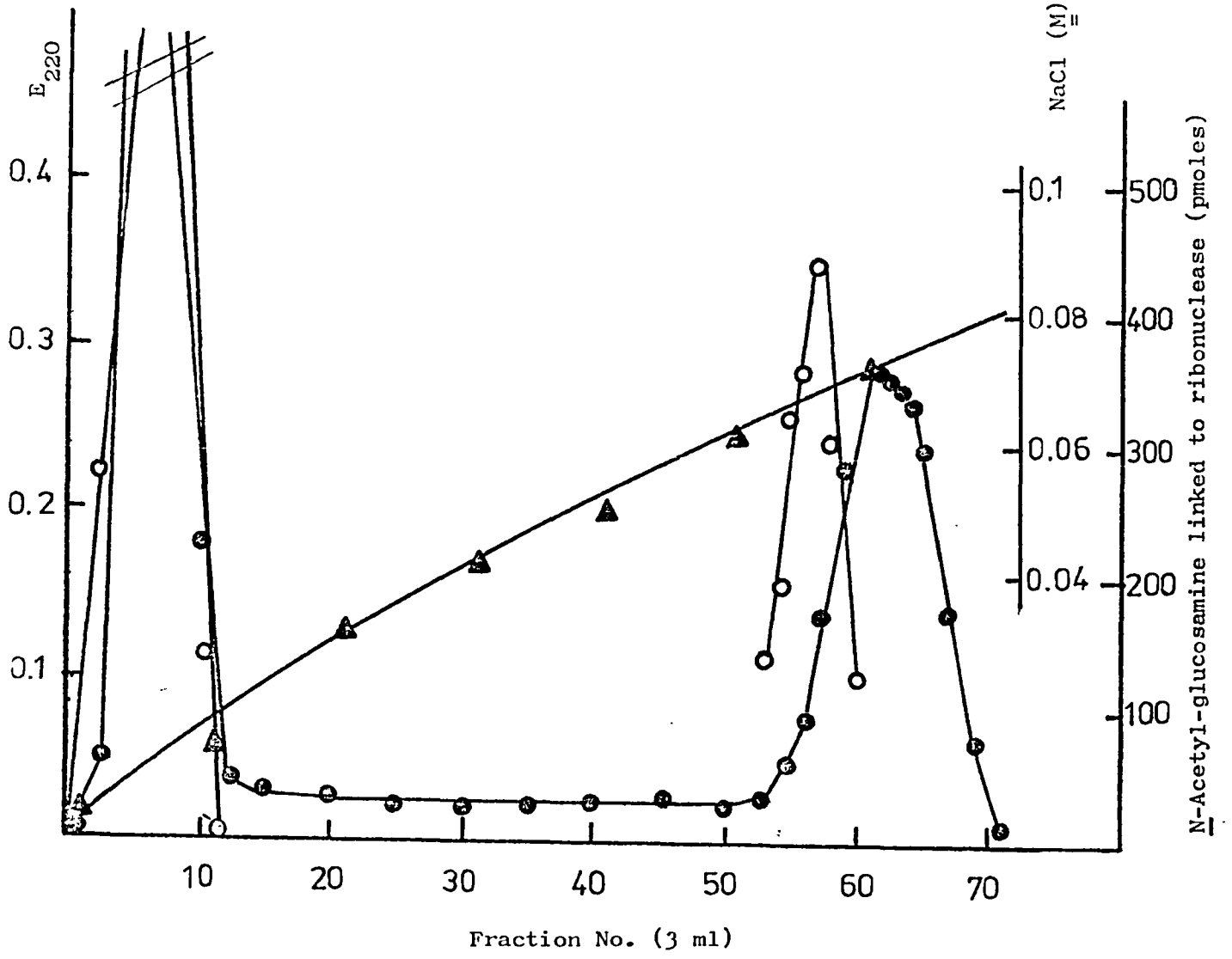
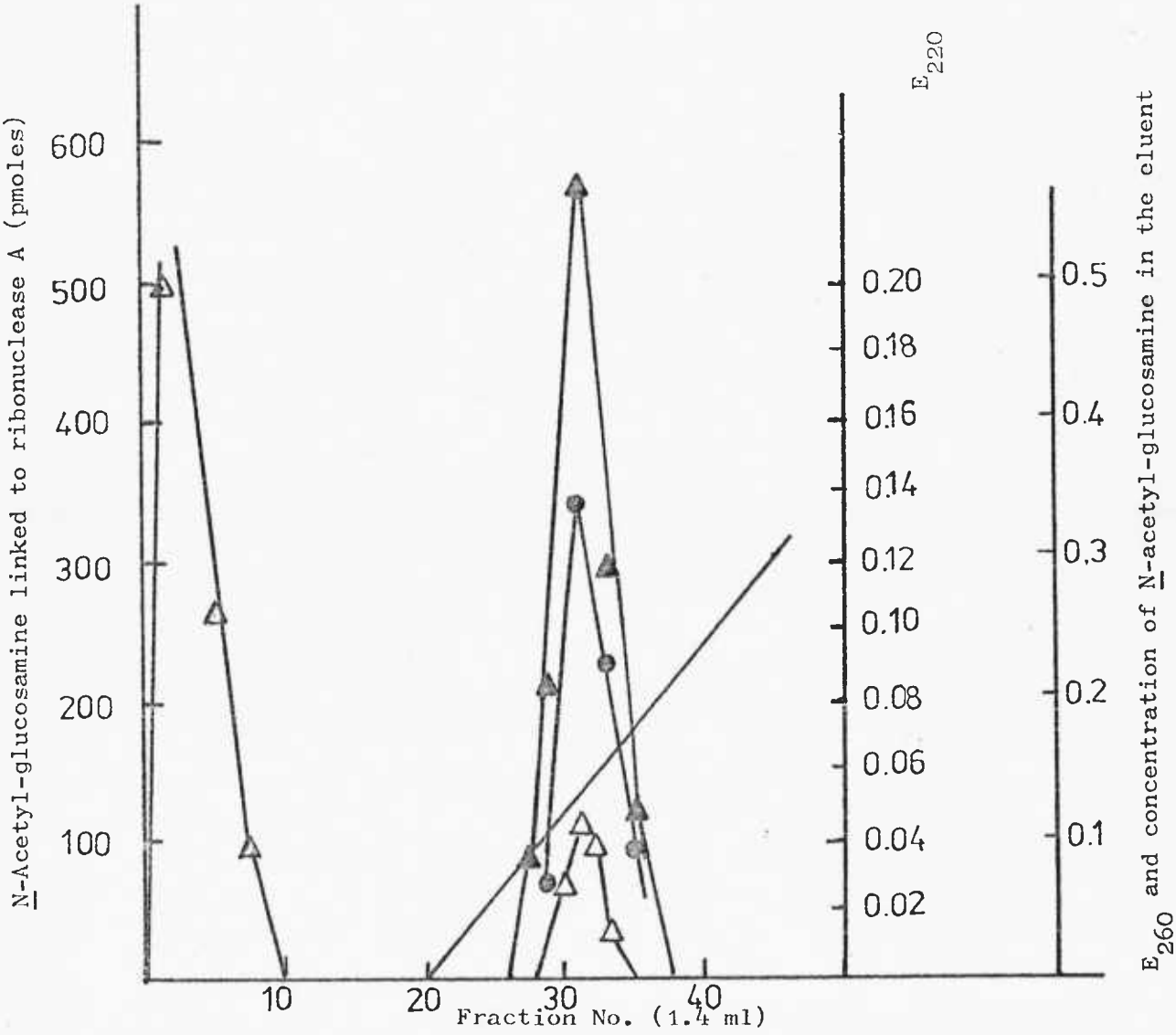


Fig 57b. Chromatography on CM-cellulose (0.9 x 9 cm) of a mixture containing ribonuclease A (1 mg) and preparation of rough endoplasmic reticulum from regenerating rat liver 48 after partial hepatectomy.



△——△ E_{220}

●——● N-acetyl-glucosamine incorporated into ribonuclease

▲——▲ Ribonuclease activity (E_{260}; Kalnitsky et al., 1959) of monoglycosylated ribonuclease

_____ Concentration of eluting sugar (N-acetyl-glucosamine)

Fig. 58. Separation by chromatography on a column of wheat-germ agglutinin-Sepharose of ribonuclease A and N-acetyl-glucosaminyl-ribonuclease

glucosamine of about 0.15mM. The radioactivity associated with this peak amounted to 1475 pmoles of N-acetyl-glucosamine. The material in this region was able to catalyse hydrolysis of ribonucleic acid as estimated by Kalnitsky's method (Kalnitsky et al., 1959).

A portion of glycosylated ribonuclease (0.3 ml of fraction 31 from the wheat-germ agglutinin Sepharose column (Fig. 58)) was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) using a reference sample of non-glycosylated ribonuclease. The gels were stained with Coomassie blue and cut into 3 mm slices which were disolved in 2 ml of 30% v/v H_2O_2 in scintillation vials (Goodman & Matzura, 1971). The solutions were evaporated to dryness, and to the residues were added 10 ml scintillation fluid. The vials were counted for radioactivity in a scintillation counter. Radioactivity was only observed in the slices which corresponded to the position of the control ribonuclease A. The radioactivity corresponded to 70 pmoles GlcNAc = 94% recovery.

5. Summary

The results of these experiments demonstrate that the level of the enzymic activity in preparations of rough endoplasmic reticulum from rat liver is extremely low. The reason for the low levels of the enzymic activity in rat liver is not known, although it is known that rat liver synthesises glycoproteins (see Molnar, 1975). Moreover, recent findings of Brown et al. (1977) show the presence in rat milk of a glycosylated form of α-lactalbumin which contains ~13.4% carbohydrate. Rat

lactalbumin is unique in that it is a glycoprotein and exists in multiple forms. Most α-lactalbumins do not contain carbohydrate, although there is good evidence (Hopper & McKenzie, 1973) for a minor form of bovine α-lactalbumin which contains carbohydrate. All multiple forms are glycoproteins and are active in the lactose synthetase reaction.

It is of interest that orosomucoid in rat serum contains relatively low levels of carbohydrate (23%) (Charlwood et al., 1976) compared with that of rabbit serum (44%). Whether the low level of carbohydrate in orosomucoid of rat is due to low levels of transferase remains unknown.

However, when rats were subjected to partial hepatectomy the enzymic activity became apparent. The rise in enzymic activity reached a maximum in the regenerating liver 48 h after partial hepatectomy. A similar rise was observed when sham operated animals were used, but the increase in the enzymic activity was not so large as it was in the hepatectomized animals. It is difficult to know whether the increase observed in vitro parallels changes in activity in vivo, mainly because there may well be several additional activators or controlling inhibitors present in the latter case. CDP-Choline, which was shown in Chapters 3 and 5 to act as an activator for the enzyme in rabbit liver and human serum under the experimental conditions used, may play a significant regulatory role. According to Schneider (1971), the level of CDP-choline rises in liver after regeneration. He measured the CDP-choline of rat liver by an isotope dilution technique and reported a value of 3.38 μmoles per 100 g liver (wet weight) for normal,

compared to a value of 6.74 μmoles per 100 g liver 12 h after partial hepatectomy. The amount 24 h after partial hepatectomy was somewhat smaller (~ 5 μmoles/100 g liver).

Care is needed in interpreting the data presently obtained, because it is not impossible that, under the assay conditions used, UDP-choline was functioning as an inhibitor for enzymes which may have been present and which might be involved in hydrolysis of the nucleotide sugar. This is an important aspect for further studies.

Another significant activator for the enzyme, spermidine, might also be involved in regulating the level of the enzymic activity. The conversion of ornithine to putresine followed by condensation with the product derived from the decarboxylation of S-adenosylmethionine in animal cells yields spermidine. The first step of the reaction, conversion to putresine, is controlled by the enzyme ornithine decarboxylase. This is the first and probably the rate-limiting step in biogenesis of polyamines (see Bucher & Malt, 1971). The enzymic activity in regenerating liver increases very markedly (10- to 70-fold during the first few hours), the extent of conversion to putresine increases 60-fold in 4 h and remains high for several hours. The increase in spermidine is ~ 2-fold after 48 h. It is therefore quite possible that this increase in the level of spermidine could stimulate the N-acetyl-glucosaminyl transferase present in the rough endoplasmic reticulum. This, of course, is unlikely to explain the increased activity observed in the present experiment, the assays for which were done *in vitro*.

It is difficult at this stage to deduce any significant implications from these findings. Much more information is needed about the mechanism of action of the transferase in question.

It is of interest that the rate of production of glycoproteins per unit weight of regenerating liver is greater than in normal liver (Akamatsu et al., 1976), although the absolute rate of incorporation of glucosamine into glycoproteins secreted by the liver is reduced (Serafini-Cessi, 1976).

CHAPTER 7

CONCLUDING REMARKS

The mechanism of assembly of sugar moieties of glycoproteins has been the subject of numerous studies. However, the matter is still but poorly understood. There is considerable evidence for the view that incorporation of some of the sugar moieties occurs while the nascent protein is still associated with the ribosomes (Simkin & Jamieson, 1967; Redman & Cherian, 1972; Parkhouse & Melchers, 1971; Melchers, 1973; Kiely et al., 1976). Indeed the release by puromycin in vitro of some of the acid-insoluble [^{14}C]-glucosamine incorporated into rat liver ribosomes in vivo (Lawford & Schachter, 1966) suggests that incorporation of some sugars occurs even before the polypeptide chain is completed.

