STUDIES ON THE ASPARAGINE TO N-ACETYLGLUCOSAMINE LINKAGE IN GLYCOPROTEINS.

A thesis submitted by
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ABSTRACT.

Present knowledge of the occurrence and structure of carbohydrate-protein linkages, and of the stability of these linkages under alkaline conditions, is reviewed. Methods that have been employed to determine the primary structure of carbohydrate moieties are discussed, and special reference is made to methods that involve treatment of glycoproteins with alkali or sodium borohydride in alkali.

The effects of alkali and alkaline borohydride on 4-N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn) were studied, and some interesting products were identified. It was found that reduced oligosaccharides may be obtained from glycoproteins containing GlcNAc-Asn as linking moiety by treatment with sodium borohydride in alkali at 100°C, and the value of the method was demonstrated by the preparation of reduced oligosaccharides from hen ovalbumin.

A hypothesis that attempts to explain why only specific L-asparaginyl residues become glycosylated is discussed. These residues are followed by a 3-hydroxyamino acid in a position next-but-one towards the C-terminus of the apoprotein. It has been suggested that hydrogen bonding occurs between the hydroxyl group of the 3-hydroxyamino acid residue and the oxygen atom of the amide group of the L-asparaginyl residue. Attempts have been made to test the validity of this hypothesis by the titration, with acid and base, of glycopeptides containing the specific amino acid sequence.

It is accepted that certain biological activities of some glycoproteins may be influenced by the stereochemical characteristics of carbohydrate moieties. The chiroptical properties of GlcNAc-Asn have been studied in order to elucidate some of these characteristics. The conformation of GlcNAc-Asn in aqueous solution has been established and compared, by X-ray diffraction studies, to those adopted in the crystalline state and when bound to lysozyme.

ABBREVIATIONS.

OSM	Ovine submaxillary mucin
PSM	Porcine submaxillary mucin
BSM	Bovine submaxillary mucin
Dns- or dansyl	1-Dimethylaminonaphthalene-5-sulphonyl
Dns-OH	1-Dimethylaminonaphthalene-5-sulphonic acid
Dns-NH_2	1-Dimethylaminonaphthalene-5-sulphonamide
GlcNAc-Asn	4-N-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine
GlcN-Asn	4-N-(2-Amino-2-deoxy-β-D-glucopyranosyl)-L-asparagine
Glc-Asn	4-N-α-D-Glucopyranosyl-L-asparagine
GlcNAc-N_3	2-Acetamido-2-deoxy-β-D-glucopyranosyl azide
GlcNAc-NH_2	2-Acetamido-2-deoxy-β-D-glucopyranosylamine
GlcNAc-NHAc	2-Acetamide-1-N-acetyl-2-deoxy-β-D-glucopyranosylamine
Glc-NHAc	N-Acetyl-β-D-glucopyranosylamine
Man-NHAc	N-Acetyl-β-D-mannopyranosylamine
β Gal-NHAc	N-Acetyl-β-D-galactopyranosylamine
α Gal-NHAc	N-Acetyl-α-D-galactopyranosylamine
Ara-NHAc	N-Acetyl-α-L-arabinopyranosylamine
Xyl-NHAc	N-Acetyl-β-D-xylopyranosylamine
GlcNol	2-Amino-2-deoxy-D-glucitol
ManNol	2-Amino-2-deoxy-D-mannitol
Chitobiose	2-Amino-2-deoxy-β-D-glucopyranosyl-(1$\rightarrow$4)-2-amino-2-deoxy-D-glucopyranose
Chitobiol	2-Amino-2-deoxy-β-D-glucopyranosyl-(1$\rightarrow$4)-2-amino-2-deoxy-D-glucitol
ORD	Optical rotatory dispersion
CD	Circular dichroism
Glycopeptide	The definition of this term has been extended to include materials that are obtained from glycoproteins and that contain carbohydrate covalently bound to only one amino acid residue. The term has been extended in this way previously (see for example Fletcher, Marks, Marshall & Neuberger, 1963).

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CONTENTS.

Page

LIST OF TABLES.

LIST OF FIGURES.

LIST OF PLATES.

Table 1.1. Some glycoproteins and their carbohydrate content.

From animal tissues:-

No.*	Type	Example	Mol.Wt.	Total no. sugar residues
1	Enzymes	Bovine pancreatic ribonuclease B	14,700	7
2		Porcine pancreatic ribonuclease	21,000	38
3		Bovine pancreatic deoxyribonuclease	31,000	7
4		Snake venom L-amino acid oxidase	70,000	40
5		Snake venom proteinase B	95,000	85
6		Bovine liver β-glucoronidase	280,000	(8%)
7	Hormones	Porcine Follicle stimulating hormone	32,000	13
8		Human chorionic hormone	27,000	41
9		Human thyroglobulin	670,000	351
10		Ovine interstitial cell-stimulating hormone	16,300	13
11	Immunoglobulins	Rabbit γG	140,000	19
12		Human γA	140,000	58
13		Human γM	890,000	457
14	Egg-white	Hen ovalbumin	45,000	8
15		Hen ovomucoid	28,000	38
16		Hen avidin	53,000	2
17		Hen ovotransferrin	80,000	14
18	Plasma	Calf fetuin	48,000	49
19		Human α_1-acid glycoprotein	44,000	89
20		Human transferrin	90,000	24
21		Human haptoglobulin 2-1	200,000	143
22		Bovine fibrinogen	330,000	42
23	Urine	Human Tamm-Horsfall protein	80,000	103
24	Connective Tissue	Dog tendon collagen		(0.8%)
25	Extra-cellular membranes	Bovine glomerular basement membrane		(9.4%)
26	Cellular membranes	M and N specific glycoproteins from red-cell membranes	30,000	

(cont. opp. p.30)

CHAPTER 1. INTRODUCTION.

PART 1. THE NATURE OF GLYCOPROTEINS.

Glycoproteins are characterised as a group of proteins that contain covalently linked carbohydrate moieties that have structural features in common and require, at least in part, similar enzymatic mechanisms for their assembly and breakdown. Several writers have noted the wide diversity of substances that may be classified as glycoproteins (see, for example, Marshall & Neuberger, 1970). Some of them have biological activity as enzymes, hormones, immunoglobulins and plasma clotting factors. They are of widespread occurrence: egg-white, milk, serum and urinary proteins often have covalently-attached carbohydrate, and they are components of connective tissue, mucinous secretions and cellular membranes (see Table 1.1). Glycoproteins may be soluble globular proteins or insoluble protein types such as collagen.

Glycoproteins are found in mammalian tissues, in plants and in bacteria. Molecular weights range between those of relatively small glycoproteins such as bovine pancreatic ribonuclease B (No. 1 in Table 1.1) and ovine submaxillary mucin (MW 1 x 10^6) (No. 27 in Table 1.1), and the carbohydrate content from less than 1% (No. 24 in Table 1.1) to greater than 80% (No. 27 in Table 1.1). Anything from one to seven types of sugar residues may constitute the oligosaccharide moiety and the size, complexity and number

Table 1.1 (cont).

No.*	Type	Example	Mol.Wt.	Total no. sugar residues
27	Mucins	Ovarian cyst fluid A substance	1,000,000	4,120
28		Sheep submaxillary gland glycoprotein	1,000,000	1,600
29	From plants	Pineapple stem bromelain	33,000	9
30		Soybean haemagglutinin	110,000	33
31		Glucoamylase from Aspergillus niger		(15%)
32	From bacteria	Envelope-specific glycoprotein from Escherichia Coli B		(4%)

* Also stipulates the references below.

1. Plummer & Hirs (1964)
2. Reinhold et al. (1968)
3. Catley, Moore & Stein (1969)
4. de Kok & Rawitch (1969)
5. Oshima, Iwanaga & Suzuki (1968)
6. Plapp & Cole (1967)
7. Cahill et al. (1968)
8. Bahl (1969)
9. Spiro & Spiro (1965)
10. Walborg & Ward (1963)
11. Graham & Neuberger (1968)
12. Dawson & Clamp (1968)
13. Miller & Metzger (1965)
14. Johansen, Marshall & Neuberger (1961)
15. Montgomery & Wu (1963)
16. Melamed & Green (1963)
17. Williams (1967)
18. Spiro (1960)
19. Eylar & Jeanloz (1962)
20. Jamieson (1965a)
21. Gerbeck, Bezkorovainy & Rafelson (1967)
22. Bray & Laki (1968)
23. Fletcher, Neuberger & Ratcliffe (1970)
24. Kefalides & Winzler (1966)
25. Spiro (1967)
26. Kathan & Adamany (1967)
27. Aminoff, Morgan & Watkins (1950)
28. Pigman & Gottschalk (1966)
29. Murachi, Suzuki & Takahashi (1967)
30. Lis, Sharon & Katchalski (1966)
31. Lineback (1968)
32. Okuda & Weinbaum (1968)

of oligosaccharide units per polypeptide chain too may vary. The oligosaccharide moiety may be as simple as the single O-β-D-galactopyranosyl residue found in collagen (Spiro, 1969), or it may be far more complex. The limit in size would appear in general to be about fifteen sugar residues, although the heterosaccharide moieties of glycosaminoglycans are believed to be much larger. For example, keratan sulphates isolated from various sources by treatment with papain and cold alkali have number-average molecular weights of 11,500 (bull-shark cartilage), 10,000 (human cartilage), 8,900 (ox cornea) and 9,700 (chicken cornea) (Mathews & Cifonelli, 1965).

It may be seen that the presence of carbohydrate does not impart any properties to a protein which are specific for glycoproteins. The structural features of glycoproteins that differentiate them from other proteins are the carbohydrate-protein linkages. The investigations to be described are concerned with several aspects of the linkage involving L-asparagine and N-acetyl-D-glucosamine.

PART 2. THE CARBOHYDRATE-PROTEIN LINKAGES.

Section 1. The amino acids and sugars involved in linkages.

A degree of specificity is exhibited in both the amino acid and sugar residues involved in carbohydrate-protein linkages. Glycosylated residues of the amino acids L-asparagine, L-serine, L-threonine, 5-hydroxy-L-lysine and L-cysteine have been found. The majority of the linkages elucidated so far fall into three distinct types.

In the first type of linkage, the amide group of an

L-asparagine residue is bound to the C_1 atom of an N-acetyl-D-glucosamine residue. This linkage is known to occur in many serum and egg-white proteins and was first described in hen ovalbumin (see Gottschalk, 1966). Glycoproteins containing this linkage are often of relatively low molecular weight and generally possess only a few heterosaccharide moieties per mole of protein. The sugar residues D-mannose, N-acetyl-D-glucosamine, D-galactose, L-fucose and sialic acid are often found in the moiety bound by this type of linkage, whereas N-acetyl-D-galactosamine seldom occurs.

The second type of linkage involves β-hydroxy amino acids, which are often bound to N-acetyl-D-galactosamine residues. Various mucins (Harbon et al., 1964; Tanaka, Bertolini & Pigman, 1964) and blood group substances (Lloyd & Kabat, 1968) contain this sugar residue linked to both L-seryl and L-threonyl residues. Glycoproteins containing this type of linkage often have relatively high molecular weights, and possess a high percentage of their weight as carbohydrate. They generally contain D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose and sialic acid, but seldom D-mannose. D-Xylose also serves as the linking sugar in proteoglycans, but is bound only to L-serine (Rodén, 1968).