Equally, there is evidence that carbohydrate moieties are first assembled on lipid and then transferred directly to endogenous proteins or glycoproteins. Whether an asparagine residue of the protein, or even the polypeptide chain itself, acts as acceptor is not certain (see Waechter & Lennarz, 1976), although highly suggestive evidence has been presented recently (Pless & Lennarz, 1977) in one case.

The present studies have revealed the presence of enzymic activity in human serum and in endoplasmic reticulum from rabbit liver, regenerating rat liver, and from yeast cell membrane which can catalyse the incorporation of N-acetyl-glucosamine into the asparagine residue of a sequon of an appropriate substrate such as ribonuclease A.

Subcellular fractionation of rabbit liver revealed the presence of the enzyme mainly in the rough endoplasmic reticulum. However, there was also activity associated with the smooth endoplasmic reticulum and the post-mitochondrial fraction. So far, no sugar transferase activity associated with the membrane of endoplasmic reticulum has been shown to be localized exclusively in the rough or smooth fractions. Mannosyl transferase was originally thought to be present only in the rough microsomes (Redman & Cherian, 1972; Wagner & Cynkin, 1971; Molnar et al., 1969), but it was later shown that the apparent absence of the enzyme in the smooth membranes was artifactual and was due to the presence of zinc-independent α-mannosidase activity associated with the Golgi fragments contaminating this fraction (Dallner & Hemming, 1974). After removal of the Golgi fragments, the mannosyl transferase activity in the smooth membranes became apparent.

In the present studies the possibility that the enzymic activity found in the smooth endoplasmic reticulum of rabbit liver resulted from contamination of the latter with rough endoplasmic reticulum must be considered. The relatively high specific activity of the post-mitochondrial fraction which is generally believed to contain nuclei, cell debris, mitochondria, unbroken cells, as well as rough and smooth microsomes, raises some questions as to the occurrence of the enzyme in other subcellular fractions such as plasma membranes. Both of these need further investigation.

The enzyme in the preparations from liver exhibits some aspects of behaviour as other membrane-bound enzymes. It could be released

by the action of non-ionic detergent such as Triton X-100, which appears to stimulate its activity. The function of Triton X-100 as stimulator could simply be the removal of the barrier permeability and hence rendering free access of the substrate molecules to the active site. It appears that the concentration of the detergent required for maximum activity of the enzyme is not highly critical, as there is no sharp maximum; but other membrane-bound enzymes differ in this respect. Thus rat liver glucose-6-phosphatase, when treated with a detergent such as deoxycholate shows a sharp maximum at $\sim$0.03% (Ernster & Jones, 1962). Behrens et al. (1971) have also shown that the dependence of glucosyl transferase activity from rat liver on Triton X-100 and deoxycholate concentration has a sharp maximum.

The liver enzyme in question appeared to require a high concentration of metal ions for the expression of its full activity, but as discussed below, it seems possible that spermidine might function equally well or even better. The reason for the requirement of a high concentration of metal ions is not clear. Most other glycosyl transferases so far studied appear to require relatively low concentrations of metal ions for expression of maximum enzymic activity. It is possible that there is an electrostatic interaction between the metal ions and the enzymes causing conformational changes in the transferase molecule, changes which expose the active sites. However, at this stage it is difficult to suggest a plausible mechanism for the action of the transferase due to the difficulties of obtaining a pure enzyme. The stability of the liver enzyme seems to be very dependent on its topographical environment, because when the

enzyme is released from the membrane it rapidly loses its activity.

The enzyme in serum exhibited similar metal ion requirements, although it was equally stimulated by naturally-occurring polyamines; among them spermidine appeared to be the best. The action of spermidine on the enzyme activity could result from a similar electrostatic interaction to that suggested for metal ions, because polyamines are polycationic substances. However, the specificity of metal ion or polyamine requires explanation. Spermidine might function as an *in vivo* regulator of the enzymic activity in this way in addition to its effects on other cellular control mechanisms (Bachrach, 1973; Moller *et al.*, 1977).

It is of interest that the enzymic transfer of *N*-acetyl-glucosamine into ribonuclease A does not go to completion. Even in the most extensive conversions seen, only 23% of ribonuclease A appeared to undergo glycosylation, and in many experiments the extent of conversion was much less. There may be product inhibition by the glycosylated ribonuclease, but there is presently no direct evidence on this point.

Ribonuclease appears to be only partially glycosylated in many species (see Table 6) such as cattle, sheep, roe deer and guinea-pig, but completely glycosylated in other species such as coypu, chinchilla, pig and horse. It is non-glycosylated in rat and red deer. In at least some of these ribonucleases the requisite sequon occurs and, as shown in the present studies, ribonuclease A may be catalytically converted to a glycosylated form. This might be due to the lack of sufficient transferase in the pancreatic cells of some species, leading to product inhibition.

There is another factor which needs consideration and which might explain why both rat and coypu do not carry a carbohydrate moiety at asparagine residue 97 and 94 respectively where an apparent asparagine sequon occurs (see Table 6). At this site the intermediate amino acid in the sequon is in each case cystine. The initial protein chain must have been formed with a cysteine residue in this position and, while Asn-Cysteine-Ser(Thr) might act as acceptor for N-acetyl-glucosamine, it seems reasonable to speculate that the protein containing Asn-Cystine-Ser(Thr) might not be the substrate for the transferase. It seems possible that disulphide bridge formation may have occurred too rapidly to have allowed the original sequon to be acted upon by the sugar transferase. This problem presents itself in connection with the similar non-glycosylated asparagine sequon occurring in the same region of the pancreatic ribonucleases of a number of species (Table 6).

There is a further sequon, Asn-Thr-Thr, in rat pancreatic ribonuclease at position 101-103 which is not glycosylated. Is this because the level of transferase activity in the pancreas of the rat is also at a low level, as seen in the liver, or are other presently unresolved problems of the acceptor substrate involved?

Other proteins with potential sites for glycosylation also set similar problems. In the collagen of the gooseberry sawfly (N-ribesii) the large number of hydroxylysine residues are not glycosylated which might suggest the absence in the silk glands of the sawfly larvae of the appropriate galactosyl transferase (Spiro et al., 1971). When the collagen is incubated with kidney

galactosyl transferase and UDP-galactose under appropriate conditions it shows a high acceptor activity.

The studies of Pless & Lennarz (1976) on glycosylation of denatured proteins are of interest. These authors used the products obtained by sulphitolysis of bovine ribonuclease A and other proteins as acceptors. Their enzyme source was a preparation of hen oviduct membrane, and their results showed that transfer of an oligosaccharide from an oligosaccharide lipid intermediate occurred. Although the site of attachment to the ribonuclease A was not established, the product nevertheless behaved as ribonuclease B upon electrophoresis in two systems. In the present studies lipid intermediates have not been investigated, but it seems reasonable to consider whether there are two mechanisms for the synthesis of the carbohydrate moiety *in vivo* which may not be mutually exclusive. The problem remains unsolved and its solution may have to await the isolation of pure enzyme. This could possibly be obtained from serum where the enzyme circulates in a soluble and apparently stable form. In some preliminary experiments the bulk of the serum enzyme was separated from albumin by ammonium sulphate fractionation. The purification procedure might be further extended by conventional chromatographic techniques.

Several glycosyl transferases, especially those in soluble form, have been purified to homogeneity. Whitehead *et al.* (1974b) have demonstrated purification by chromatography on agarose beads of an *N*-acetyl-galactosaminyl transferase from human plasma. This enzyme catalyses the transfer of *N*-acetyl-galactosamine only to glycoprotein and oligosaccharide acceptors which have blood

group A activity. Fraser & Mookerjea (1976) obtained a pure preparation of galactosyl transferase from rat serum by affinity chromatography on Sepharose 4B coupled with α-lactalbumin. The authors also attempted purification of rat liver microsomal galactosyl transferase by incubating the microsomes with saline solution followed by further purification on Sephadex G-200 column. The purified enzyme from serum was further purified by polyacrylamide gel electrophoresis and had properties similar to those of both the soluble and membrane-bound transferases.

Myllyla et al. (1977) isolated a collagen glucosyl transferase as an homogenous protein from chick embryos by a procedure consisting of ammonium sulphate precipitation, affinity chromatography and gel filtration. The specific activity of the purified enzyme was 32,000 times higher than that of the 15,000 g supernatant of the embryo homogenate. Their purified enzyme appeared homogenous upon gel electrophoresis, had a molecular weight of 72,000-78,000 and consisted of one polypeptide chain.

It is interesting that although a large number of glycosyl transferases are known to be present in membranes of animal cells, only a few of them have been obtained as pure preparations. The difficulty with the membrane-bound enzymes is that many of them lose activity readily upon solubilization. This was observed when attempts were made to purify the transferase under study from liver preparations. Release of enzyme from the membrane was accompanied by increased instability. A UDP-galactose - xylose galactosyl transferase and other transferases which are probably involved in vivo in the formation of chondroitin sulphate chains

were successfully solubilized with the use of Nonidet P-40 (70% of the activity) from embryonic chick cartilage membrane (Schwartz & Rodén, 1975). The alkaline detergent method of Helting (1971) was also used successfully. The procedures described might have wide applicability.

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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: $Co^{2+} > Mn^{2+} > Ni^{2+}$. 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*-acetylglucosaminylation 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 ···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 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 UPD-*N*-acetyl-D-[U-^{14}C]-glucosamine (>200 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, Wollsery, 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 (17mg) was dissolved in 1.5ml of sodium citrate buffer (0.05M in citrate) containing 0.013M-$CaCl_2$, pH6.0. The solution was placed at 4°C on a column (9.0cm ×0.9cm) of CM-cellulose, which had been pre-equilibrated with the same buffer. The column was eluted with 14ml of this buffer, followed by 30ml of 0.1M-sodium citrate buffer, pH6.0, containing 0.013M-$CaCl_2$. Flow rate was 5ml/h and the eluate was collected in 2ml volumes. Two u.v.-absorbing peaks (280nm) appeared. The second one (fractions 13–25) contained trypsin. The solution was desalted by eluting in 0.2M-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 10ml 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 (2g) was cut up finely and homogenized in the cold in a Potter–Elvehjem homogenizer with 4–5ml of buffer (0.05M-Tris–HCl, pH7.4 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 10g of rabbit liver were used.

Glucosamine. This was determined after acid hydrolysis (4M-HCl; 3–6h; 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, pH3.25 (0.2M-sodium citrate), 55min; pH4.25 (0.2M-sodium citrate), 110min; pH6.65 (1.0M-sodium citrate), 140min. Programme II allowed the separation of leucine and glucosamine, pH4.25 (0.2M-sodium citrate), 100min; pH6.65 (1.0M-sodium citrate), 140min. 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 related to the amount of ribonuclease present in the incubation mixture. A column 9cm×0.9cm was used for up to 20mg of ribonuclease, but one of 18cm×0.9cm was used for 41mg of ribonuclease. The samples, either ribonuclease solution or an incubation mixture after centrifugation (2000*g*; 10min; 4°C), was applied to the column previously equilibrated with 0.01M-Tris–HCl, pH7.0 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 2×200ml volumes, one containing buffer only and the other 0.1M-NaCl in the starting buffer. The gradient was prepared from 2×100ml 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 (1–18mg) 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 4h in water adjusted to pH8.0. Adjustment to pH8.0 was made at frequent intervals with 0.02M-NaOH. The mixture was then left for 12h 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*; 15min; 4°C). The pellet was taken up in 0.2M-acetic acid and the mixture was re-centrifuged under the same conditions. The supernatant was added to the previous (pH3.3) 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 (4M-HCl; 3h;

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.

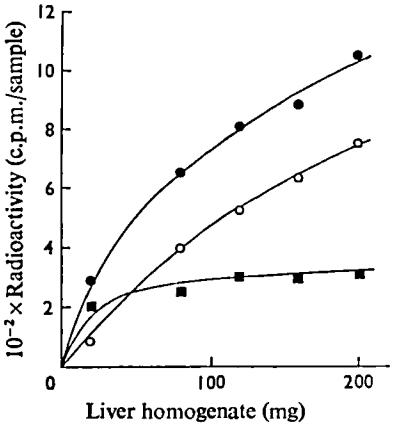


Fig. 1. *Relationship between the amount of N-acetylglucosamine transferred from UDP-N-acetylglucosamine to bovine pancreatic ribonuclease A in the presence of increasing amounts of rabbit liver homogenate*

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 ■.

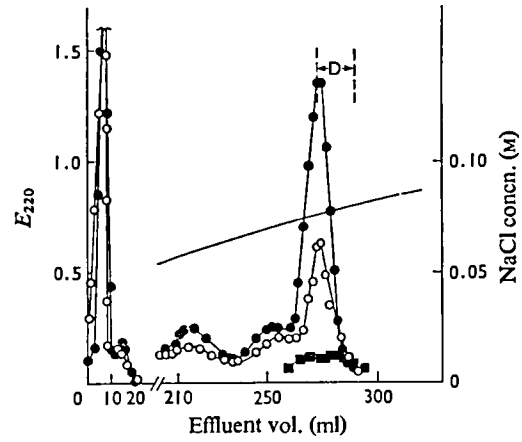


Fig. 2. *Chromatography on a CM-cellulose column* (9.0 *cm* × 0.9 *cm*) *of an incubation mixture of ribonuclease A, UDP-N-acetyl-D-glucosamine, $MnCl_2$ and liver homogenate as described in column* 3, *Table* 2 (●, E_{220})

The chromatographic conditions are given in the text. In one control experiment, no added ribonuclease at all was used (■, E_{220}) and in another 2.6 mg of ribonuclease A was added after incubation (○, E_{220}). 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 2000*g* 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 0.074M. 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 2.6mg 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 0.14mol 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 0.23mol 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 (41.7mg) was incubated with a portion of the rough endoplasmic reticulum in the presence of UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine (for conditions see Table 1, column 4). Chromatography on CM-cellulose (Fig. 3*a*) 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 0.08mol 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

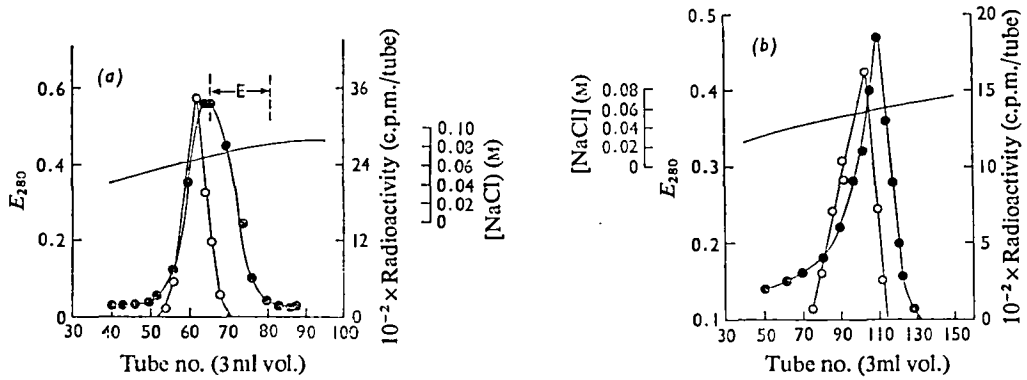


Fig. 3. *Partial separation of the ^{14}C-labelled monoglycosylated ribonuclease from ribonuclease A by chromatography on CM-cellulose*

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-[^{14}C]glucosamine which led to the production of further ^{14}C-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. 3*a*) 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 UPD-*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 0.05mol of *N*-acetyl-D-glucosamine had been incorporated into 1mol of ribonuclease. Again the peak of radioactivity was displaced towards the early part of the ribonuclease peak (Fig. 3*b*).

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. 3*a*) 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+}, 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.

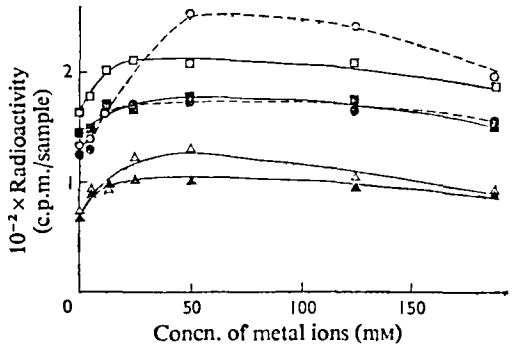


Fig. 4. *Effect of metal ions on the incorporation of N-acetyl-D-[^{14}C]glucosamine into ribonuclease A*

(i) Co^{2+}: ○, pancreatic ribonuclease added; ●, no ribonuclease added. (ii) Mn^{2+}: □, ribonuclease added; ■, no ribonuclease added. (iii) Ni^{2+}: △, ribonuclease added; ▲, no ribonuclease added.

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. 3*b*) 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; 40min; in acetic acid–formic acid–water buffer (78g of acetic acid+25g 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 20.2cm 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 (150cm × 0.9cm) 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 (200cm × 0.6cm) 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),

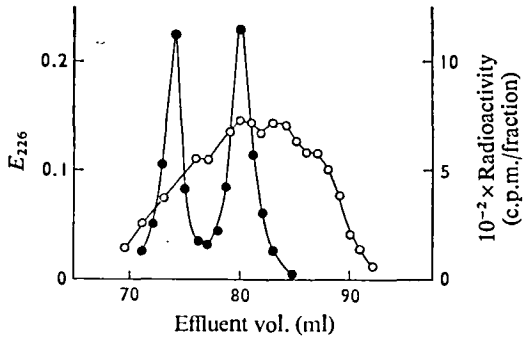


Fig. 5. *Rechromatography in* 0.2M-*acetic acid on Sephadex G-25 (200cm × 0.6cm) of part of the effluent from Sephadex G-25 of a tryptic hydrolysate of monoglycosylated ribonuclease A*

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 ··· Met-Met-Lys-Ser-Arg-Asn-Leu-Thr-Lys-Asp-Arg-Cys-Lys··· 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-Ala-Lys···).

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

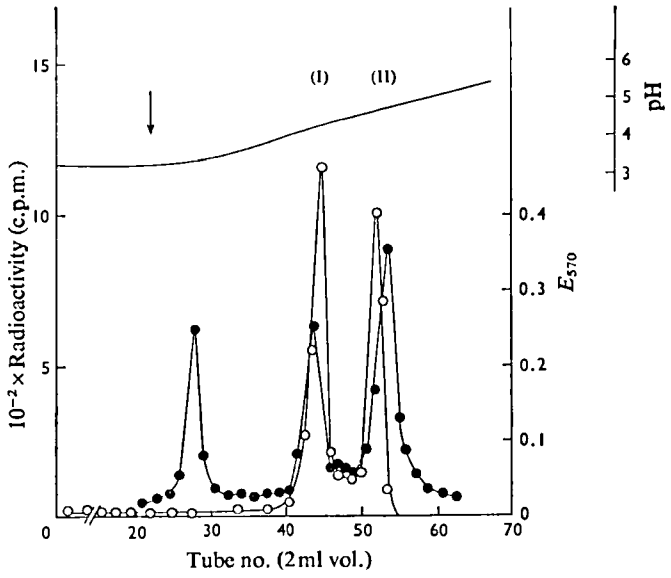


Fig. 6. *Chromatography on Amberlite CG-120 of combined fractions 72–84 from the Sephadex G-25 column (Fig. 5)*

The fractions were combined and freeze-dried. The residue was dissolved in 1.0ml of pyridine-formate buffer (0.2M-pyridine, pH3.26) and applied to the column (7cm×0.9cm), which had been equilibrated with this buffer at room temperature (24°C). Elution was effected first with 50ml of the same buffer and then with a gradient made with two 75ml vessels: in the first was the starting buffer, in the second was pyridine-formate buffer (0.4M-pyridine, pH6.5). Fractions (2ml) 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).

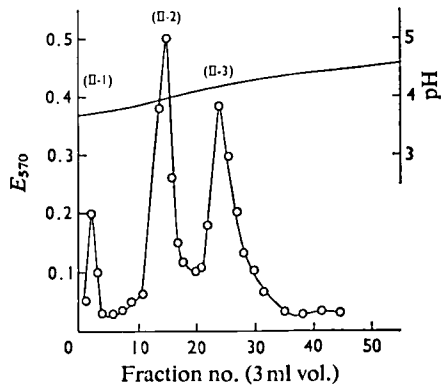


Fig. 7. *Rechromatography of fraction (II) (Fig. 6) on a column (12cm×1.6cm) of sulphopropyl-Sephadex C-25*

Elution was effected with a pyridine-formate gradient (100ml of pyridine-formate buffer, pH3.75, 0.2M in pyridine: 100ml of pyridine-formate, pH5.25, 0.4M 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 reseparated on CM-cellulose and digested with trypsin

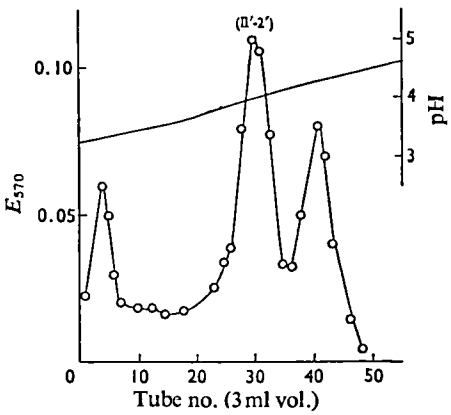


Fig. 8. *Chromatography on sulphopropyl-Sephadex C-25 (under the conditions described in the legend of Fig. 7) of a peptide from ribonuclease A corresponding in behaviour to the glycopeptide (II) obtained from monoglycosylated ribonuclease*

○, 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 corresponding 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 recepter 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).

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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],

*Dedicated to the memory of Professor Edward J. Bourne.

†Presented, in part, at the Third International Symposium on Glycoconjugates, University of Sussex, July 6–12th, 1975.

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[i]
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. [i]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.

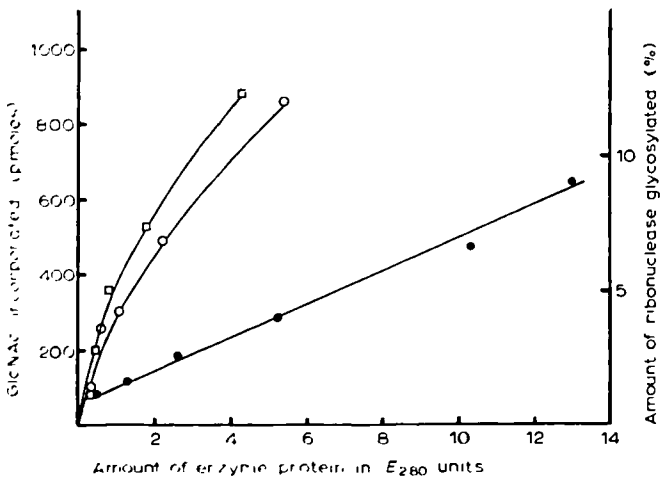


Fig. 1. Relationship between the amount of enzyme protein used and the extent of enzymic incorporation of *N*-acetyl-D-glucosamine into ribonuclease A. The conditions of the experiment are described in the text, and the incubation conditions in Table II (column 1): —●—●—, dialysed whole serum; —○—○—, 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).

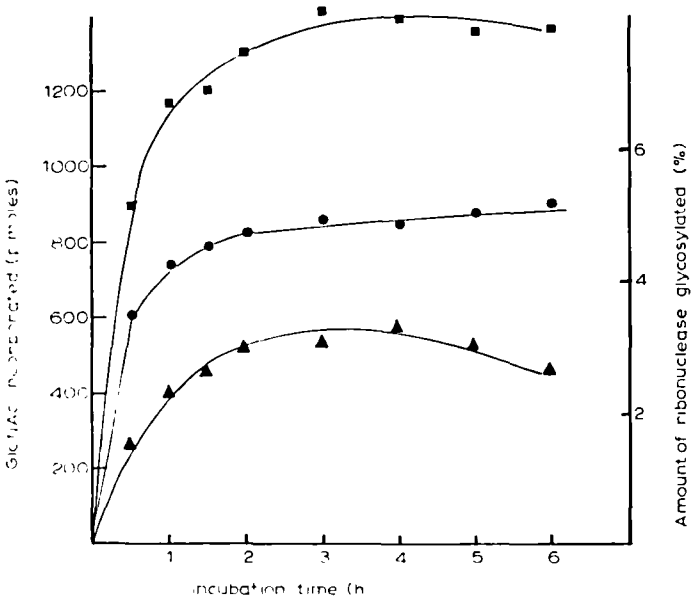


Fig. 2. Rate of incorporation of *N*-acetyl-D-glucosamine into ribonuclease A. Incorporation into protein precipitable by phosphotungstic acid/trichloracetic acid mixture (see Methods) when ribonuclease A was added to the incubation mixtures under the conditions of incubation described in Table II (column 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

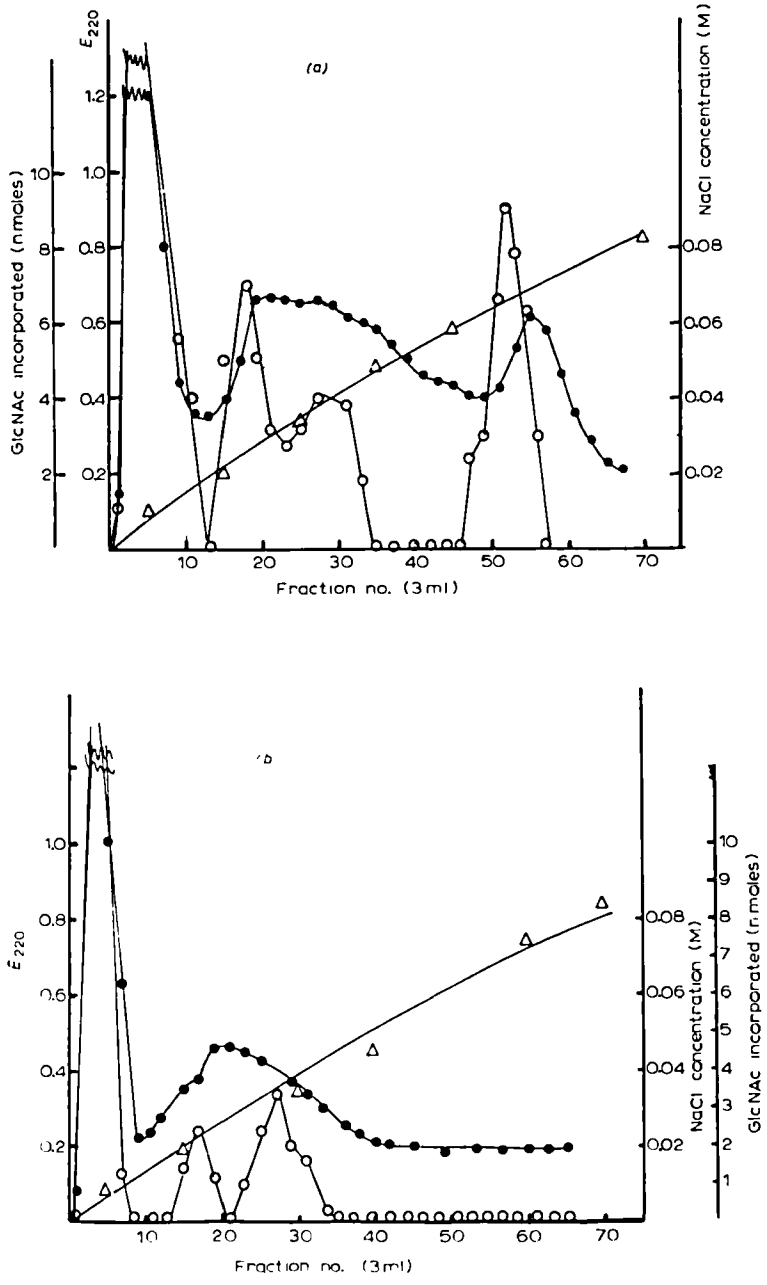


Fig. 3. Chromatography on a CM-cellulose[12] column (9 × 0.9 cm) of a mixture incubated as described in Table II (column 3). The chromatographic conditions are described in the text. E_{220} is represented as —●—●—; *N*-acetyl-D-glucosamine incorporated as - - ○ - ○ - -, and NaCl gradient as —△—△—. Results 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.