A third type of glycoprotein linkage has been found in guinea pig skin collagen (Butler & Cunningham, 1966; Spiro, 1969) and in ox glomerular basement membranes (Spiro, 1967). It involves an O-glycosidic linkage between a D-galactose residue and the hydroxyl group of 5-hydroxy-L-lysine.

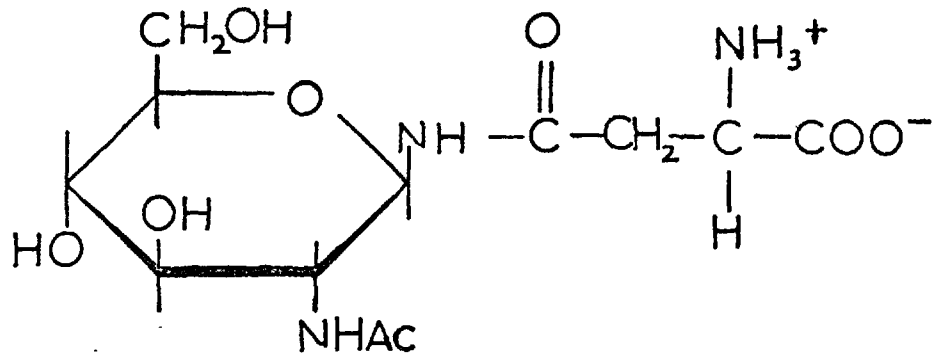
Glycopeptides which contain glycosylated residues of

L-cysteine have been reported as components of red cell membranes (Weiss, Lote & Bobinski, 1971). The chemical natures of the structures involving some of these amino acids and the sugars to which they are most commonly bound are described in Fig. 1.1.

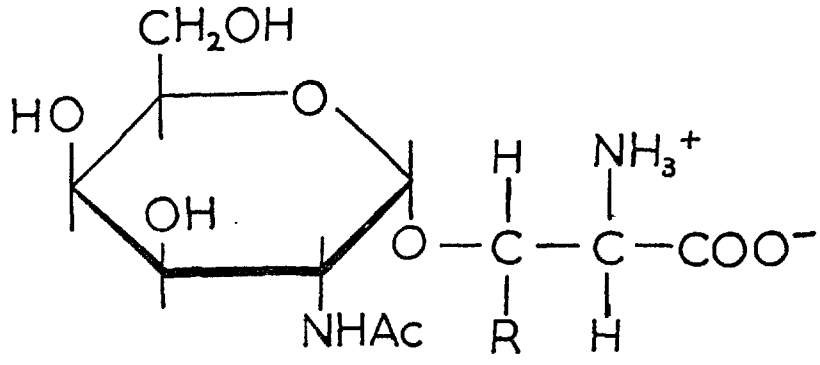
Occasionally other sugars have been found. L-Serine and L-threonine may be linked to D-galactose in earthworm cuticle collagen (Lee & Lang, 1968), to L-arabinose in a hyaluronic acid-polypeptide complex from ox vitreous humor (Hardi, Allen, Turner & Stary, 1969) or with D-mannose in the cuticle collagen of Annelida (Spiro & Bhyroo, 1971) and in an enzyme mycodextranase secreted by Penicillium melinii (Samuel & Nordin, 1971). 4-trans-Hydroxy-L-proline is found in plant extensin (Lamport, 1969), and has been found bound to L-arabinose. The structure O-D-galactopyranosyl-(1→3)-O-D-galactopyranosyl-L-cysteine has been determined in a glycopeptide isolated from human urine (Lote & Weiss, 1971).

A Schiff-base type of linkage involving a sugar and an N-terminal L-valine residue has been proposed for the β-chain of one of the minor haemoglobins (A_{Ic}), which is found in increased amounts in patients with diabetes mellitus. After reduction of the glycoprotein with sodium [^{3}H]borohydride at pH 7 or pH 3.5, followed by acid hydrolysis, an isotopically labelled substance was isolated. Esterification and acetylation yielded a compound with a similar, but not identical, mass spectrum to that obtained with 1-N-(2,3,4,5,6-penta-O-

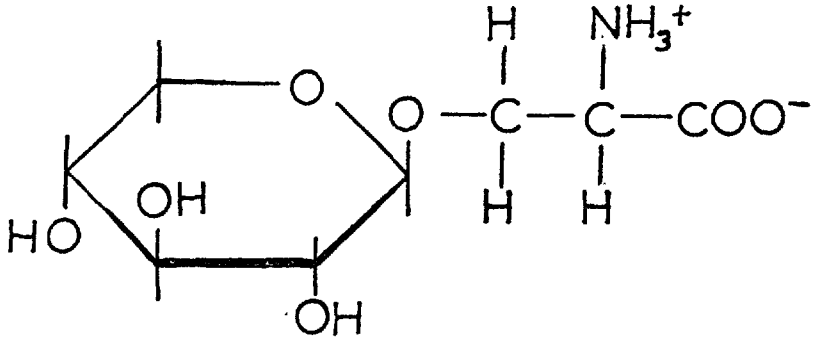
Fig. 1.1. The structures of some carbohydrate-protein linkage moieties.



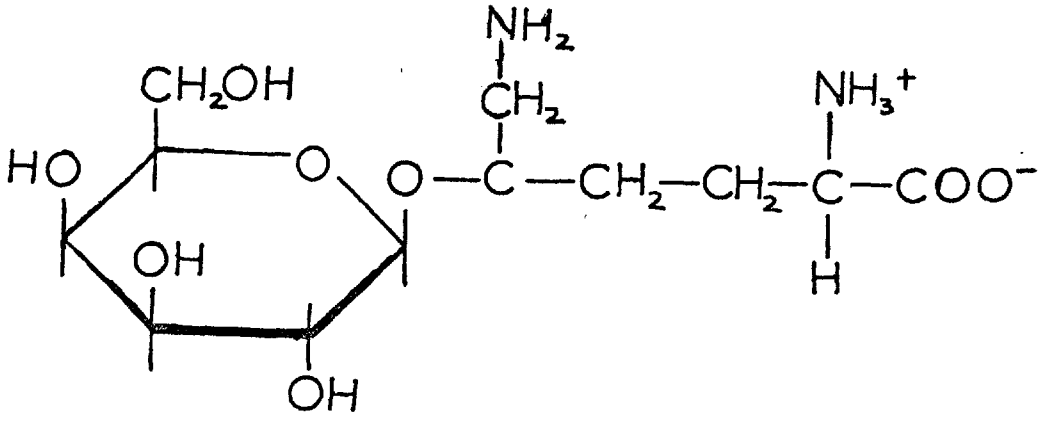
4-N-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine



3-O-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-L-serine (R = H) or -L-threonine (R = Me)



3-O-β-D-Xylopyranosyl-L-serine



5-β-D-Galactopyranosyloxy-L-lysine

acetyl-1-deoxy-D-galactitol-1-yl)-L-valine ethyl ester (Bookchin & Gallop, 1968).

It was suggested originally, partly because cleavage of the carbohydrate-protein linkage occurred readily in mild alkali (Graham, Murphy & Gottschalk, 1963), that a number of proteins contain glycosidic ester linkages, but this suggestion overlooked the likelihood that O-glycosidic bonds involving peptide-linked L-serine and L-threonine residues would be cleaved readily under these conditions by a β-elimination reaction. Further evidence for glycosidic esters in BSM and OSM was presented by Murphy & Gottschalk (1961), who observed that reduction by lithium borohydride in tetrahydrofuran of glycopeptides that had been solubilised in this solvent by digestion with trypsin and treatment with phenylisothiocyanate resulted in the loss of 83% of the total dicarboxylic acids and the production of 2-amino-4-hydroxy-L-butyric acid (L-homoserine) and 2-amino-5-hydroxy-L-pentanoic acid. These observations have not been fully explained, but it was suggested that diborane was formed during the decomposition of excess lithium borohydride with an anhydrous solution of hydrogen chloride in methanol. Diborane will reduce carboxylic acids (Gottschalk & König, 1968). The Hestrin hydroxylamine reaction for esters has been applied to OSM, BSM and PSM, and no glycosidic ester could be found, although the BSM used contained acetyl esters linked to sialic acid residues (Bertolini & Pigman, 1967).

Section 2. Glycoproteins with more than one type of carbohydrate-protein linkage.

Some glycoproteins are known to contain two types of carbohydrate-protein linkage. Thomas & Winzler (1969) showed that 25% of the carbohydrate in M and N-specific glycoproteins from erythrocyte membranes is linked via alkali-labile bonds. They have also isolated a glycopeptide (see p.5) that contains only L-aspartic acid (Thomas & Winzler, 1971). The chondromucoprotein prepared from ox nasal-septa without the use of alkali or proteolytic enzymes gave, on treatment with 0.5M-NaOH at 25°C for 24 hr, protein-free chondroitin sulphate, and an alkali-resistant glycopeptide containing keratan sulphate (Partridge & Elsden, 1961). Chondroitin sulphate and keratan sulphate may be linked, therefore, to the same polypeptide chain. Ox corneal keratan sulphate also appears to be involved in a linkage that is stable to 0.5M-KOH for 48 hr (Seno, Meyer, Anderson & Hoffman, 1965)&that may involve L-asparagine and N-acetyl-D-glucosamine (Greiling, Stuhlsatz, Kisters & Plagemann, 1968). Renal glomerular basement membrane may contain a heteropolysaccharide unit bound to L-asparagine as well as the disaccharide units bound to 5-hydroxy-L-lysine (Spiro, 1967). Linkages involving L-asparagine and L-serine or L-threonine appear to be present on the H-chain of rabbit γG immunoglobulin (Smyth & Utsumi, 1967) and in the human γA (Bra) myeloma protein (Dawson & Clamp, 1968). A heavy-chain disease protein (Cra)(Clamp, Dawson & Franklin, 1968), lactotransferrin (Spik & Montreuil, 1968) and γA lactoglobulin

Table 1.2

Glycoproteins which are known to contain GlcNAc-Asn.

Hen ovalbumin (Johansen, Marshall & Neuberger, 1961)

Hen ovomucoid (Tanaka, 1961)

Human α_1-acid glycoprotein (Eylar, 1962)

Guinea-pig α_1-acid glycoprotein (Cunningham & Simkin, 1966)

Human transferrin (Jamieson, 1965a)

Human lactotransferrin (Spik & Montreuil, 1968)

Bovine fibrinogen (Mester & Vaas, 1964)

Calf fetuin (Spiro, 1964)

Human ceruloplasmin (Jamieson, 1965

Rabbit γG globulin (Nolan & Smith, 1962a)

Human γG globulin (Rothfus & Smith, 1963)

Bovine γG globulin (Nolan & Smith, 1962b)

Human myeloma γA protein (Bra) (Dawson & Clamp, 1968)

Heavy chain disease protein (Cra) (Clamp, Dawson & Franklin, 1968)

Mouse Bence Jones protein (MOPC 46) (Melchers, 1969)

Human thyroglobulin (Spiro, 1965)

Bovine ribonuclease B (Plummer, Tarentino & Maley, 1968)

Horse and pig aorta glycoproteins (Noczàr, 1968)

Corneal keratan sulphate (Baker, Cifonelli, Mathews & Rodén, 1969)

Bovine glomerular basement membrane (Spiro, 1967)

Taka-amylase A (Anai, Ikenaka & Matsushima, 1966)

External yeast invertase (Gascón, Neumann & Lampen, 1968)

Soybean haemagglutinin (Lis, Sharon & Katchalski, 1966)

Pineapple stem bromelain (Takahashi, Yasuda & Murachi, 1969)

(Descamps, Monsigny & Montreuil, 1968) are other examples in which these two types of linkages occur.