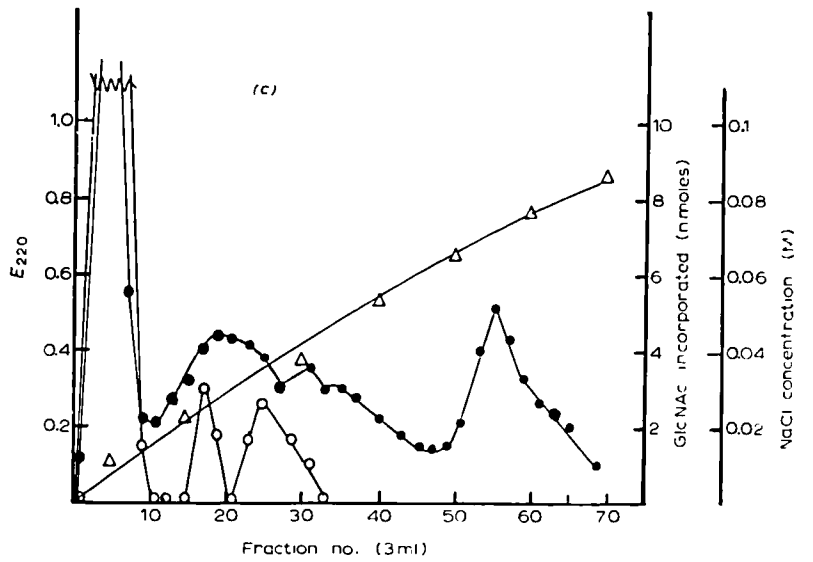


Fig. 3. (*Legend on p. 00 below*).

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

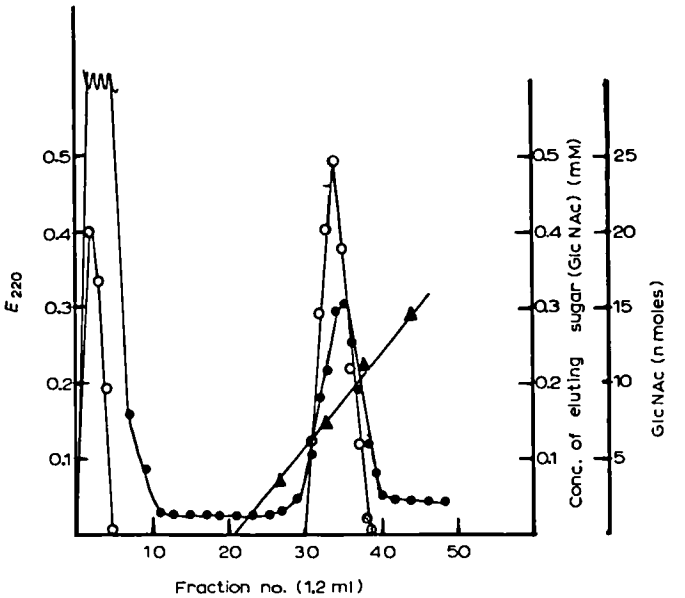


Fig. 4. Separation by chromatography on a column (20 × 0.3 cm) of wheat-germ agglutinin–Sepharose of ribonuclease A and ribonuclease GlcNAc: —●—●—, E_{220}; —○—○—, *N*-acetyl-D-glucosamine-^{14}C; —▲—▲—, *N*-acetyl-D-glucosamine assayed by a Morgan–Elson procedure[14].

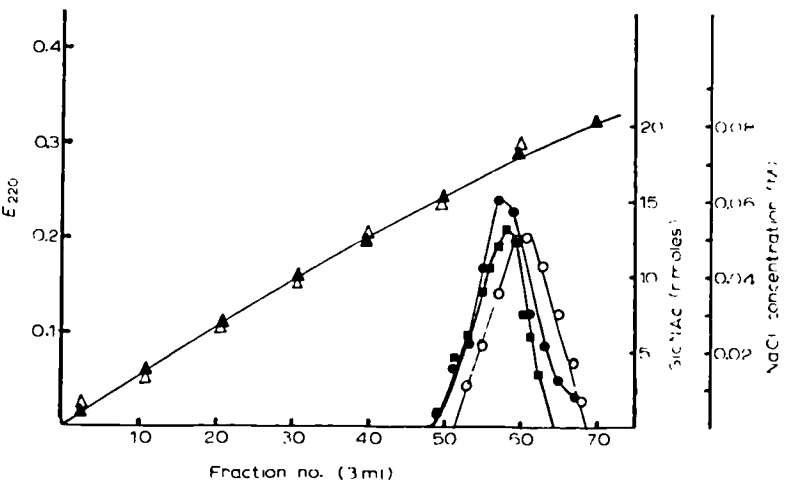


Fig. 5. Chromatography on a CM-cellulose column[12] (9 × 0.9 cm) of either 1.5 mg of ribonuclease-GlcNAc (—●—●—, E_{220}; —■—■—, amount of *N*-acetyl-D-glucosamine covalently bound to ribonuclease; —▲—▲—, NaCl gradient) or 1.0 mg of ribonuclease A (—○—○—, E_{220}; —^—^—, NaCl gradient). The conditions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

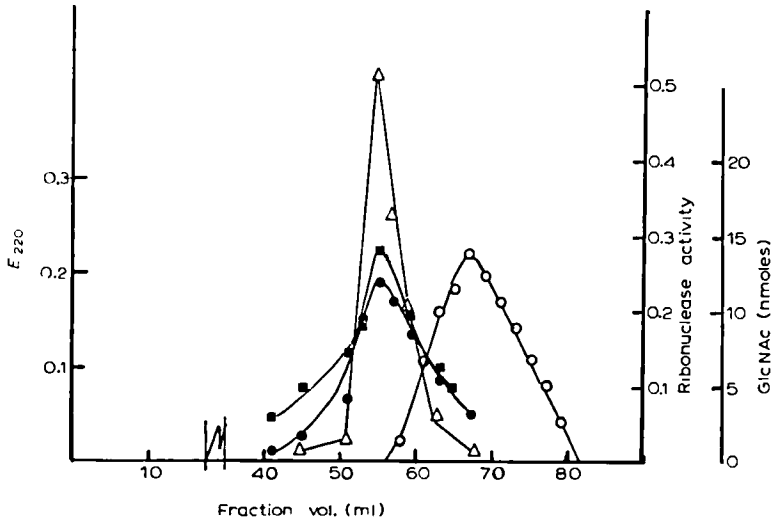


Fig. 6. Chromatography on an Amberlite CG-50 column[18] (45 × 0.9 cm) of either 0.5 mg of ribonuclease-GlcNAc (—●—●—, E_{220}; —■—■—, amount of *N*-acetyl-D-glucosamine covalently bound in ribonuclease-GlcNAc; —△—△—, enzyme activity of ribonuclease-GlcNAc), or of 0.8 mg of ribonuclease A (—○—○—, E_{220}).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

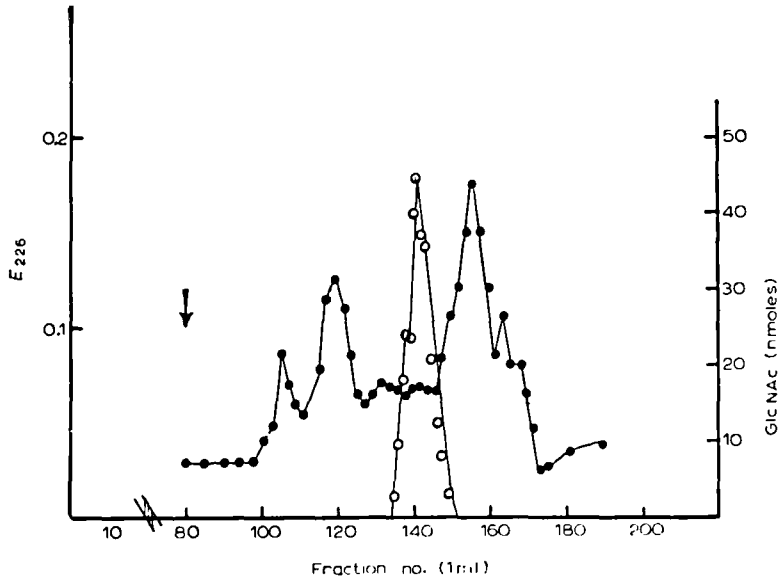


Fig. 7. Fractionation at room temperature of tryptic digest of carboxyethyl derivative of ribonuclease-GlcNAc-^{14}C (4.5 mg) on Sephadex G-25 medium [two connected, parallel columns (each 125 × 1.0 cm)]. The eluting buffer was 0.1M acetic acid: —●—●—, E_{226}; —○—○—, *N*-acetyl-D-glucosamine incorporated.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

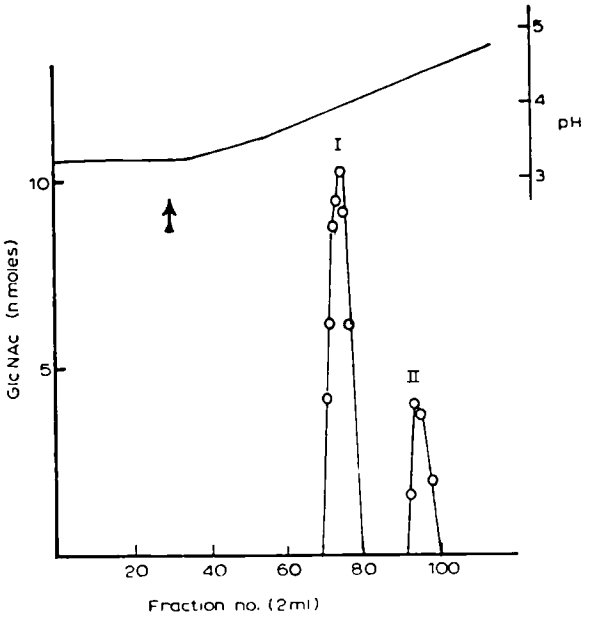


Fig. 8. Chromatography on an Amberlite CG-120 column (7 × 0.9 cm) of a measured aliquot (~ 200 nmoles of D-glucosamine-^{14}C) of combined Fractions 139–146 from the Sephadex G-25 column (Fig. 7): —○—○—, *N*-acetyl-D-glucosamine incorporated. Elution was effected with a linear pH gradient (- - - - - - - -) 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

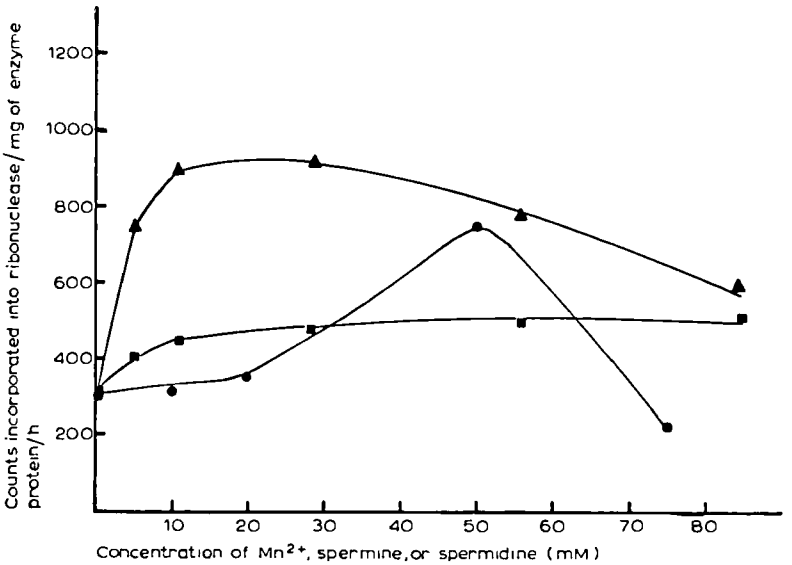


Fig. 9. Effects of Mn^{2+} (—●—●—), spermine (—■—■—), and spermidine (—▲—▲—) on the transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A. The conditions of incubation are described in Table II (column 6).

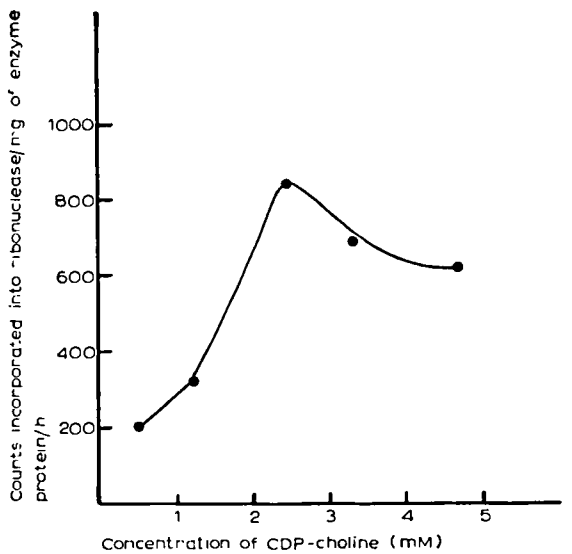


Fig. 10. Effect of CDP-choline on the incorporation of *N*-acetyl-D-glucosamine into ribonuclease A, with UDP-*N*-acetyl-D-glucosamine as the donor. The incubation conditions are described in Table II (column 7).

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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Glycosylation *in vitro* of an Asparagine Sequon Catalysed by Preparations of Yeast Cell Membranes

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(*Received* 1 *June* 1976)

Preparations of yeast cell membranes can catalyse *in vitro* the *N*-acetyl-β-D-glucosaminylation of the asparagine sequon at residues 34–36 of bovine pancreatic ribonuclease A. The relevant glycopeptides were isolated from tryptic hydrolysates of the glycosylated ribonuclease and analysed. The donor used was UDP-*N*-acetyl-D-glucosamine, although the mechanism of the transfer is unknown. Mn^{2+} ions at concentrations of 25mM double the activity of the enzymic transfer.

An asparagine residue in an apoprotein may act as the site for attachment of a carbohydrate moiety (Kohno & Yamashina, 1973; Khalkhali & Marshall, 1975, 1976) through a linkage of the form 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn; Marshall & Neuberger, 1972). The accepting asparagine moiety must occur in a sequence of the form Asn-X-Thr or Asn-X-Ser (Marshall, 1967; Neuberger & Marshall, 1968) which has been termed a sequon (Marshall, 1974) in order that the residue may function as an acceptor for the sugar.

Glycosylation of appropriate asparagine residues of serum glycoproteins seems to occur at the polyribosomal level, at least in part (Molnar *et al.*, 1965; Lawford & Schachter, 1966; Simkin & Jamieson, 1967; Redman & Cherian, 1972), and an enzyme with the requisite substrate specificity has been shown to be present in the rough endoplasmic reticulum of rabbit liver (Khalkhali & Marshall, 1975). There is also similar enzymic activity in serum, and a reason for its presence there has been suggested (Khalkhali & Marshall, 1976).

The results described below show that there is enzymic activity in yeast cell-membrane preparations, which is able to catalyse *in vitro* the glycosylation of asparagine-34 of bovine pancreatic ribonuclease A.

Experimental

Materials

The sources of many of the materials have been described (Khalkhali & Marshall, 1976). The ribonuclease A was purchased from Boehringer Corp. (London) Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, U.K. (cat. no. 15410) and was chromatographed on CM-cellulose before use (Taborsky, 1959). It was shown to be free of glucosamine (Khalkhali & Marshall, 1975) before use. The yeast mutant x 2180-1A-5mnn2 was supplied by Dr. C. E. Ballou, Department of Biochemistry, University of California, Berkeley, CA 94720, U.S.A. and tunicamycin was kindly provided by Dr. G. Tamura, Laboratory for Microbiology, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku 113, Tokyo, Japan.

Methods

Preparation of yeast cell membranes. *Saccharomyces cerevisiae*, mutant x 2180-1A-5mnn2, was grown at 30°C in a medium similar to that described previously (Van Rijn *et al.*, 1972). Adenine (0.37mM) was added and half the amount of $(NH_4)_2SO_4$ was replaced by the addition of 40ml of phosphate-free casamino acid solution for each litre of medium. The casamino acid solution was prepared by dissolving 40g of casamino acids (Difco Laboratories, Detroit, MI, U.S.A.) and 6g of $MgSO_4,7H_2O$ in 300ml of water. The pH of the preparation was adjusted to 8.0 (25%, v/v, satd. NH_3 in water, sp. gr. 0.88) and the volume made up to 400ml. The mixture was kept at 0°C for 3h and filtered. The solution contained less than 0.1mM-phosphate.

Cells were harvested in the late-exponential phase and 1.5×10^{10} cells/20ml of Tris/HCl buffer (0.05M in Tris), pH7.0, containing 1mM-mercaptoethanol, were mechanically disrupted at 0°C in a cell homogenizer (MSK, Braun, Melsungen, German Federal Republic), by shaking them for 5min in the presence of 25ml of Ballotini beads (0.25–0.30mm diameter). Cell debris was removed as a precipitate after centrifuging the homogenate (10min, 1000*g*, 4°C). The supernatant was centrifuged (30min, 40000*g*, 4°C), and the pellet, consisting of membranes, was used as

a suspension in the Tris/HCl buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. It contained 21 mg of protein/ml, the protein being assessed by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Microsomal preparation from rabbit liver. This was prepared by the procedure of Keller & Zamecnik (1956). It was finally prepared for use as a suspension in the same buffer and at the same concentration as the yeast cell-membrane preparation.

Tryptic digestion of glycosylated ribonuclease. This was carried out by procedures closely similar to those used earlier (Khalkhali & Marshall, 1975), the crystalline trypsin used (Boehringer; cat. no. 15330) having been freed of extraneous proteinases by the technique of Maroux *et al.* (1962).

All other methods used were described previously (Khalkhali & Marshall, 1976).

Results

The first series of experiments involved measuring the extent of incorporation of labelled glucosamine into bovine pancreatic ribonuclease A catalysed by preparations of yeast cell membranes. Incubations were carried out both in the presence and in the absence of added ribonuclease A, under the conditions described in Table 1, column (*a*), and the differences in the amounts of radioactivity incorporated were assessed (Fig. 1). There is clearly extensive incorporation of glucosamine into endogenous material in the yeast cell membranes as well as into added ribonuclease A. After incubation for 1 h under the conditions used, about 3.3 nmol of glucosamine was incorporated into endogenous glycoproteins/mg of enzyme protein, and about 7.9 nmol/mg of ribonuclease A.

Table 1. *Conditions used in the incubation medium for incorporation of glucosamine into bovine pancreatic ribonuclease A*

The temperature was in all cases 37°C. In (*a*) the enzyme was a preparation of yeast cell membranes, and in (*b*) it was a preparation of either yeast cell membranes or rabbit liver microsomal fraction (see under 'Methods' in all cases).

Assay conditions	(*a*)	(*b*)
Tris/HCl buffer, pH 7.2 (mM)	45	45
$MnCl_2$ (mM)	25	25
UDP-GlcNAc (mM)	0.355	2.84
(c.p.m./nmol)	222	78.3
Ribonuclease A (μM)	71.4	714
(mg)	0.2	10
Triton X-100 (%)	0.4	0.4
Total volume (ml)	0.2	1
Amount of enzyme used (μl)	20	800
Incubation time (h)	0–4	1

The effects of Mn^{2+} concentration on the rate of glycosylation of ribonuclease A were studied. Incubation mixtures were set up under conditions almost identical with those described in column (*a*), Table 1,

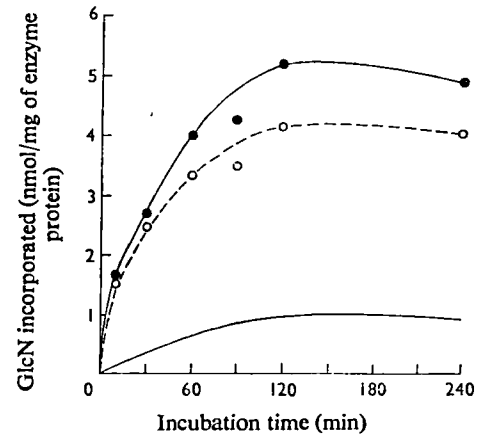


Fig. 1. *Rate of incorporation of N-acetyl-D-glucosamine from UDP-N-acetyl-D-glucosamine into ribonuclease A catalysed in vitro by yeast cell membranes*

The conditions of incubation were as described in column (*a*) of Table 1. Incubation was effected either in the presence of added ribonuclease A (●) or in its absence (○). The incorporation into added ribonuclease A is indicated by ——. Replicate values generally agreed within 6% and incorporation of radioactivity was always greater in those samples that contained added ribonuclease A.

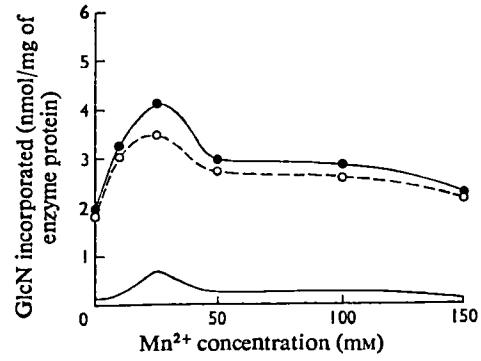


Fig. 2. *Effect of Mn^{2+} concentration on the rate of incorporation of N-acetyl-D-glucosamine from UDP-N-acetyl-D-glucosamine into ribonuclease A*

The conditions of incubation were identical with those in column (*a*), Table 1, apart from the variation in Mn^{2+} concentration. Time of incubation was 1 h. Incubation was effected either in the presence of added ribonuclease A (●) or in its absence (○). The incorporation into added ribonuclease A is indicated by ——.

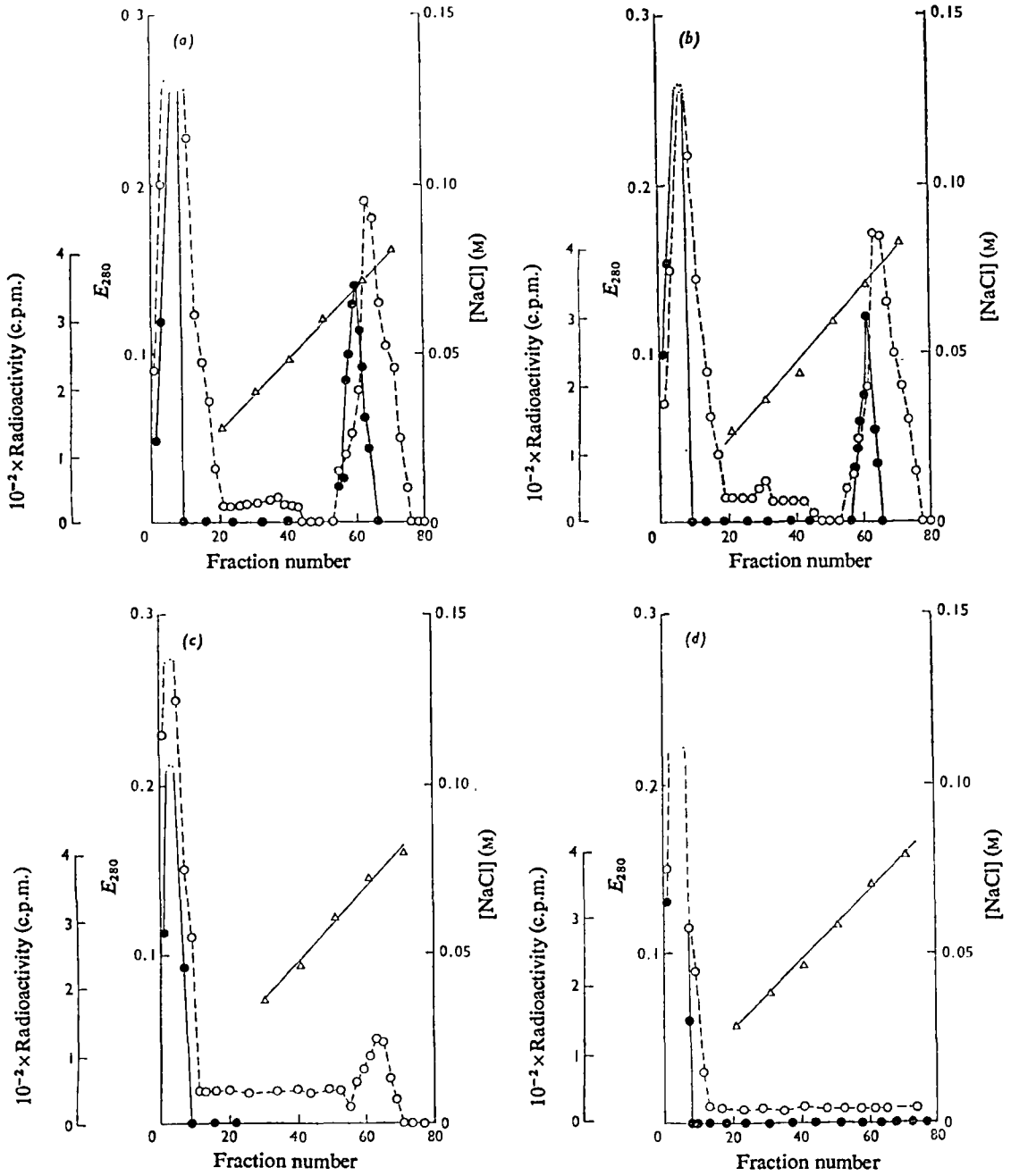


Fig. 3. *Chromatography on CM-cellulose columns* (0.9 cm × 9 cm) *of incubation mixtures prepared under the conditions described in column* (*b*), *Table* 1

The mixtures were incubated with: (*a*) ribonuclease A (10 mg) and a rabbit liver microsomal preparation; (*b*) ribonuclease A (10 mg) and a preparation of yeast cell membranes; (*c*) yeast cell membranes and ribonuclease A (2.64 mg) was added, but only after incubation; (*d*) yeast cell membranes but no added ribonuclease A at all. Other details of the experiments are described in the text. ○, E_{280}; ●, radioactivity; △, [NaCl]; 3 ml fractions were collected.

apart from having Mn^{2+} concentrations over the range 0–125mM. The results show that the optimum concentration of Mn^{2+} is of the order of 25mM (Fig. 2).