Section 3. A genetic relationship between the amino acids involved.

There appears to be no obvious relationship between the amino acids that are found glycosylated, but a phylogenetic connection has been proposed by Jamieson & Jett (1971), linking L-asparagine, the β-hydroxy-L-amino acids and L-lysine (which undergoes hydroxylation prior to glycosylation; Rosenbloom, Blumenkrantz & Prockop, 1968). The nucleoside base codons for L-asparagine (AAU and AAC) can give the codons for L-serine (AGU and AGC), for L-threonine (ACU and ACC) and L-lysine (AAA and AAG) by a single base sequence change. L-Asparagine was suggested to be the most primitive acceptor amino acid, the other types having arrived by single mutations. It should be noted, however, that a change to L-cysteine or L-proline would require two base changes.

PART 3. METHODS USED FOR IDENTIFICATION OF THE STRUCTURE OF THE LINKAGE MOIETY.

Section 1. The L-asparagine to N-acetyl-D-glucosamine linkage. Isolation of glycopeptides.

Glycoproteins which are known to contain GlcNAc-Asn as linking component are listed in Table 1.2. Identification of this type of linkage normally involves the isolation of glycopeptides (see p.5) containing L-aspartic

acid as the major, if not the only, amino acid after proteolytic digestion of the protein chain with non-specific enzymes such as subtilisin, papain or pronase. Activities in addition to its proteolytic activity have occasionally been ascribed to some preparations of pronase. Thus, cleavage of the carbohydrate-protein N-acyl-glycosylamine type of linkage in orosomucoid (Kamiyama & Schmid, 1962), in thyroglobulin (Spiro, 1965) and in soybean haemagglutinin (Sharon, 1972), and also partial cleavage of the O-glycosidic linkage (Carubelli, Bhavanandan & Gottschalk, 1965), has been described. Even with pronase, it is often difficult to remove completely the amino acids adjacent to the carbohydrate moiety, and repeated digestion is often necessary; the presence of sialic acid appears to inhibit digestion of some glycoproteins (Cunningham & Simkin, 1966). If peptide material remains attached to the α-carboxyl group of the glycosylated L-asparagine residue, it may sometimes be removed by protecting the free α-amino group with a benzyloxycarbonyl group, followed by treatment with carboxypeptidase (Fletcher, Marks, Marshall & Neuberger, 1963; Kaverzneva & Bogdanov, 1962).

Syntheses of GlcNAc-Asn.

4-N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine, GlcNAc-Asn, has been synthesised and characterised (Marshall & Neuberger, 1964; Bolton, Hough & Kahn, 1966; Tsukamoto, Yamamoto & Miyashita, 1964). The

Table 1.3. Half-lives of some glycosides in 2M-HCl at 100°C.

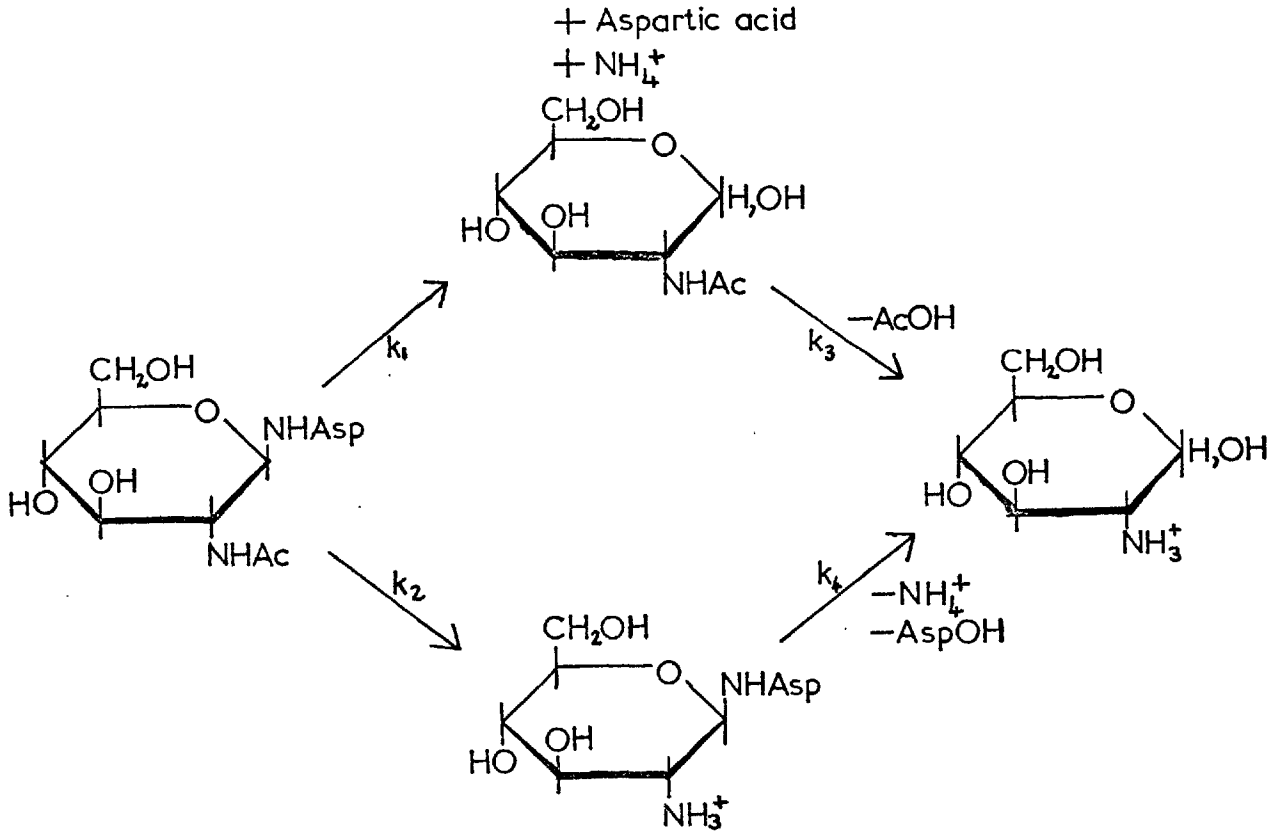
	$t_{\frac{1}{2}}$ (min)	Reference
Methyl α-D-mannopyranoside	3	Overend, Rees & Sequeira (1962)
Methyl α-D-galactopyranoside	1	Overend, Rees & Sequeira (1962)
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	10	From data of Moggridge & Neuberger (1938)
Mannose from hen ovalbumin	6	Graham & Neuberger (1968)
GlcNAc-Asn	17	Marshall (1969)

protected intermediates, 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine and α-benzyl-N-benzyloxycarboxyl-L-aspartate, were condensed in the presence of dicyclohexylcarbodiimide. The protected amino acid derivative required laborious separation by counter-current distribution or by the solvent extraction procedure described by Le Quesne & Young (1952) and, overall, eleven stages were required for synthesis starting from D-glucosamine and L-aspartic acid. Part of the present work was devoted to simplifying the procedure required for this synthesis and to characterising GlcNAc-Asn with special regard to its optical rotation in solution and to its hydration phenomena in the crystalline state.

Isolation and identification of GlcNAc-Asn from glycopeptides.

Isolation of GlcNAc-Asn from glycopeptides containing only aspartic acid (see page 5) may be achieved by partial acid hydrolysis. As can be seen from the half-lives measured in 2M-HCl at 100°C (Table 1.3), the rate of hydrolysis in acid of GlcNAc-Asn is somewhat slower than the rates of hydrolysis of those types of glycosidic bonds that are often found in glycoproteins. However, low yields of GlcNAc-Asn might be expected. The destruction of GlcNAc-Asn proceeds via two competitive pathways in 2M-HCl at 100°C (see Fig. 1.2). One of these pathways involves N-deacetylation. Owing to the presence of a positive charge adjacent to the acylamido group, the rate of hydrolysis of N-deacetylated GlcNAc-Asn is relatively slow and this compound may

Fig. 1.2. The two major pathways by which GlcNAc-Asn may be hydrolysed in 2M-HCl at 100°C



$k_1 = 20 \times 10^{-3} min^{-1}$
$k_2 = 22 \times 10^{-3} min^{-1}$
$k_3 = 21 \times 10^{-3} min^{-1}$
$k_4 = 7 \times 10^{-3} min^{-1}$
$k_1 + k_2 = 42 \times 10^{-3}$

From Marshall (1969).

accumulate in acid hydrolysates of glycopeptides (Fletcher, Marshall & Neuberger, 1963; Monsigny, Adam-Chosson & Montreuil, 1968). GlcNAc-Asn may also be isolated from glycopeptides containing L-aspartic acid as the only amino acid by the sequential periodate degradation method developed by Smith and co-workers (Abdel-Akher, Hamilton, Montgomery & Smith, 1952) in which periodate oxidation is followed by reduction with borohydride, and release of the polyalcohols produced by mild acid hydrolysis.

The material isolated may be compared to the synthetic compound by its physical and chemical properties and by the chromatographic behaviour of itself and its derivatives. Further acid hydrolysis of the material should yield one mole of ammonia per mole of L-aspartic acid released.

The highly fluorescent dansyl derivative of GlcNAc-Asn has proved useful in its identification in hydrolysates of dansylated glycopeptides from ribonuclease B (Plummer, Tarentino & Maley, 1968) and soybean haemagglutinin (Lis, Sharon & Katchalski, 1969).

An amido hydrolase that will cleave the linkage quantitatively providing that the α-amino and carboxyl groups of the glycosylated L-asparagine residue are free has been isolated from a number of sources (Ohgushi & Yamashina, 1968). The enzyme was found to have no activity on L-asparagine, and to cleave the model linkage compound GlcNAc-Asn to give L-aspartic acid and 2-acetamido-2-deoxy-β-D-glucopyranosylamine. The latter compound decomposes at neutral pH to give N-acetyl-D-glucosamine and ammonia (Makino, Kojima, Ohgushi &

Yamashina, 1968; Mahadevan & Tappel, 1967). The amido hydrolase has been used to characterise GlcNAc-Asn in glycopeptides from ribonuclease B (Tarentino & Maley, 1969) and stem bromelain (Okumura & Yamashina, 1970). Large amounts of GlcNAc-Asn are excreted in the urine of some mentally retarded patients (Jenner & Pollitt, 1967), and an examination showed that this amido hydrolase was absent from the seminal fluid.

The stability to alkali of a glycopeptide prepared by tryptic digestion of hen ovalbumin was investigated by Neuberger (1938). Half of the total nitrogen content was liberated as ammonia by 1M-NaOH at 100°C after 16 hr. Later studies (Marks, Marshall & Neuberger, 1963) showed that 1.5 moles of ammonia per mole of glycopeptide containing L-aspartic acid and L-leucine as the only amino acids were liberated by 0.2M-NaOH at 100°C after 400 min, and a first order rate constant of $7.2 \times 10^{-3} min^{-1}$ was calculated for the initial part of the reaction. This value was similar to that obtained for the rate of evolution of ammonia from Glc-Asn ($5.5 \times 10^{-3} min^{-1}$) under the same conditions (Marks & Neuberger, 1961). Treatment of a glycopeptide containing several amino acids, which had been prepared from hen ovalbumin, with 10% (w/v) barium hydroxide at 100°C for 4 hr in the absence of oxygen was found to change the D-mannose : D-hexosamine ratio from 1.6 : 1.0 to 4.0 : 1.0 (Clamp & Hough, 1965).