Separation of glycosylated ribonuclease A

It was shown that the additional radioactivity incorporated into protein when ribonuclease A was in the incubation mixture did in fact represent glycosylation of the added pancreatic enzyme. The following experiments were done in order to examine this aspect.

Three incubation mixtures were prepared under the conditions described in column (*b*), Table 1, and each was incubated at 37°C for 1h. The first of these contained 10mg of added ribonuclease A and the enzyme used in this case, for comparative purposes, was a rabbit liver microsomal preparation. The second contained 10mg of ribonuclease A and as enzyme the yeast cell-membranes preparation. The third incubation mixture was devoid of added ribonuclease A and it contained the yeast cell-membranes preparation. The mixtures were centrifuged (2000*g*, 10min, 4°C) after incubation and the third was divided equally into two parts, to one of which was added 2.64mg of ribonuclease A at that stage. The four preparations [*a*, with ribonuclease A (10mg) incubated in presence of rabbit liver microsomal preparation; *b*, with ribonuclease A (10mg) incubated in presence of yeast cell membranes; *c*, with ribonuclease A (2.64mg) added after incubation; *d*, without any added ribonuclease A] were subjected to chromatography on CM-cellulose. In all cases the weights of ribonuclease A used were not corrected for either water or ash content.

The results obtained show that preparations of yeast cell membranes as well as rabbit liver microsomal preparations contain enzymic activity which can catalyse the incorporation of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine into ribonuclease A. If no ribonuclease A was present in the original incubation mixture, no peak of protein was eluted from the column at a NaCl concentration of about 0.07M (Fig. 3*d*), which are the conditions required for elution of ribonuclease A from the CM-cellulose column under the conditions used (Taborsky, 1959). In the experiment in which ribonuclease A was added to the incubation mixture, but only after incubation, the pancreatic enzyme was eluted at the expected position (Fig. 3*c*) in an amount of 1.8mg, assuming $E^{1\%}_{280} = 7.2$ (Scheraga & Rupley, 1962), but no radioactive glucosamine appeared in this region.

On the other hand, when the preparation of yeast cell membrane was incubated with ribonuclease A and UDP-*N*-acetyl-D-glucosamine under the conditions described in column (*b*), Table 1, chromatography of the products on CM-cellulose led to the appearance of the ribonuclease A in the eluate at an NaCl concentration of about 0.07M (Fig. 3*b*), and a peak of radioactive glucosamine was also eluted in this region. The radioactive substance is almost certainly monoglycosylated ribonuclease A, in view of the results reported below. Moreover, it was shown previously that this latter substance chromatographs in that position (Khalkhali & Marshall, 1975, 1976). The amount of glucosamine incorporated is about 16.1 nmol/7.7mg of ribonuclease A, which is equivalent to 0.029 residue/molecule. In the control experiment using a rabbit liver microsomal preparation as the enzyme source (Fig. 3*a*), 26.0nmol of glucosamine was incorporated/7.9mg of ribonuclease A, which is equivalent to 0.046residue/molecule. The latter value is of the order found previously when rough endoplasmic reticulum, isolated from rabbit liver, was used as the enzyme source.

Identification of the position at which ribonuclease A is glycosylated

It was shown by isolation of the radioactive tryptic peptide from the glycosylated ribonuclease A that the pancreatic enzyme had undergone substitution at residue 34, where the asparagine sequon -Asn-Leu-Thr- occurs. The tryptic digest of fractions 56–65 (Fig. 3*b*; 1220c.p.m.≡15.6nmol of glucosamine, 3.98mg of total ribonuclease, equivalent to 0.055 residue/molecule of ribonuclease) was subjected to gel filtration on Sephadex G-25 (1cm×250cm) in 0.1M-acetic acid. Fractions (1ml) were collected and the only radioactive peak was in fractions 80–87 inclusive (1061c.p.m., 87% recovery). These fractions were combined and the solution was freeze-dried. The residue was subjected to chromatography on Amberlite CG-120 under conditions identical with those described earlier (Khalkhali & Marshall, 1975). Of the three peaks that were apparent by ninhydrin assay, only one, the middle one, was radioactive (1016c.p.m., 96% recovery). Analysis of acid hydrolysates (4M-HCl; 100°C; 4h) of this peak on the amino acid analyser showed the presence of aspartic acid, leucine, threonine and lysine only, in amounts of 1.0, 0.99, 1.07 and 1.03mol respectively/0.062mol of glucosamine. These results show that the peak analysed consisted of a mixture of about 94% of the peptide Asn-Leu-Thr-Lys and about 6% of its glycosylated derivative, and it is known from earlier studies that this peptide and the glycopeptide co-chromatograph under the conditions used (Khalkhali & Marshall, 1975). This is a relative composition almost identical with that of the mixture of ribonuclease A and its glycosylated derivative which was originally subjected to tryptic digestion. The mixture of peptide and glycopeptide must be derived from residues 34–37 of the original ribonuclease A, where the sequence is Asn-Leu-Thr-Lys and the asparagine residue is preceded by an arginine residue.

Discussion

The results of the above experiments show that there is enzymic activity in preparations of yeast cell membranes which can catalyse *in vitro* the *N*-acetyl-β-D-glucosaminylation of the single asparagine sequon in bovine pancreatic ribonuclease A, that at residues 34–36. The results demonstrate that this is the only asparagine residue in the pancreatic enzyme that was found to undergo glycosylation.

The mechanism of transfer of the sugar is unresolved because preparations of yeast cell membranes can catalyse the transfer of *N*-acetyl-D-glucosamine 1-phosphate from UDP-*N*-acetyl-D-glucosamine to dolichol phosphate (Lehle & Tanner, 1975). Dolichol pyrophosphate *N*-acetyl-D-glucosamine can be used *in vitro* as the donor for the glycosylation of endogenous glycoproteins of yeast cell-membrane preparations (C. Habets-Willems, unpublished work), but whether the sugar becomes directly linked to an asparagine residue in the protein chain(s) of the acceptor is unknown. In any case the main question is whether a sugar-lipid is an obligatory intermediate in the transfer of the sugar from UDP-*N*-acetyl-D-glucosamine to an asparagine sequon. There was found to be extensive (80%) inhibition of the formation of glycosylated ribonuclease by the preparations of yeast cell membranes in the presence of tunicamycin (12 μM). This antibiotic has the facility to inhibit *in vitro* the enzymic transfer of the sugar from UDP-*N*-acetyl-D-glucosamine to an acceptor present in calf liver microsomal preparation with the formation of a polyisoprenyl *N*-acetyl-D-glucosaminyl pyrophosphate (Tkacz & Lampen, 1975). Further experiments are needed to establish whether the inhibition of glycosylation of ribonuclease is due to a lower rate of synthesis of a lipid intermediate.

We thank Mr. J. Hofsteenge and Dr. A. Reinking for some technical assistance and for helpful discussions. We are grateful for the financial assistance of the Medical Research Council and of the British Council. Z. K. is a British Council Scholar on leave of absence from Tehran University Nuclear Centre, Tehran, Iran.

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The UDP-*N*-acetylglucosamine–Asparagine-Sequon *N*-Acetyl-β-D-glucosaminyltransferase Activity in Preparations of Rough Endoplasmic Reticulum from Regenerating Rat Liver

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The UDP-*N*-acetylglucosamine–asparagine-sequon *N*-acetylglucosaminyltransferase activity in preparations of rough endoplasmic reticulum from rat liver is less than 1% of that in preparations from rabbit liver. The activity of the enzyme is increased about 20-fold in preparations from regenerating rat liver within 48h after partial hepatectomy. A smaller, but still marked, increase (8–10-fold) occurs in preparations of rough endoplasmic reticulum from sham-operated rats.

Synthesis of the glycoproteins of serum and of the structural glycoproteins of the hepatocyte must involve the enzymic formation of the carbohydrate–protein linking moiety, 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine. The asparagine residue to which the sugar becomes attached must occur in a sequence of the form ...Asn-X-Thr... or ...Asn-X-Ser... (Marshall, 1967; Neuberger & Marshall, 1968), which is termed an asparagine sequon (Marshall, 1974). There is known to be enzymic activity, in preparations of rough endoplasmic reticulum from rabbit liver, which can catalyse formation of the 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine linkage (Khalkhali & Marshall, 1975). Attempts (Z. Khalkhali, F. Serafini-Cessi & R. D. Marshall, unpublished work) to demonstrate such an enzymic activity in similar preparations from rat liver yielded equivocal results, although glycoproteins are known to be synthesized by rat liver (Molnar, 1975).

There is an increased rate of synthesis, per unit wet wt. of liver, of serum glycoproteins by regenerating rat liver (Mutschler & Gordon, 1966; Akamatsu *et al.*, 1976), as well as an increased activity of glucosamine phosphate isomerase (glutamine-forming) (EC 5.3.1.19; Akamatsu & Maeda, 1971). The latter enzyme may be involved in the regulation of glycoprotein biosynthesis. It seemed possible that the increased rate of production of glycoproteins by regenerating liver might require higher activities than normal of that sugar transferase involved in the formation of 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine linkages. It was decided therefore to examine the activity of UDP-*N*-acetylglucosamine–asparagine-sequon *N*-acetylglucosaminyltransferase in preparations of rough endoplasmic reticulum isolated from regenerating rat liver.

Experimental

Materials

The sources of most of the materials used were described previously (Khalkhali & Marshall, 1976). Wheat-germ agglutinin bound to Sepharose was from Miles Laboratories (Stoke Poges, Bucks., U.K.).

Female rats

Female rats (200–250g) of the Wistar strain, fed on laboratory chow and water *ad lib.*, were used. Partial hepatectomy (Higgins & Anderson, 1931; Waynforth, 1969) was performed under diethyl ether anaesthesia. Sham operation consisted of laparotomy and palpation of the liver.

Methods

Preparations of rough endoplasmic reticulum. These were isolated from the livers of control animals, as well as from sham-operated and partially hepatectomized rats 24, 48 and 72h after operation. The technique of Dallner (1963), as described by Lawford & Schachter (1966), was used. The subcellular fractions were prepared for use by washing them with 0.9% KCl, and finally suspending them in 50mM-Tris/maleate buffer (50mM final concentrations of both Tris and maleate, adjusted to pH7.1 with NaOH).

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

Measurements of enzyme activity. Incubation mixtures of 100μl volume contained 50mM-Tris/maleate, pH7.1, 50mM-$MnCl_2$, 0.506mM-UDP-*N*-acetyl[U-^{14}C]glucosamine (1096d.p.m./nmol), 0.4% Triton X-100, 100μg of bovine pancreatic ribonuclease A and appropriate amounts of the various preparations of rough endoplasmic reticulum. In all cases control incubation mixtures were also set up, and these did not contain added ribonuclease A. Incubations were done for measured periods of time at fixed temperatures, and reactions were stopped by the addition of 2ml of a cold solution containing 1% phosphotungstic acid in 0.5M-HCl. The precipitates formed were collected on glass-fibre filters (Whatman GF/A, 2.1cm diam.), carefully washed with the precipitating acid (10×5ml), then with 5ml of 5% (w/v) trichloroacetic acid and air-dried. The incorporation of *N*-acetyl-D-glucosamine into the precipitates was determined by radioactivity counting.

Radioactivity counting. This was done by placing the glass-fibre filters in 6ml of methylCellosolve and adding 10ml of scintillator [2vol. of toluene, containing 0.5% 2,5-diphenyloxazole and 0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene, and 1 vol. of Triton X-100]. A Packard Tri-Carb scintillation counter was used and the efficiency of ^{14}C counting in the solvent used was 62%. Corrections were applied for quenching by protein.

Identification of the product formed by enzymic glycosylation. Ribonuclease A (1mg) was incubated at 37°C for 1h in presence of 50mM-Tris/maleate, pH7.1, 50mM-$MnCl_2$, 0.506mM-UDP-*N*-acetyl[U-^{14}C]glucosamine (1096d.p.m./nmol) and a preparation of rough endoplasmic reticulum (1mg of protein) isolated from rat liver 48h after partial hepatectomy, all in a final volume of 1ml. A control experiment was also done in which ribonuclease A was omitted from the incubation mixture.

The reaction was stopped by cooling and the mixture was centrifuged (3000*g*, 10min, 2°C). The supernatant was applied to a column (9cm×0.9cm) of CM-cellulose and chromatographed as described before (Khalkhali & Marshall, 1976). The fractions containing both ribonuclease A and the putative monoglycosylated ribonuclease were combined, dialysed against water and freeze-dried. The residue was dissolved in 1ml of Tris/HCl, pH7.0 (10mM in Tris), and the solution was applied to an affinity column (2.0cm×0.3cm) of wheat-germ agglutinin–Sepharose and chromatography was performed as described previously (Khalkhali & Marshall, 1976).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This was carried out by the procedure of Marshall & Zamecnik (1969) (see also Khalkhali & Marshall, 1976).

Ribonuclease activity. This was assayed by the method of Kalnitsky *et al.* (1959).