In the work to be described, the degradation in alkali and in alkaline borohydride of the N-acylglycosylamine

linkage in model compounds and in glycopeptides has been comprehensively studied.

Section 2. The O-glycosidic linkages to L-serine and L-threonine.

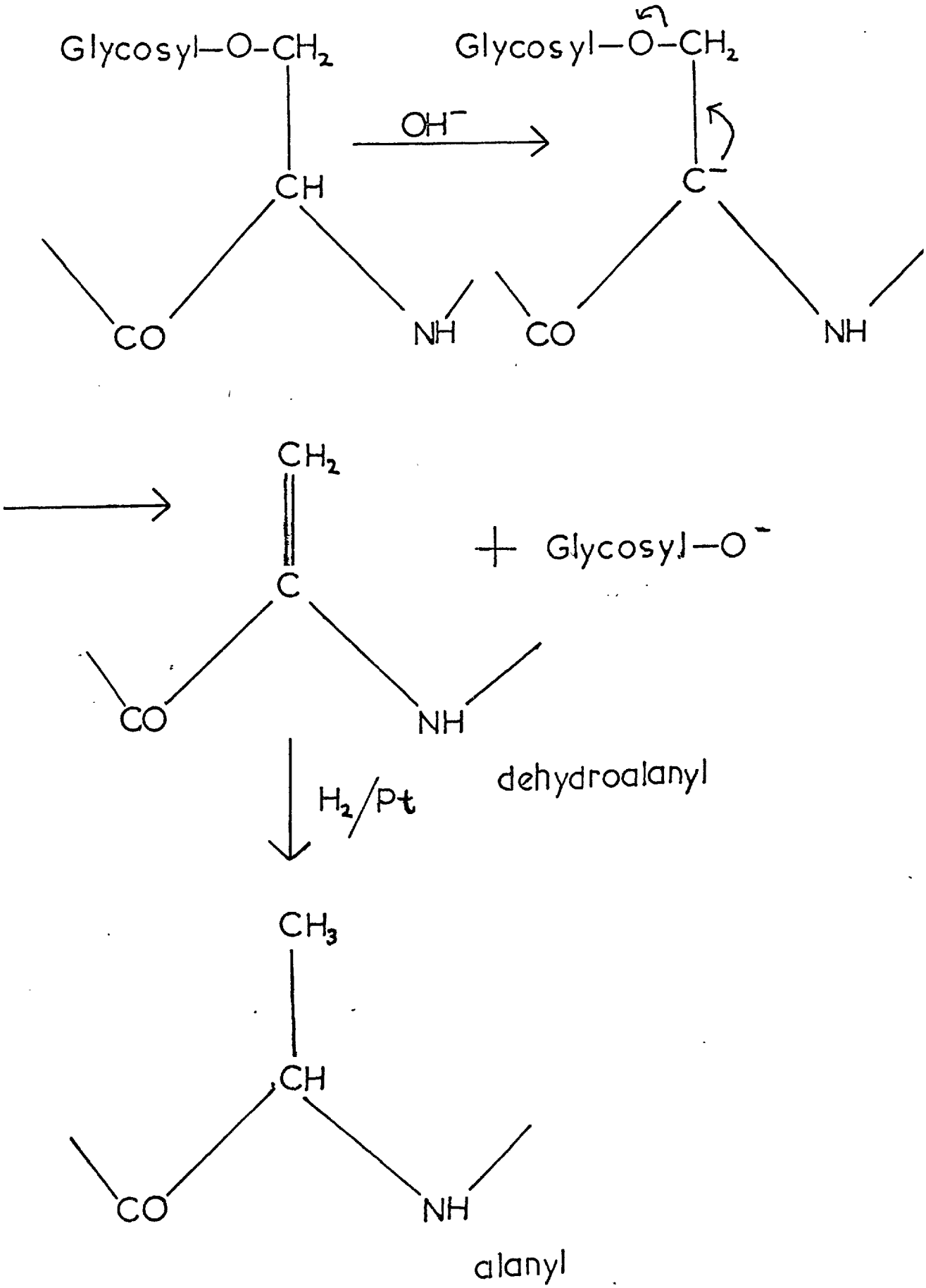
β-Elimination in alkali.

O-Glycosidic linkages involving L-seryl and L-threonyl residues usually undergo facile cleavage in the presence of alkali by β-elimination (see Fig. 1.3). This reaction results in destruction of the L-threonyl and L-seryl residues, the concomitant production of 2-amino crotonic and acrylic acids, and in the release of oligosaccharides.

This type of linkage has been identified by studying the products of treatment of glycoproteins with alkali. Anderson, Hoffman & Meyer (1963) provided evidence for the involvement of the hydroxyl group of L-serine in linkage to chondroitin 4 and 6 sulphates by measuring, after acid hydrolysis, the destruction of this amino acid resulting from treatment of the chondromucoprotein with alkali. Anderson et al., (1964) treated BSM, blood group A + H substance and shark cartilage keratan sulphate with 0.5M-NaOH at 4°C for 2 days and proteoglycans containing keratan sulphate from a number of different sources with 0.45M-KOH at room temperature for 20 hr. In each case the alkaline treatment was followed with acid hydrolysis. Substantial losses of L-threonine and L-serine were found. In separate experiments, hydrogenation over a catalyst of

Fig. 1.3. β-Elimination of glycosyloxy residues from carbohydrate-protein linkages involving L-seryl or L-threonyl residues.

Reduction results in the formation of alanyl or 2-aminobutyryl residues.



10% platinised charcoal reduced the unsaturated amino acid residues of the alkali-treated glycoprotein to alanyl (20% yield) and α-aminobutyryl (35% yield) residues (see Fig. 1.3). Carubelli, Bhavanandan & Gottschalk (1965) showed that there was an increase in the spectrophotometric absorption at 241nm during the alkaline degradation of OSM. This increase was attributed to the strong absorbance at 241nm of α-amino-acrylic acid and α-aminocrotonic acid.

The effect of substitution of the α-amino and carboxyl groups of L-seryl derivatives on the rate of β-elimination has been investigated (Derevitskaya, Vafina & Kochetkov, 1967). 3-O-(β-D-Glucopyranosyl)-N-benzyl oxycarbonyl-L-serine methylamide is split to the extent of 95% at pH 11 at 37°C after 24 hr. Neither 3-O-(β-D-glucopyranosyl)-L-serine nor 3-O-(β-D-glucopyranosyl)-L-serine methylamide is split under these conditions. Surprisingly, 3-O-(β-D-glucopyranosyl)-N-glycyl-L-serine methylamide is also completely stable.

<u>β-Elimination in the presence of sodium borohydride</u>.

Tanaka, Bertolini & Pigman (1964) studied the β-elimination reaction in the presence of alkaline borohydride. BSM was treated for periods of up to 9 days with 0.1M-NaOH and 0.3M-$NaBH_4$ at 5°C. A total yield of 74% alanine was formed from the L-serine destroyed but only about 13% α-amino butyric acid was formed from the L-threonine destroyed. The loss of about 70% of the

carbohydrate upon dialysis showed that this had been released from the protein.

The sugar involved in the linkage is often reduced to the corresponding alcohol in alkaline borohydride, and may be identified as such.

At higher temperatures, almost quantitative release of carbohydrate moieties may occur. Carlson (1968) treated pig submaxillary mucin at 25°C and also at 45°C for 15 hr in 1M-$NaBH_4$, both in the presence and in the absence of 0.05M-KOH. Most (90%) of the oligosaccharides were released at 45°C in the presence of $NaBH_4$ alone, or of $NaBH_4$ together with KOH, while only 20% was released at 25°C. The high yields of oligosaccharides released contrasted with the low yields obtained under similar conditions of alkali, but lower temperatures, by other workers (Lloyd, Kabat, Layug & Gruezo, 1966).

In addition, under conditions of raised temperatures, high concentrations of borohydride, and low concentrations of glycoprotein, reduction may proceed at a much faster rate than degradation of the released carbohydrate. All of the oligosaccharides isolated by Carlson (1968) had N-acetyl-D-galactosaminitol as the reduced terminal residue, whereas the oligosaccharides isolated by Lloyd, Kabat, Layug & Gruezo (1966) were terminated by galactitol, or by hex-3-ene-1,2,5,6-tetrol.

Bertolini & Pigman (1967) treated BSM and OSM with conditions that were different to those of Carlson's(0.3M-$NaBH_4$ in 0.1M-NaOH at 45°C) and measured the loss of various components of the material in the dialysis bag after

dialysis. At the end of 12 hr of alkaline reduction, approximately 75% of the L-seryl and L-threonyl residues were lost, and a total of not more than 75% of the N-acetyl-D-hexosamine residues were reduced. As protein was also lost from the dialysis bag, it was suggested that cleavage of peptide bonds occurred under these conditions.

Weber & Winzler (1969) treated avian mucin, canine and porcine submaxillary mucins, and M and N blood-group active sialoglycopeptides from erythrocytes, with 0.4M-$NaBH_4$ in 0.02M-NaOH at 45°C for 10 hr, and with 0.2M-$NaBH_4$ in in 0.2M-NaOH at 25°C for 48 hr. The decrease in the number of moles of L-serine and L-threonine was greater than that of D-galactosamine in every case, and at 45°C significant amounts of aspartic acid were lost. Alkaline reduction of the mucins resulted in production of hexosaminitols in almost quantitative yield from the hexosamines lost, while only 60% of the hexosamines destroyed in the M and N active glycoproteins were reduced to the corresponding hexosaminitols. It was concluded that the application of these conditions would give only qualitative identification of the amino acids and amino sugars involved in linkage.

Section 3. The effect of alkali on other types of carbohydrate-protein linkages.

The structural entity involving 5-β-D-galactopyranosyloxy-L-lysine is very stable to alkaline conditions and the 5-hydroxy-L-lysine-linked carbohydrate units, containing either the monosaccharide 3-β-D-galactopyranosyl or the disaccharide 2-O-(α-D-glucopyranosyl)-β-D-galacto-

pyranosyl, can be obtained in high yields by treatment of glycoproteins with 2M-NaOH for 16-20 hr at 90-105°C. The products may be quantitated directly on the auto-analyser (Spiro & Fukushi, 1969). The material obtained from the basement membrane was degraded further by 0.5M-H_2SO_4 for 28 hr at 100°C and 5-S-β-D-galactopyranosyl-oxy-L-lysine was isolated and characterised (Spiro, 1967).

Complexes containing 4-hydroxy-L-proline and L-arabinose were isolated from glycopeptides from tomato cell-walls (Lamport, 1969) by hydrolysis with 0.22M-$Ba(OH)_2$ $8H_2O$ at 105°C for 6 hr. The 4-hydroxy-L-proline residue had free imino and carboxyl groups, and it was deduced that the 4-hydroxyl group was involved in a glycosidic linkage.

PART 4. THE IMPORTANCE OF STRUCTURAL AND STEREOCHEMICAL EXAMINATION OF CARBOHYDRATE MOIETIES OF GLYCOPROTEINS.

Section 1. The functional significance of carbohydrate moieties.

The carbohydrate moiety may influence the function of a number of biologically active glycoproteins.

Human chorionic gonadotrophin and follicle-stimulating hormone were found to lose their hormonal activity after treatment with neuraminidase when tested by *in vivo* methods (Mori, 1969). That this loss of activity may be connected with changes in turn-over times was shown by Morell *et al.*, (1971). They found that removal of sialic acid from the two gonadotrophic hormones, and orosomucoid, fetuin, ceruloplasmin, haptoglobin, α_2-macroglobulin and thyroglobulin

resulted in the rapid removal of these materials from the circulation and concentration of these materials in the parenchymal cells. This increase in the rate of catabolism appears to depend on the integrity of the exposed, terminal D-galactose residues of the asialoproteins as treatment with β-galactosidase or galactose oxidase increases the survival time to that of the native protein.

Certain glycoproteins in the blood of several species of Antarctic fish have the property of lowering the freezing point of the blood (De Vries, Komatsu & Feeney, 1970). The glycoproteins lower the freezing point of water to the same extent as equal weights of sodium chloride. This glycoprotein contains disaccharides, (β-D-galactopyranosyl-(1 ⟷ 4)-2-acetamido-2-deoxy-α-D-galactopyranosyl) linked to every L-threonyl residue in repeating sequences (-THR-Ala-Ala-THR-Ala-Ala) (De Vries, Vandenheede & Feeney, 1971). The ability of the glycoproteins to depress freezing point is destroyed by proteolytic digestion. Acetylation of 35% of the sugar hydroxyls, oxidation of 80% of the D-galactose residues by periodate, or treatment with 0.15M-sodium borate destroys the anti-freeze activity, whereas oxidation at C_6 of the D-galactose residue has no effect. Results suggest that the activity is related to the cis-2,3-hydroxyls of the D-galactose residues, as well as the integrity of the whole glycoprotein.

Certain glycoproteins, such as the submaxillary gland mucins, act as protective agents and as lubricants. In many cases, these glycoproteins are rich in sialic acid, and it is these constituents, as well as the high molecular

weight of the glycoproteins, that endow mucous secretions with a high degree of viscosity (Gottschalk & Thomas, 1961).

Certain studies suggest that the fibrinogen-fibrin interconversion involves the release of carbohydrate (Chandrasekhar & Laki, 1964). The works of others do not agree with these findings; Raisys, Molnar & Winzler (1966) concluded from experiments on rabbit fibrinogen, in which sialic acid and D-glucosamine residues were labelled with ^{14}C, that release of carbohydrate during clotting does not occur.

As a large number of extracellular proteins are glycoproteins, whereas many intracellular ones are not, it was suggested some years ago that glycosylation of a protein is necessary for its export from the cell (Eylar, 1965). Thus, external yeast invertase contains 50% mannose, while the internal invertase contains less than 3% (Gascón, Neumann & Lampen, 1968). There are, however, many exceptions to this suggestion; for example, serum albumin is not a glycoprotein. Winterburn & Phelps (1972) have recently discussed the exceptions to Eylar's theory, and have suggested that glycosylation determines the extracellular fate of a protein.

Section 2. Biological activities of the carbohydrate moieties of glycoproteins.

The carbohydrate portions of certain glycoproteins are sometimes involved in forming the antigenic determinants of these macromolecules.

Substances with serological specificities related to

the A, B, O and Lewis blood-group systems have been isolated from various tissue fluids and secretions. These substances may contain non-reducing terminal N-acetyl-α-D-galactosaminyl residues and α-D-galactosyl residues, which largely determine the A and B specific activities, respectively, and the Lewis specific activities are, in part, determined by the nature of the non-reducing α-L-fucosyl residues (Watkins, 1966).

Certain cell-membrane antigens are glycoproteins. The M and N blood-group specificities of human erythrocytes reside in sialoglycopeptides which are released by trypsin from a glycoprotein isolated from erythrocyte stroma (Winzler et al., 1967). This glycoprotein also bears the receptor sites which bind with myxoviruses (Springer, Nagai & Tegtmeyer, 1966). These activities appear to depend on the presence of sialic acid residues on the membrane glycoproteins (Springer & Ansell, 1958). Considerable evidence has accumulated that the recognition of one cell by another involves the carbohydrate moieties of membrane glycoproteins (see for example Cox & Gesner, 1968). Roseman (1970) has proposed that intercellular adhesions are a result of interactions between the glycosyl transferases of the membrane of one cell with glycosyl residues of the adjacent cell.

There may also be a connection between the rate of growth and the size of glycopeptides of cell-surface components in both control and virus-transformed cells (Buck, Glick & Warren, 1971).

A synthetic antigen has been prepared by linking up to 13% of the β-carboxyl groups of poly-L-aspartic acid with di-N-acetyl chitobiosylamine, followed by cross-linking with ethylenimine. The product was found to elicit an immune response in mice (Shier, 1971). The antibody formed cross-reacts with the presumed receptor sites for wheat-germ agglutinin on tumour-cell surfaces, and 5 times the number of mice immunised by this antigen reject myeloma tumours compared to control mice.

Section 3. The heterogeneous nature of carbohydrate moieties.

Carbohydrate appears to be an important source of heterogeneity in glycoproteins. The polypeptide chain may occur naturally with or without attached carbohydrate, as with ribonuclease A and B (Plummer & Hirs, 1964) or with rabbit γ G globulin (Smyth & Utsumi, 1967). The carbohydrate moiety on a specific amino acid residue in a protein may exist in more than one form. Thus, for example, in collagen the prosthetic group may consist of a single β-D-galactosyl residue, or of a disaccharide α-D-glucosyl-(1 → 2)-β-D-galactose (Spiro, 1969).

The microheterogeneity found in the carbohydrate moiety of hen ovalbumin (Cunningham, 1968) may be of a different type where the carbohydrate chain may terminate in different sugar moieties. Chromatography of the glycopeptide prepared by pronase digestion on AG50-WX2 (Na^+) resins by elution with sodium acetate buffers

(pH 2.6, 1m$\underline{M}$ in Na^+, then pH 6.0, 50m$\underline{M}$ in Na^+; Huang, Mayer & Montgomery, 1970) yielded fractions, the compositions of which were as follows (expressed as moles per mole of $\underline{L}$-aspartic acid):

Component	Moles %	Man	GlcNAc
A	5.0	6.01	5.00
B	12.5	5.10	5.08
C	35.8	5.92	4.05
D	27.0	5.94	2.18
E	19.7	5.01	1.96

The origin of this heterogeneity may be the biosynthetic mechanism whereby carbohydrate moieties are built up by the attachment of monosaccharide residues in a stepwise manner. It should be noted however that α-$\underline{D}$-mannosidases and $\underline{N}$-acetyl-β-$\underline{D}$-glucosaminidases

are present in egg-white. This type of microheterogeneity has been established in a number of glycoproteins including α-amylase (McKelvry & Lee, 1969) and fetuin (Oshiro & Eylar, 1968), and is probably a general feature.

Section 4. The effect of carbohydrate moieties on the conformation of glycoproteins.

Although a direct functional role for the carbohydrate moieties has been established for only a limited number of glycoproteins, complete correlation of the structure, conformation and role of these macromolecules can be achieved only when knowledge of the constitution, primary structure and stereochemistry of each of the carbohydrate moieties has been obtained, and the effect of these on the overall tertiary and quaternary structure of the protein determined. For example, Shimizu *et al.*, (1971a) has suggested that the large amounts of carbohydrate in IgM molecules may create spaces between subunits, prevent steric interference between the multiple binding sites, and thus promote their more effective utilization. The role of hydroxy-L-lysine linked disaccharide units in decreasing the morphological organization of collagen has been discussed (Spiro, 1967). Excessive glycosylation of collagen molecules would permit them to participate in fibril formation only if these collagen molecules were located on the periphery of the fibril,

while the disaccharide unit located in the α chain of rat-skin collagen may fit into the "hole" region between neighbouring collagen molecules when packed in a quarter-stagger array (Morgan, Jacobs, Segrest & Cunningham, 1970).

PART 5. METHODS FOR DETERMINATION OF THE PRIMARY STRUCTURE OF CARBOHYDRATE MOIETIES.

Section 1. Methylation.

The preparation of fully methylated oligo- and polysaccharides is one of the most useful techniques. The methylated sugars are most easily separated and measured by gas-liquid partition chromatography.

Section 2. Periodate oxidation.

A molecule containing free neighbouring hydroxyl groups will be cleaved by periodate. Malaprade (1928) showed that the reaction will proceed as follows:

$$\begin{array}{l} CH_2OH \\ | \\ (CHOH)_n \\ | \\ CH_2OH \end{array} + (n+1)HIO_4 \longrightarrow \begin{array}{l} 2HCHO + nHCOOH + H_2O \\ \qquad\qquad + (n+1)HIO_3 \end{array}$$

Formic acid is formed from secondary hydroxyl groups and formaldehyde from primary hydroxyl groups. Measurement of these products, the amount of periodate uptake, and the sugars destroyed, gives much information concerning the number and types of terminal

residues, and the degree and position of substitution of internal residues.

The glycopeptide from hen ovalbumin has been subjected to periodate oxidation (Fletcher, Marks, Marshall & Neuberger, 1963), and to investigation by methylation techniques (Montgomery, Lee & Wu, 1965). On the basis of the results obtained, a structure for the carbohydrate moiety has been proposed (see Fig. 1.4).

Section 3. Isolation of fragments from glycopeptides by partial acid hydrolysis.

Hydrolysis of glycopeptides or oligosaccharides by treatment with dilute mineral acid, or water-soluble polystyrene sulphonic acid resins (Painter, Watkins & Morgan, 1962), may lead to the isolation of di- or trisaccharides which are more amenable to structural determination. Artifacts may occasionally be formed by acid reversion (see, for example, Manners, 1959).

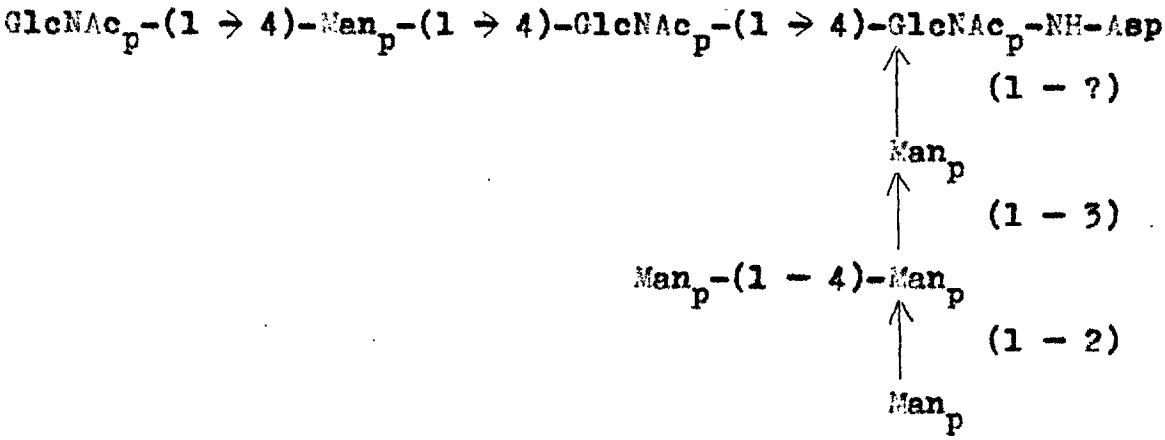
Hydrolysis with 0.1M-HCl at 100°C for 3 hr of a dinitrophenylated glycopeptide prepared from hen ovalbumin (Montgomery, Lee & Wu, 1965) led to isolation of a fragment in low yield containing one residue of D-mannose and one residue of N-acetyl-D-glucosamine per mole of L-aspartic acid.