Results

Preparations of rough endoplasmic reticulum isolated from regenerating rat liver 48h after partial hepatectomy were found to contain higher activities of UDP-*N*-acetylglucosamine–asparagine-sequon *N*-acetylglucosaminyltransferase than did those from rats which had been subjected to laparotomy and palpation of the liver. There was no direct proportionality between the extent of incorporation of *N*-acetylglucosamine into the acceptor substrate, ribonuclease A, and the amount of rough endoplasmic reticulum used (Fig. 1). Comparative estimates of activity may nevertheless be made by considering the results obtained in the presence of relatively small amounts of enzyme protein (for example 20μg), and it is suggested that the rough endoplasmic reticulum from the hepatectomized animal was about 4–5 times as active as that from the laparotomized rat. There

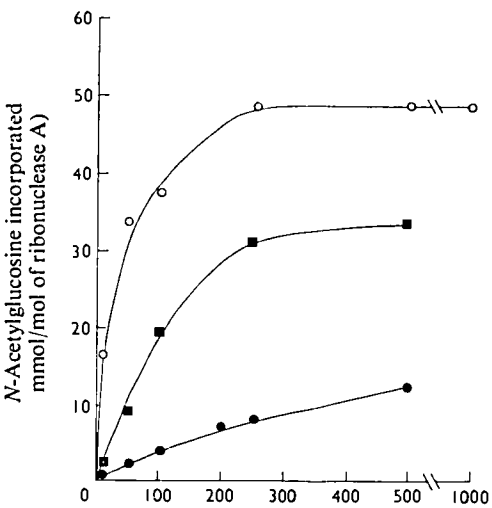


Fig. 1. *Incorporation of N-acetylglucosamine into ribonuclease A catalysed by preparations of rough endoplasmic reticulum*: ○, *from rabbit liver*; ■, *from regenerating rat liver, 48h after partial hepatectomy*; ●, *from sham-operated rat liver, 48h after operation*

Incubations (see under 'Methods') were for 1h at 37°C, and the UDP-*N*-acetylglucosamine concentration in the experiments in which preparations of rabbit liver were used was 0.28mM. Control experiments were always done, where ribonuclease A was omitted from the incubation mixtures, and corrections were applied for glycosylation of endogenous glycoproteins. Replicate values with a given preparation of rough endoplasmic reticulum generally agreed within 6%.

was even higher enzymic activity in preparations of rough endoplasmic reticulum from rabbit liver (Fig. 1), which was tested at a lower concentration (0.28mM) of UDP-*N*-acetylglucosamine than was used in the experiments with preparations from rat liver. Activities of rabbit liver preparations in the presence of 100μg of rough-endoplasmic-reticulum protein at concentrations of UDP-*N*-acetylglucosamine of 0.1, 0.2, 0.28, 0.4 and 0.5mM were in the proportions 0.54, 0.84, 1.0, 1.16 and 1.26 respectively.

Preparations of rough endoplasmic reticulum from normal rat liver appeared to contain extremely low

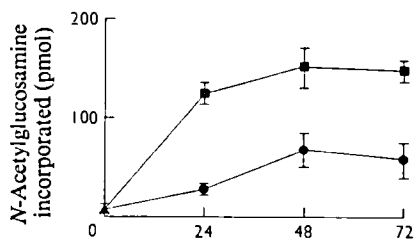


Fig. 2. *N-Acetylglucosaminyltransferase activities of preparations of rough endoplasmic reticulum made from regenerating rat liver* (■) *or from the livers of sham-operated animals* (●)

▲, Results with control animals. Assays were done as described in the legend to Fig. 1, except that the results are in all cases for 100μg of rough-endoplasmic-reticulum protein.

4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine synthetase activity, which increased to a maximum value about 20-fold greater in regenerating liver within 48h after partial hepatectomy (Fig. 2).

In the experiments described so far, it has been assumed that the differences obtained in incorporation of *N*-acetylglucosamine into acid-precipitable protein in the presence or absence of added ribonuclease A could be interpreted as an indication that the latter was indeed the acceptor of the sugar. This reasonable assumption was confirmed by isolation and identification of the glycosylated ribonuclease.

The products obtained when 1mg of bovine pancreatic ribonuclease A was glycosylated, with a preparation of rough endoplasmic reticulum made from regenerating liver 48h after partial hepatectomy as the enzyme source (see under 'Methods'), were chromatographed on CM-cellulose (Fig. 3). Ribonuclease that had undergone glycosylation (fractions 52–60) is known to be eluted, under the conditions of chromatography used, immediately before and overlapping with ribonuclease A (Khalkhali & Marshall, 1975, 1976). Fractions 50–70 (Fig. 3; 1752pmol of *N*-acetylglucosamine, equivalent to 2.5% glycosylation of the ribonuclease A used) were combined and subjected to chromatography (see under 'Methods') on wheat-germ agglutinin–Sepharose. The radioactive compound was eluted from the affinity column at a relatively low concentration (0.15mM) of the *N*-acetylglucosamine eluting agent. This concentration is near to that found earlier to be needed to release monoglycosylated ribonuclease from an affinity column (Khalkhali & Marshall

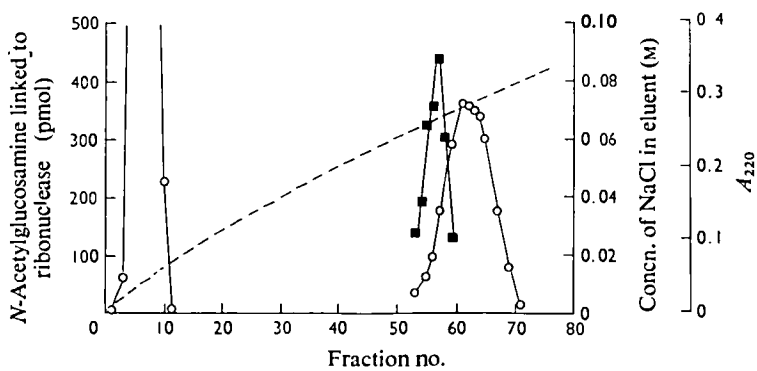


Fig. 3. *Chromatography on a column* (9cm×0.9cm) *of CM-cellulose of a mixture incubated as described under 'Methods'*

About 2.5% conversion of ribonuclease A (eluted from the column at 0.07M-NaCl) into *N*-acetylglucosaminyl-ribonuclease (eluted at 0.067M-NaCl) had occurred. ○, A_{220} (protein); ■, *N*-acetylglucosamine incorporated into ribonuclease (i.e. monoglycosylated ribonuclease); ----, concn. of NaCl in the eluent. Fractions of volume 3ml were collected.

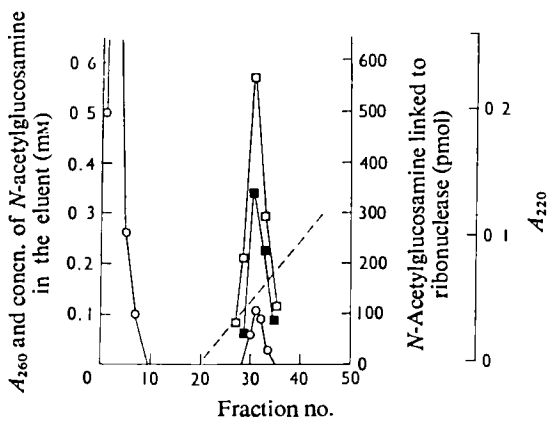


Fig. 4. *Separation by chromatography on a column* (2.0 *cm* × 0.3 *cm*) *of wheat-germ agglutinin–Sepharose of ribonuclease A and N-acetylglucosaminyl-ribonuclease*

For details see under 'Methods'. ○, A_{220}; ■, *N*-acetylglucosamine incorporated into ribonuclease; □, ribonuclease activity (A_{260}; Kalnitsky *et al.*, 1959) of monoglycosylated ribonuclease; ----, concentration of sugar (*N*-acetylglucosamine) in the eluent. Fractions of volume 1.4ml were collected. There was no radioactive bound glucosamine in fractions 1–25 or in fractions 37–45. Only fractions 26–37 were tested for ribonuclease activity (Kalnitsky *et al.*, 1959), to assess whether the radioactivity-labelled protein eluted at that position had ribonuclease activity.

1976). Moreover, this peak is associated with ribonuclease activity (Fig. 4). This peak of ribonuclease contained 1475pmol of bound *N*-acetylglucosamine, which corresponds to glycosylation of 2.1% of the ribonuclease A originally used.

A portion (0.30ml) of the radioactive fraction 31 from the wheat-germ agglutinin–Sepharose column (Fig. 4) was subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969). The gel was cut laterally into 3mm slices and these were dissolved in 2ml of aq. 30% (v/v) H_2O_2 in scintillation vials (Goodman & Matzura, 1971). The solutions were evaporated to dryness and the residues were counted for radioactivity. In only one of them, that corresponding to the position of ribonuclease A run as a control, was there any radioactivity (70pmol; 94% recovery).

All these results show that preparations of rough endoplasmic reticulum from regenerating rat liver or from the liver of injured rats can catalyse the incorporation of glucosamine into ribonuclease A, with UDP-*N*-acetylglucosamine as the donor. It is likely from our previous studies (Khalkhali & Marshall, 1975, 1976; Khalkhali *et al.*, 1976) that asparagine-34 is the glycosylated residue.

Discussion

Preparations of rough endoplasmic reticulum, made from rat liver by the procedure of Dallner (1963), appear to have extremely low UDP-*N*-acetylglucosamine–asparagine-sequon *N*-acetylglucosaminyltransferase activity. The results obtained by our standard assay procedure suggest that preparations from rabbit liver have transferase activity that is about 100 times that in normal rat liver. The reasons for the observations are unknown and the results serve solely to explain why we were unable, in earlier experiments, to isolate monoglycosylated ribonuclease from appropriate incubation mixtures containing rat liver rough endoplasmic reticulum as a source of enzyme, whereas the product could be isolated in a reasonable yield when rabbit liver rough endoplasmic reticulum was used (Khalkhali & Marshall, 1975). It is possible that the low proportion of carbohydrate (23%) in orosomucoid in rat serum (Charlwood *et al.*, 1976) compared with that in rabbit serum (44%) is due, in part at least, to the lower activity of the asparagine-sequon-glycosylating enzyme in rat liver, but there may be other explanations.

There is an increased transferase activity in the rough endoplasmic reticulum of the livers of rats which have undergone laparotomy and palpation of the liver, over the course of a few days after the operation. There are also higher activities of the enzyme in regenerating rat liver, with an apparent peak of activity about 48h after partial hepatectomy. The implications of our findings with respect to the increases in the contents of serum glycoproteins seen in a number of pathological states cannot be usefully speculated on until much more knowledge is available about the mechanism of action of the transferase in question.

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Properties of membrane-bound UDP-N-acetylglucosamine — asparagine sequon N-acetylglucosaminyl transferase in preparations of endoplasmic reticulum from rabbit liver and from regenerating rat liver.

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Synopsis

The mechanism of *in vivo* incorporation of asparagine-linked carbohydrate moieties of glycoproteins is poorly understood. There is enzymic activity in preparations of rough endoplasmic reticulum from rabbit liver and in regenerating rat liver which can catalyse *in vitro* the conversion of ribonuclease A to its ^{34}Asn-monoglycosylated form. The enzyme appears to require the structural integrity of the membrane for its activity.

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The mechanism and control of formation of the carbohydrate moieties which are covalently bound to asparagine residues of glycoproteins are but poorly understood. There is evidence that assembly of the carbohydrate units of the myeloma protein MOPC-104E (Parkhouse & Kelchers, 1971; Kelchers, 1973) and of hen's egg albumin (Kiely et al., 1976) occurs in several distinct steps, and some of the sugar residues are added to the nascent protein chains whilst the latter are still attached to the ribosomes. There are also data (see Waechter & Lennarz, 1976) which suggest that in a number of eukaryotic cells, including hepatocytes, the carbohydrate moiety is added, at least in part, by direct transfer, to an appropriate asparagine residue, of the oligosaccharide from the lipid-sugar complex

$$(\alpha\text{-Man})_n\text{-}\beta\text{-D-Man-}(1\rightarrow4)\text{-}\beta\text{-D-GlcNAc-}(1\rightarrow4)\text{-}\beta\text{-D-GlcNAc-P-P-dolichyl}$$

There is present, in preparations of rough endoplasmic reticulum from rabbit liver, enzymic activity which can catalyse *in vitro* the attachment of an N-acetylglucosamine residue to the asparagine moiety in the sequon ...Asn.Leu.Thr..., present in bovine pancreatic ribonuclease A (Khalkhali & Marshall, 1975). The level of activity of the transferase in preparations

of rough endoplasmic reticulum from rat liver appears to be very much lower than in that from rabbit liver. The activity is increased considerably in regenerating rat liver (Khalkhali et al., 1977).

The transferase in rough endoplasmic reticulum from rabbit liver has a pH optimum (7.4) slightly different from that from regenerating rat liver (pH 6.9, Fig 1a). The other sugar transferases which have been shown in vitro to catalyse the addition of a sugar to an amino acid residue of a polypeptide chain, namely the xylosyl (—> Ser) transferase from embryonic chick epiphyseal cartilege (Stoolmiller et al., 1972), the N-acetylgalactosaminyl (—> Ser, Thr) transferase from sheep submaxillary gland (McGuire & Roseman, 1967) and the galactosyl (—> Hyl) transferase from rat kidney cortex (Spiro & Spiro, 1971), all exhibit pH optima in the region of pH 7.

There is a marked increase in activity as the temperature of assay is increased, up to 37°C (Fig 1b). At higher temperatures the activity is reduced.

For the rabbit liver enzyme the apparent K_m value for UDP-N-acetyl-glucosamine at 37° was 2.27×10^{-4} M but the presence of phosphatases or pyrophosphatases in the membrane preparations needs to be taken into consideration. The K_m value at 37° for ribonuclease A was about 2.2×10^{-5} M for UDP-N-acetylglucosamine —— asparagine sequon N-acetyl-β-D-glucosaminyl activity in preparations of rough endoplasmic reticulum from both rabbit and regenerating rat liver. The values found by others for carp swim-bladder ichthyocol and for disaccharide-free bovine renal glamerular basement membrane with rat kidney cortex galactosyl transferase were 6.4×10^{-3} M and 4×10^{-4} M respectively (Spiro & Spiro, 1971). The protein acceptor core of sheep submaxillary mucin has a K_m value of 2×10^{-3} M with the N-acetylgalactosaminyl transferase (McGuire & Roseman, 1967).