Section 4. Structural determination by the use of glycosidases.

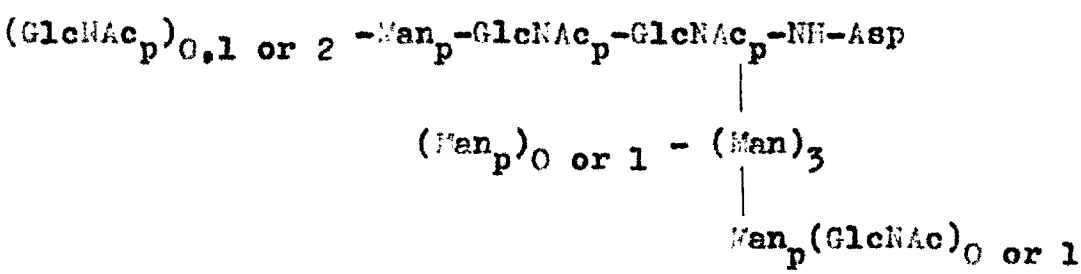
The structure of glycopeptides may be investigated by identification of the sugar residues that are cleaved by specific, purified glycosidases. The components of hen ovalbumin glycopeptide that were separated by chrom-

Fig. 1.4. The structure of hen ovalbumin glycopeptide.

The structure proposed by Montgomery, Lee & Wu (1965):-



The structure proposed by Huang, Mayer & Montgomery (1970):-



atography on AG50-WX2 resins have been subjected to the actions of α-D-mannosidase and N-acetyl-β-D-glucosaminidase (Huang et al., 1970) and a sequence of sugar residues proposed (Fig. 1.4). A complex with the composition ($Man_3(GlcNAc)_2Asn$) has been isolated from hen ovalbumin glycopeptide by treatment with the glycosidases mentioned above. The final D-mannose residue is cleaved from the complex by a β-D-mannosidase prepared from Achatina fulica (Sugahara, Okumura & Yamashina, 1971).

Section 5. Alkaline degradation of oligosaccharide moieties. Mechanism of β-alkoxy carbonyl elimination with neutral reducing terminal hexoses.

Glycosidic bonds are normally stable to alkaline hydrolysis, but a stepwise degradation may take place from the reducing end of oligosaccharide moieties, and alkaline degradation has been used to obtain oligosaccharide fragments from glycoproteins with alkali-labile carbohydrate-protein linkages. The mechanism involved was described as a β-alkoxy carbonyl elimination (Corbett, Kenner & Richards, 1953a,b). The proton α to a carbonyl group in the open-chain form of the reducing neutral hexose may be removed by hydroxide ion, followed by elimination of the β-alkoxy substituent. A C_3 substituent will be readily eliminated. C_4 substituents are less readily eliminated; the mechanism assumes that a carbonyl group is formed in the 2-position by a Lobry de Bruyn transformation, so that the C_4 substituent will be in a βposition relative to it. C_6 substituents are relatively stable, and C_2 substituents very stable. The eliminated alkoxide anion is an oligosaccharide, which may have an alkali-labile linkage at the reducing end. Further degradation may result and sugar units will be removed in a stepwise manner until a sugar unit involved in an alkali-stable linkage is exposed.

Mechanism of degradation of oligosaccharides with reducing terminal N-acetyl hexosamine residues.

Mild treatment with alkali of N-acetyl-D-hexosamine has been considered in the elucidation of the Morgan-

Elson (1934) reaction. The structures of the products that are formed by treatment of N-acetylhexosamine residues with alkali have been determined (Kuhn & Krüger, 1956, 1957). These products have been called "chromogens". The two principal chromogens appear to be formed by the loss of one and two molecules respectively of water to give an anhydro sugar in the 5-membered ring form, and finally 5-dihydroxyethyl-3-acetamidofuran (structures I and II in Fig. 1.5). Leaback & Walker (1963) showed that under the acidic conditions of the condensation step with p-dimethylaminobenzaldehyde, the furan derivative (structure II) is formed quantitatively from the anhydro sugar (structure I). Aminoff, Morgan & Watkins (1952) have discovered some of the more satisfactory conditions to use for quantitative estimation of N-acetylhexosamine. These workers found that maximum colour intensity was produced by heating N-acetyl-D-glucosamine at 100°C with 0.025M-Na_2CO_3 (pH 10.8) for 4 mins, followed by treatment with p-dimethylaminobenzaldehyde. The chromogens formed by treatment with alkali possessed ultra-violet absorption (λ_{max} 230nm), which decreased with continued heating, as did the colour produced by the addition of p-dimethylaminobenzaldehyde. This suggested that the chromogen itself was unstable in alkali. It was also found that enhanced colour production was achieved in the presence of borate buffers.

The colour produced by 3-O-substituted N-acetyl-D-hexosamine residues, such as that in lacto-N-biose I (Gal 1 → 3 GlcNAc) was greatly enhanced compared to that produced by free N-acetyl-hexosamines (Kuhn, Gauhe & Baer, 1954a,b). It was proposed that simultaneous β-elimination of the 3-substituent

rendered conversion to the anhydro sugar almost quantitative. A reaction mechanism is shown in Fig. 1.5. Treatment with alkali (pH 10.5) at 37°C of a cell-wall glycopeptide containing the disaccharide 4-O-N-acetyl-β-D-glucosaminyl-N-acetyl-D-muramic acid, the D-lactyl residue of which is linked to a tetrapeptide, results in elimination of the D-lactyl residue and formation of 2-acetamido-2-deoxy-β-D-glucopyranosyl-3-ene. The product is a chromogen which reacts directly with p-dimethylamino-benzaldehyde (Tipper, 1968).

Treatment of 4-O-substituted or 4,6-disubstituted N-acetylhexosamine residues with alkali does not result in chromogen formation. The 4-substituent prevents the pyranose-furanose interconversion (Kuhn, Gauhe & Baer, 1954a,b).

It should be noted that epimerisation of N-acetylhexosamines may also occur in alkali. Spivak & Roseman (1959) obtained a 20% yield of N-acetyl-D-mannosamine by the treatment of N-acetyl-D-glucosamine with a dilute aqueous solution of sodium hydroxide (pH 11; 2 days; room temperature).

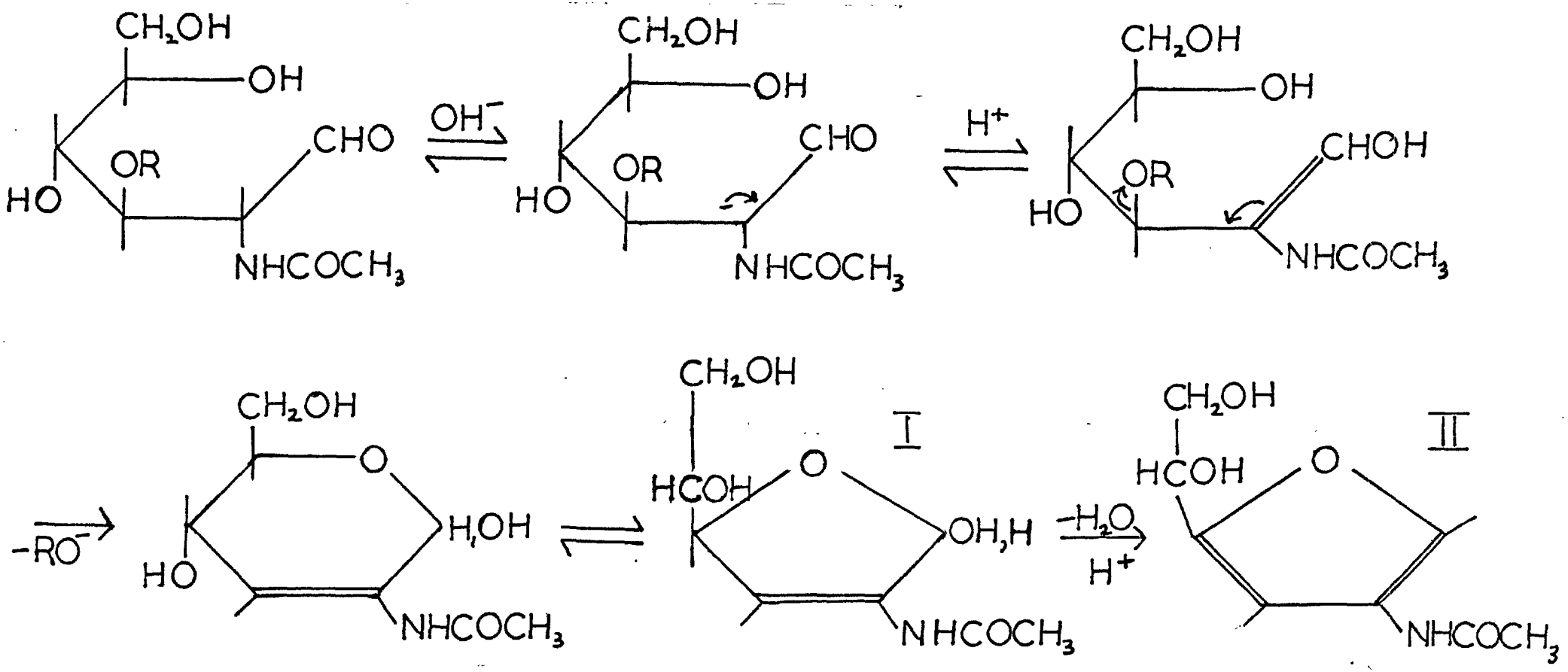
Other reactions of sugars in alkali.

Pyranose-furanose interconversions, condensations and fragmentation reactions may occur as a result of the action of alkali on sugars. Nef (1914) isolated 100 different products from glucose by treatment with alkali. Saccharinic acids, resins and other high molecular weight products of unknown compositions, may be formed.

Pyrazine glyoxaline derivatives are formed by the

Fig. 1.5. Formation of chromogens from 3-O-substituted N-acetyl-D-glucosamine residues by treatment with alkali.

Conversion of I to II occurs in acid solution.



anhydro-N-acetyl-D-glucosamine (pyranose form)

anhydro-N-acetyl-D-glucosamine (furanose form)

5-dihydroxyethyl-3-acetamidofuran

RO^- = eliminated glycosyloxy residue

action of ammonia on D-glucose at 37°C for 2 weeks (Hough, Jones & Richards, 1952). These derivatives have a characteristic ultraviolet spectrum with λmax at 275nm. Similar compounds have been separated from the reaction products of aqueous ammonia on D-glucosamine (Taha, 1961).

De-amination of hexosamines may occur in hot alkali. This reaction has been adopted for use as a method for determining glucosamine (Tracey, 1952). However, whereas saturated phosphate/borate buffer (pH 11.4) at 100°C yielded 100% ammonia from glucosamine, saturated sodium carbonate yielded 80%, 0.25M sodium carbonate 50%, and saturated sodium tetraborate only 25%. It is clear that we may expect a variety of products when glycopeptides are treated with alkali.

Section 6. Alkaline degradation with borohydride reduction.

Stepwise degradation in alkali normally proceeds until an alkali-stable linkage is reached and has been used to isolate (1→2) linked disaccharides. Thus, lacto-N-fucopentaose I yielded 52% α-L-fucosyl-(1→2)-D-galactose and 16% of the corresponding talose derivative—formed by epimerisation - when treated at 100°C for 20 min with 0.025M-Na_2CO_3 (Kuhn, Baer & Gauhe, 1958). Derivatives containing 3-O-substituted reducing terminal sugars have been isolated by interrupting the alkaline degradation with an alkaline resin [poly(vinylbenzyl-triethylammonium) hydroxide] by dialysing away the fragments

(Marr, Donald & Morgan, 1968).

Another method of interrupting this process of alkaline degradation is to use sodium borohydride, as discussed earlier. By this method, Carlson (1968) isolated from pig submaxillary mucin a pentasaccharide; 2-acetamido-2-deoxy-α-D-galactopyranosyl (1→3)-[α-L-fucopyranosyl-(1→2)-]β-D-galactopyranosyl-(1→3)-[N-glycolylneuraminyl-(2→6)]-2-acetamido-2-deoxy-D-galactitol as well as a number of smaller reduced saccharides, all of which contained N-acetyl-D-galactosaminitol. Lloyd, Kabat, Layug & Gruezo (1966) showed that the action of sodium hydroxide and sodium borohydride on blood group substances produced complex mixtures of products ranging from monosaccharides to oligosaccharides containing more than six monosaccharide residues. 2-O-α-L-Fucopyranosyl-D-galactitol was isolated and characterised, as well as a reduced tetrasaccharide α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)hex-3-ene-1,2,5,6-tetrol.

Thomas & Winzler (1969) have prepared reduced oligosaccharides from M or N active human erythrocyte membranes using 0.1M-NaOH and 0.4M-$NaBH_4$ at room temperature for 24 hr. The most complex product was a reduced tetrasaccharide, N-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[N-acetylneuraminyl-(2→6)]-N-acetyl-D-galactosaminitol. Other smaller saccharides were formed. Some of these had structures that suggested they had arisen from carbohydrate moieties containing

3 or less sugar residues. In addition, reduced products such as N-acetylneuraminyl-(2 → 3)-D-galactitol were isolated; these may have arisen from a peeling reaction from a larger oligosaccharide initially released. Free sialic acid, galactitol, N-acetyl-D-glucosaminitol, a 3-deoxy-D-glycitol, and the reduced form of the Kuhn & Krüger chromogen (see page 60), were also formed.

A mechanism for the production of a 3-deoxy-D-glycitol in these reactions has been proposed (Lloyd, Kabat & Licerio, 1968) in which unsubstituted 3-deoxyhexulose, formed by elimination of a substituent at C_3 from a reducing terminal D-galactose residue via a peeling reaction, is further reduced at the 2-keto group by borohydride. These workers also isolated substituted and unsubstituted 3-hexenetetrols by the action of alkaline borohydride on blood group Le substance. The unsaturated derivatives decolourised bromine and potassium permanganate solutions. A reduced form of the Kuhn & Krüger chromogen has also been obtained by the action of alkaline sodium borohydride on β-D-Gal-(1 → 3)-D-GlcNAc (Lloyd & Kabat, 1969).

Section 7. The present work.

It may be seen that, although the results of methods such as periodate oxidation and cleavage with glycosidases can give readily-interpreted data concerning the structure of non-reducing terminal sugar residues, alkaline degradation, especially in the presence of reducing agents,

provides useful information concerning the primary structure at the reducing end of the oligosaccharide moiety, especially when overlapping fragments can be obtained. So far, this latter method has been used to obtain oligosaccharides from linkages involving L-threonine and L-serine. Part of the work to be described will be devoted to determining the conditions for alkaline degradation of the L-asparagine to N-acetyl-D-glucosamine linkage, so that structural determinations of this type may be performed.

PART 6. DETERMINATION OF THE CONFORMATION OF GLYCOPROTEINS.

A large number of physical techniques have been applied to the study of protein conformation. Of these, high-resolution X-ray crystallography has not been employed successfully with a glycoprotein so far. Once obtained, electron density data may be difficult to interpret because of the inherent heterogeneity of carbohydrate moieties. Of other methods, acid-base titration and chiroptical methods will be discussed here.

Section 1. Acid-base titration.

The hydrogen-ion titration curve of a protein molecule is a measure of the total number of protons bound to, or dissociated from, all of the acidic or basic groups of the protein, as a function of pH. The results may be analysed according to the Linderstrøm-Lang (1924) model to determine the intrinsic dissociation

of the groups involved; group counting may be compared to the amino acid analysis to discover groups that are inaccessible to titration, and the effect of denaturing solvents, high and low pH etc. on these may be evaluated.

Titration of glycoproteins.

The first electrometric titration curve of protein to be reported in the literature was that of ovalbumin and the first discovery of a phenolic group inaccessible to titration was made with this protein (Crammer & Neuberger, 1943). Other glycoproteins have been titrated, including ovine luteinizing hormone (Ward, Walborg & Adams-Mayne, 1961), bovine fibrinogen (Mihalyi, 1970), haptoglobins (Waks & Alfsen, 1966), fetuin (Spiro, 1960) and glycoprotein I from kidney bean (Pusztai, 1968), a protein of about 60,000 molecular weight containing 50 monosaccharide residues. In 0.15M-NaCl, the kidney bean glycoprotein showed irreversible behaviour above pH 9, and storage in alkali, or titration in 5M-guanidine and 1.2M-urea exposed 15 more carboxyl groups (but with lower than expected intrinsic dissociation contents), 10 more lysyl residues and 4 more tyrosyl residues. Storage at pH 12 for longer periods than 1 hr increased the titrating groups to a number higher than theoretical, suggesting that degradation had occurred. It was suggested that these abnormalities might, in part, be explained by intramolecular interaction between carbohydrate side-chains and the polypeptide chains, or by simple steric effects caused by the carbohydrate moieties

themselves.

Interpretations of this kind would be helped by knowledge of the behaviour of the prosthetic groups during titration and at extremes of pH.

Section 2. Chiroptical methods.

Denatured proteins generally show much less negative rotations than native proteins at wavelengths above about 220nm, and Moffitt & Yang (1956) suggested that this was due to a loss of helical content. Several equations have been used to determine helical content from ORD curves, including a single term Drude equation (Kauzmann, 1957) and the several term equations of Moffitt & Yang (1956) and of Schechter & Blout (1964).

Glycoproteins studied containing relatively little carbohydrate such as hen ovalbumin (Tomimatsu & Gaffield, 1965) and avidin (Green & Melamed, 1966) are probably open to interpretation in terms of their polypeptide chain conformations alone. However, glycoproteins containing a higher density of prosthetic groups on the polypeptide chain, such as in haptoglobulins I and II (Gerbeck, Bezkorovainy & Rafelson, 1967), fetuin (Waks & Alfsen, 1966), γM immunoglobulin (Dorrington & Tanford, 1968) and α_1-acid glycoprotein (Yamagami & Schmid, 1967), have also been studied, and parameters of the multi-term Drude equation (Schechter & Blout, 1964) used to determine the helical content of these glycoproteins.

The CD spectra of "freezing point" depression glycoproteins from antarctic fish show positive peaks at 218nm and negative peaks at 194nm (De Vries, Komatsu & Feeney, 1970). The 2-acetamido chromophore of the

N-acetyl-D-galactosamine residues, which are bound to every third amino acid residue (L-threonine), will make important contributions to the CD pattern, but it is difficult to predict the sign and magnitude of the effect. The CD spectra of red cell membranes have also been interpreted in terms of α-helical content (Glaser & Singer, 1971).

A complete interpretation of these data necessitates an evaluation of both the direct and indirect effects of the carbohydrate moieties on the chiroptical properties of glycoproteins.

ORD/CD studies of oligosaccharides.

Free, neutral sugars and glycosides give plain ORD curves down to 200nm (Listowsky, Avigad & England, 1965). A rule was proposed to predict the contribution of hydroxy and methoxy groups in a number of sugars, somewhat along the lines of the octant rule of Moffitt et al., (1961), considering the ring oxygen atom, which has its absorption maximum below 190nm, as the principal chromophore. Accessible chromopheres in sugar derivatives such as lactones (Okuda, Nagaya & Kiyomoto, 1964) and azides (Paulsen, 1966) have been examined by ORD and CD, and octant rules formulated.

The chromophoric acetamido group has been examined in the glycosides of N-acetyl-D-glucosamine and N-acetyl D-galactosamine (Beychok & Kabat, 1965; Listowsky, Avigad & England, 1968). The ORD curves showed that the α-anomer had a more positive rotation than the β-anomer, but both had a trough at about 220nm and a peak at 200nm. Variation in the magnitude of the Cotton

effect for the various derivatives were interpreted using the octant rule (Beychok & Kabat, 1965) and the preferred orientation of the 2-acetamido group postulated to be the same as that observed in the lysozyme N-acetyl-D-glucosamine complexes (Phillips, 1967). CD spectra of these derivatives were also obtained down to about 210nm (Kabat, Lloyd & Beychok, 1969). In the same paper, the ORD/CD spectra of several oligosaccharides containing mono- and di-substituted N-acetyl-D-hexosamine residues, and of N-acetylneuraminic acid, colominic acid and teichoic acid, were presented. Some rules were proposed which may allow the nature and position of substitution of N-acetyl-D-hexosamine residues to be predicted. Substitution, especially on the 3-position, appears to enhance the higher wavelength negative Cotton effect/CD band and an α-L-fucosyl substituent has a dominant effect. The CD spectra of mucopolysaccharides show two bands in the region of the amide and carboxyl chromophores. The negative peak at higher wavelengths is common to all polymers, while the characteristics of the other band depend on the polymer structure (Stone, 1971).

Conformational aspects in the present work.

Much stereochemical information has been obtained from CD and ORD of well defined and easily available chromophores. For example, the carbonyl group in the rigid environments that exist in steroids or triterpenoids has been much studied. The wavelengths at which chromophores of naturallyoccurring carbohydrates absorb have only recently been penetrated by modern spectropolarimeters and CD machines. Consequently, much remains to be done

before the interpretation of data becomes straightforward.

The examination of the stereochemistry of carbohydrate moieties, and of their conformation with respect to the polypeptide chain, will be approached in this work by determining the stereochemistry of the linking moiety GlcNAc-Asn. The chiroptical properties of GlcNAc-Asn, glycopeptides containing this compound, as well as a number of analogous synthetic compounds, in aqueous solution will be described and interpreted.

The stereochemistry of GlcNAc-Asn in two other environments will be described. Large crystals of GlcNAc-Asn.$3H_2O$ and Glc-Asn.H_2O were grown, and the three-dimensional structures studied, using X-ray diffraction methods, by Dr. L.Delbaere in the Department of Chemical Crystallography, Oxford.

The stereochemistry of GlcNAc-Asn will also be described when bound to an enzyme, hen egg lysozyme. It was felt that the type of environment presented by an enzyme might be more akin to that experienced in an <u>in vitro</u> situation than in either aqueous solution or in the solid state. The experiments were performed in the laboratory of Professor D. Phillips at the Department of Molecular Biophysics, Oxford, by Drs. C. Beddell and L. Johnson, and we were able to take part in constructing difference electron density maps and interpreting them with Kendrew model building. The results of these experiments will be described in terms of a comparison of the conformation in the three different environments.