The intracellular distribution of the N-acetylglucosaminyl transferase is of interest. The levels of activities of the enzyme in preparations of both rough and smooth endoplasmic reticulum from rabbit liver were compared. The procedure of Dallner (1963) as described by Lawford & Schachter (1966) was used for isolating the preparations from rabbit liver which had been frozen on removal from the animal.

The respective fractions contained RNA(Ceriotti, 1954): protein (Lowry, et al, 1951; with bovine serum albumin as standard) ratios of 0.57 and 0.10 respectively. The rates of incorporation of N-acetylglucosamine into ribonuclease was 390 ± 26 nmoles per mole ribonuclease per h per mg enzyme protein when catalysed by the preparation of rough endoplasmic reticulum and 94 ± 14 nmoles when catalysed by the smooth endoplasmic reticulum. The higher activity exhibited by the preparation of rough endoplasmic reticulum might suggest that addition of N-acetylglucosamine to the asparagine residue occurs whilst the nascent protein is associated with polysomes. But final conclusions must await information concerning the substrate specificity of UDP-N-acetylglucosamine — asparagine sequon N-acetyl-β-D-glucosaminyl transferase and its mechanism of action.

The enzyme in the rough endoplasmic reticulum, being membrane bound, is activated by Triton-X 100 over the concentration range 0.04% to 1.0%. Sonication leads to considerable losses of activity if continued for more than about 30 sec (Fig 2a). Treatment of the membrane with phospholipase A_2 (EC 3.1.1.4) leads to an extensive loss in activity of the sugar transferase (Fig 2a). It seems likely that the structural integrity of the membrane is important for the activity of the sugar transferase.

The enzyme in rabbit liver rough endoplasmic reticulum is activated to a small extent by CDP-choline (Fig 2b). The effects of the nucleotide might be due to inhibition of a pyrophosphatase. On the other hand CDP- choline

might function to stimulate incorporation of N-acetylglucosamine into one or more lipid-linked intermediates (Mookerjea et al., 1972) which might participate in the reaction. N-Acetyl glucosamine at concentrations up to 20 mM does not appear to inhibit the enzyme.

The mechanism of in vivo incorporation of asparagine- linked carbohydrate moeities of glycoproteins is unresolved generally. Further studies of the N-acetylglucosaminyl transferase are likely to help in understanding this important biosynthetic pathway.

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Legends to Figures

Fig 1 (a) Effect of pH on N-acetylglucosaminyl (—> asparagine sequon) transferase activity in preparations of rough endoplasmic reticulum from rabbit (-●-) and regenerating (48 h post hepatectomy) rat (-■-) liver. Assays were done as described earlier (Khalkhali et al., 1977). The concentration of UDP-GlcNAc used in the rabbit liver experiments was 0.28 mM (291 dpm/nmole) and in those with rat liver was 0.506 mM (1096 dpm/nmole). An amount of 250 µg enzyme protein was used in the experiments and incorporation of GlcNAc into ribonuclease A was linear over the period of time studied. Results are given also for the enzyme activity present in the 35-45% ammonium sulphate fraction isolated from pooled, AB, Rh positive plasma, (-o-; assayed by a procedure closely similar to that in Table II, column 2 of Khalkhali & Marshall, 1976; enzyme protein amount was ~~0.25 mg~~ 250 µg per tube) and for the enzyme activity in preparations of yeast cell membranes (-Δ-; assayed by a similar method to that described in Table 1, column (a) of Khalkhali et al., 1976; 1 h incubation). Assays were done in Tris-maleate buffer (0.05 M in each ion, adjusted to the various pH values with MNaOH).

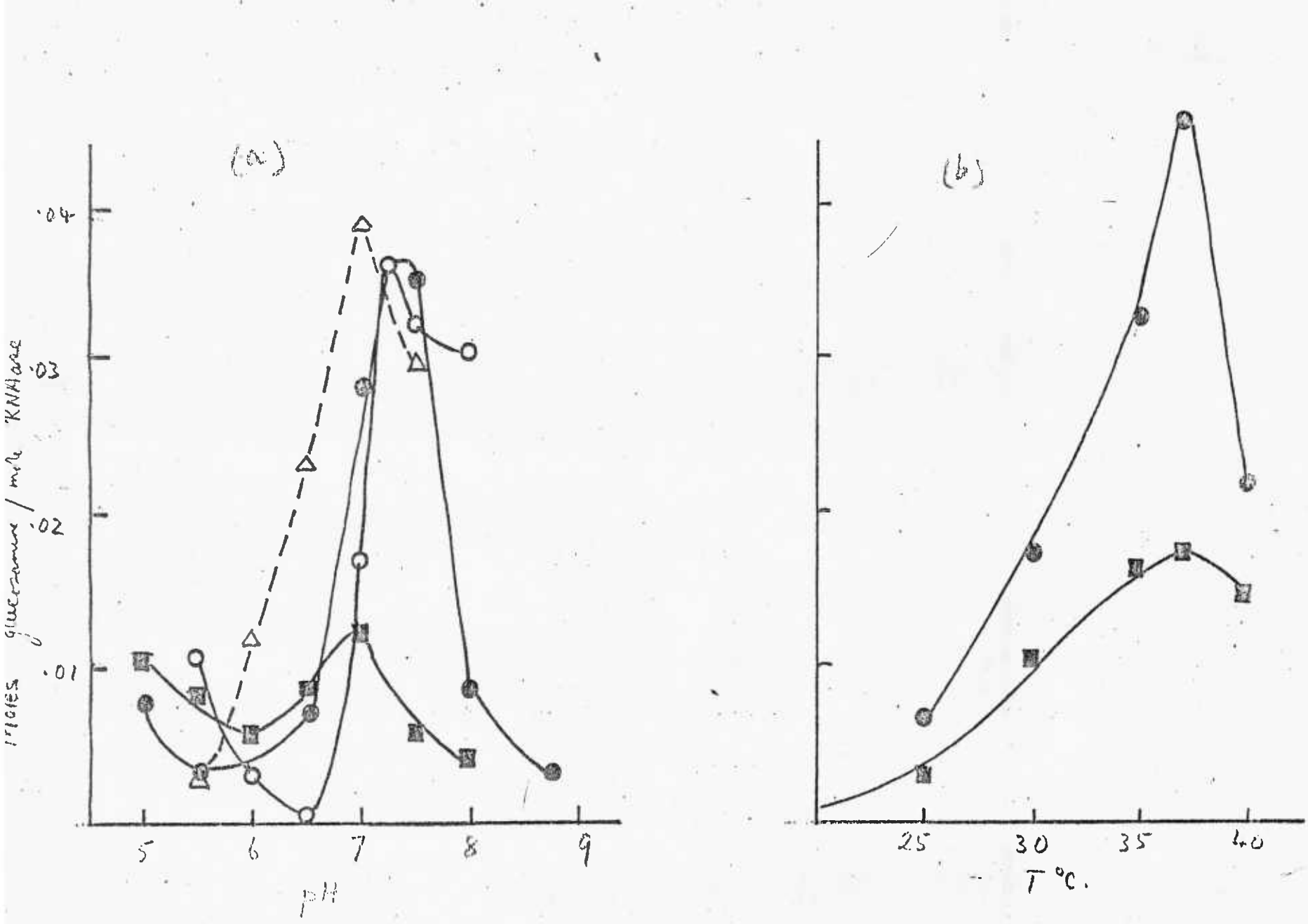
1 (b) Effect of temperature on transferase activity in preparations of rough endoplasmic reticulum from rabbit (-●-; at pH 7.4) and regenerating (48 h post-hepatectomy) rat (-■-; at pH 7.1) liver.

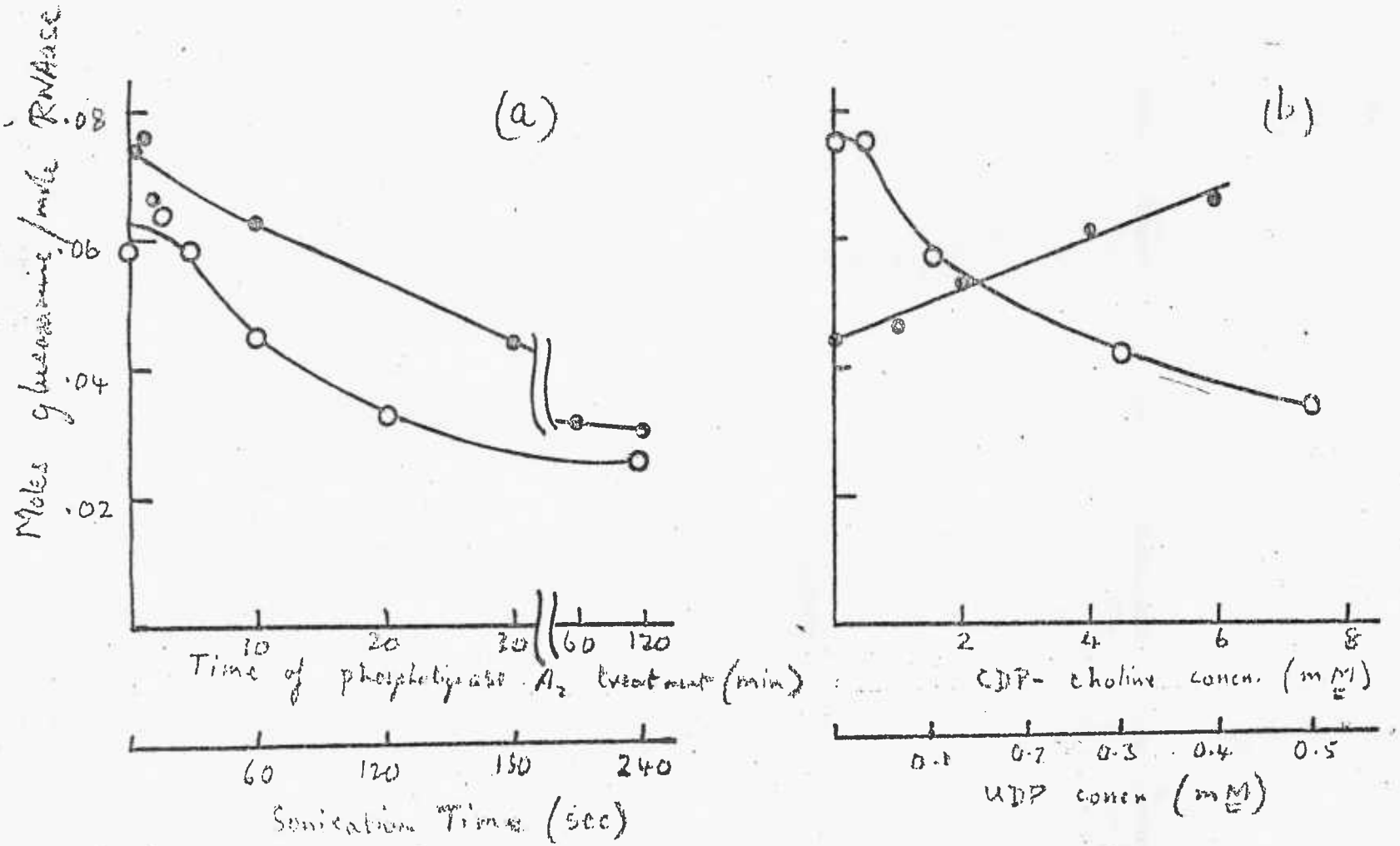
2 (a) Effect of sonication time (-o-; 0.2 ml vols of a suspension of rough endoplasmic reticulum (10 mg protein/ml) in cold (2°C) 0.05 mM Tris-maleate buffer, pH 7.4 were sonicated at 1A (MSE Ultrasonic Power Unit)) on transferase activity of preparation of rough endoplasmic reticulum from rabbit liver. Assay conditions as in legend to Fig 1A using 50 µl of sonicated samples.

The effect of treatment of the membrane with phospholipase A_2 (Boehringer,

cat. no. 15057) on the enzyme activity is also shown (-●-; 4 mg of rough endoplasmic reticulum protein in 0.4 ml 0.05 M Tris buffer, pH 8.0 containing 40 µg (8 I.U.) phospholipase A_2, were incubated at 25°. Reaction was stopped by adding EDTA to aliquots removed for assay (25 µl ≙ 250 µg enzyme protein) to a final concentration of 5 mM; EDTA at the concentration used causes a loss of about 7% of the transferase activity). Assays were done as in the legend to Fig 1a.

2 (b) Effects of CDP- choline (-●-) and of UDP (-o-) on the UDP-N-acetyl-glucosamine —— asparagine sequon N-acetyl-β-D-glucosaminyl transferase activity of preparations of rough endoplasmic reticulum from rabbit liver. Assays were done as in the legend to Fig 1a, with 250 µg enzyme protein in each case.


(a)
·04
·03
·02
·01
5
6
7
8
9
pH
(b)
25
30
35
40
T °C.


(a)
Moles glucosamine / mole RNAase
.08
.06
.04
.02
10
20
30
60
120
Time of phospholipase A2 treatment (min)
60
120
180
240
Sonication Time (sec)
(b)
2
4
6
8
CDP-choline concn. (mM)
0.1
0.2
0.3
0.4
0.5
UDP concn (mM)

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