The significance of the conformation of GlcNAc-Asn

as a factor in determining the mechanism of biosynthesis of glycoproteins will be discussed below.

PART 7. THE BIOSYNTHESIS OF CARBOHYDRATE-PROTEIN LINKAGES.

Section 1. The cellular location of biosynthesis.

Roseman (1962) outlined the two possible pathways for the biosynthesis of the prosthetic groups of glycoproteins. The polypeptide chain may be formed on the ribosome, released and then the carbohydrate inserted at specific points, or the oligosaccharide may first be linked to a specific amino acid, which is then activated, and the complex incorporated into the growing peptide chain. It now appears from a number of studies that most sugars are added in a sequential manner after the formation of the polypeptide chain, while it is on its way through the endoplasmic reticulum.

Section 2. Biosynthesis of N-acyl glycosylamine carbohydrate-protein linkages.

The mechanism of assembly of inner N-acetyl-D-glucosamine and D-mannose residues remains to be elucidated. That the formation of the N-acyl glycosylamine linkage is coded for only in a direct way is unlikely, as no transfer RNA carrying N-acetyl-D-glucosamine has been found (Sinohara & Sky-Peck, 1965). The work of some suggests that the internal N-acetyl-D-glucosamine

residue is attached while the protein is still on the ribosome (Molnar, Robinson & Winzler, 1965; Lawford & Schachter, 1966), but other studies do not agree with this finding (Sarcione, 1964; Bouchilloux & Cheftel, 1966; Melchers, 1970), although the studies were performed on different types of cell. The sugar donor is likely to be UDP-N-acetyl-D-glucosamine, and the enzyme responsible similar to those transferases studied by Leloir (1964). As the glycosylated L-asparagine residue in ribonuclease B corresponds to an L-asparagine residue (No. 34) in ribonuclease A (Plummer & Hira, 1964), it is probable that L-asparagine residues rather than L-aspartic acid residues are the acceptors of N-acetyl-D-glucosamine.

UDP-N-Acetyl-D-glucosamine transferases have been isolated (e.g., from goat colostrum by Johnston. McGuire, Jourdain & Roseman, 1966), but appear to be active only with terminal D-mannose acceptor residues of glycoproteins. The enzyme specific for formation of the N-acyl glycosylamine carbohydrate-protein linkage remains to be isolated.

Section 3. Biosynthesis of O-glycosidic carbohydrate-protein linkages.

Direct enzymic addition of N-acetyl-D-galactosamine on to protein receptors formed by removal from BSM of the carbohydrate prosthetic groups with periodate has been shown (Hagopian & Eylar, 1968). Other sugars,

Table 1.4 Sequences of peptides containing glycosylated asparagine residues.

Human orosomucoid	Cys-Ala-Asn-Leu-Val-Pro-Val-Pro-Ile-Thr-ASN-Ala-Thr-Leu-Asp-Arg-Ile-Thr-Gly-Lys-Trp-Phe. Tyr-Ile-Ala-Ser-Ala-Phe-Arg-Asn-Glu-Glu-Tyr-ASN-Lys-Ser-Val-Gln- Glu-Ile-Gln-Ala-Thr-Phe-Phe-Tyr-Phe-Thr-Pro-ASN-Lys-Thr-Glu-Asp-Thr-Ile-Phe-Leu-Arg- Glu-Tyr-Gln-Thr-Arg-Gln-Asp-Gln-Cys-Ile-Tyr-ASN-Thr-Thr-Tyr- Leu-Asn-Val-Gln-Arg-Glu-ASN-Gly-Thr-Ile-Ser-Arg-Tyr [1]
Rat Yoshida ascites tumour α_1-glycoprotein	Thr-Glu-ASN [2]
Human Plasma Ba-α_2-glycoprotein	ASN-(Ala,Thr,Pro,Val) [3] Asn-(ASN,Gly)-Ser [4*] ASN-Asn-Thr [4]
Human fibrinogen	Asp-Ile-Leu-His-Gln-Val-Glu-ASN-Lys-Thr-Ser-Glu-Val-Lys-Gln [5]
Ox fibrinogen	ASN-Lys-Thr-Ser [6,7,8] Gly-Glu-ASN-Arg [6]
Human γG-myeloma protein (Eu)(constant portion)	Pro-Arg-Glu-Gln-Gln-Tyr-ASN-Ser-Thr-Tyr-Arg-Val [9]
Human γG myeloma protein (Cor)(variable portion)	Asp-Asp-Lys-Tyr-Tyr-ASN-Thr-Ser-Leu-Glu-Thr [10]
Human γG globulin	Glu-Glu-Asp-Tyr-ASN-Ser-Thr [11, 12]
Rabbit γG globulin	Pro-Leu-Arg-Glu-Gln-Gln-Phe-ASN-Ser-Thr-Ile-Arg-Val-Val-Ser [13, 14] (ASN,Gly,Ser) [15]
Ox γG globulin	Glx-Glx-Glx-Phe-ASN-Ser-Thr-Tyr-Arg [16,17]
Mouse Bence-Jones K-chain (MOPC 46)(variable portion)	Ala-Ser-Gln-ASN-Ile-Ser-Asn-Asn-Leu-His [18]
Human Bence-Jones K-chain (HBJ 10)	(Ile,Glx)-ASN- - -Thr [19]
Human Bence-Jones λ-chain (Ful)	Cys-Ser-Gly-ASN-Ser-Ser [19]
Human Bence-Jones K-chain (HBJ 4)	Ala-Ser-Glx-ASN-Val-Ser-Asx [19]
Human Serum myeloma K-chain (Dup)	ASN-Glx-Ser-Gly- Thr-ASN-Phe-Thr [19]
Human Serum myeloma K-chain (Mor)	Val-Asn-Trp-ASN-Trp-Ser [19*]
Waldenström macroglobulin (Ou & Di) μ-chain (constant portion)	Phe-Ser-Trp-Lys-Tyr-(ASN,Asx,Ser,Asn,Lys) [20,21] Leu-Thr-Phe-Gln-Glx-ASN-Ala-Ser-Ser-Met [20,21] Val-Lys-Thr-His-Thr-ASN [20,21] Ile-Ser-Glx(ASN,Ser,His,Pro) [20,21] Leu-Tyr-ASN-Val-Ser-Leu-Val-Met [20,21]
Human transferrin	Ala-Glu-Asn-Tyr-ASN-Lys-Ser [22,23] Cys-Asp-ASN-Leu [23] Phe-Gly-Ser-Tyr-ASN-Lys-Ser [22,23]

peptides and free L-serine and L-threonine were inactive as receptors. D-Xylose is not added to free L-serine, but is transferred to a preformed protein receptor (Robinson, Telser & Dorfman, 1966) during the biosynthesis of chondromucoproteins. Similarly, a UDP-D-galactosyl transferase purified from the skin of embryonic guinea pig was active with a receptor prepared by Smith-degradation of collagen, whereas 5-hydroxy-L-lysine had much reduced receptor activity (Bosmann & Zylar, 1968). These experiments show that the carbohydrate-protein linkages form after the completion of the polypeptide chains.

Section 4. Amino acid sequence requirements for the formation of the L-asparagine to N-acetyl-D-glucosamine linkage.

A preferred sequence in the neighbourhood of glycosylated L-asparagine residues has been noted (Neuberger & Marshall, 1969). An examination of amino acid sequences in glycopeptides of this type shows that a β-hydroxy amino acid, L-threonine or L-serine, occurs in the position next but one towards the C-terminus of the protein (see Table 1.4). In all but one, or possibly two, cases the following sequence occurs:

-ASN-X-Thr(Ser)-

where ASN is the glycosylated asparagine residue, and X may be any amino acid except L-proline, L-cysteine, L-cystine, L-tyrosine, L-tryptophan, L-phenylalanine

or L-aspartic acid, which have not been unambiguously identified in this position. The occurrence of this sequence shows that the carbohydrate is attached after the completion of the polypeptide chain, that the transferase that initiates glycosylation requires this sequence as the receptor and that the necessary site for glycosylation is coded for directly in the genome, as well as indirectly via the production of the transferase enzymes.

The occurrence of non-glycosylated receptor sequences.

The amino acid sequence described above may occur in proteins where the L-asparagine residue is not glycosylated. Examples such as human calcitonin N (Kiniker et al., 1969), guinea pig immunoglobulin K-chain (Lamm & Listowska-Bernstein, 1968) and bovine trypsinogen (Mikeš, Holeyšovský, Tomášek & Šorm, 1966) are included in a list of the 101 occurrences of this sequence tabulated in the Atlas of Protein Sequences and Structure (1969) (Hunt & Dayhoff, 1970). Thus the sequence is a necessary, but not sufficient condition for glycosylation to occur. Other factors such as the availability of UDP-N-acetyl-D-glucosamine, contact of apoprotein with transferase, kinetic factors and the total stereochemistry of the receptor site are also presumably important.

A hydrogen-bonded model for the receptor site.

Several mechanisms might be suggested for the ability of the amino acid sequence shown above to specify

Table 1.4 (Cont).

Human thyroglobulin	Ala-Leu-Glu-ASN-Ala-Thr-Arg [24]
Bovine thyrotropin	Val-Pro-Lys-ASN-Ile-Thr-Ser-Glx-Ala [25]
	Arg-Val-Glx-ASN-His-Thr-Glu-Cys-His 25
	Leu-Thr-Ile-ASN-Thr-Thr-Val-Cys-Ala 25
Ovine luteinising hormone	Val-Pro-Lys-ASN-Ile-Thr-Ser-Glu-Ala 26
	Arg-Val-Glx-ASN-His-Thr-Glx-Cys-His 26
	Glu-Pro-Ile-ASN-Ala-Thr-Leu-Ala-Ala 26
Hen ovalbumin	Glu-Glu-Lys-Tyr-ASN-Leu-Thr-Ser-Val-Leu 27,28,29
Hen ovomucoid	ASN-Thr-Asp [30,31]
	Thr-ASN 30,31
	Pro-Pro-Ala-ASN 31
	Ala-ASN-Thr 32
Hen egg-white avidin	Leu-Gly-Ser-ASN-Met-Thr-Ile-Gly-Ala-Val [33]
Hen ovoglycoprotein	ASN-Thr-Ser [30]
	ASN-Gly 30
	Thr-ASN 30
	ASN-Ser 30
Hen serum/ovo transferrin	Gly-Leu-Ile-His-ASN-Arg-Thr-Gly-Thr-Cys [34,35]
Hen ovo transferrin	Ala-ASN-Leu-Thr-Gly [34,35]
Hen egg-white vitellin	ASN-Thr-Ser-(Ala,Gly,Val)-Ile [36]
Hen egg-yolk phosvitin	Ser-ASN-Ser-Gly-$(SerP)_8$-Arg-Ser-Val [37]
Ox pancreatic ribonuclease B	Lys-Ser-Arg-ASN-Leu-Thr-Lys-Asp-Arg-Cys [38]
Pig pancreatic ribonuclease	Ser-Arg-Arg-ASN-Met-Thr-Lys-Asp-Arg-Cys-Lys [39]
	Ser-Ser-Ser-Ser-ASN-Ser-Ser-Asn 39
	Tyr-Gla-Ser-ASN-Ser-Thr-Met 39
Bovine pancreatic ribonuclease	Ser-ASN-Ala-Thr [40]
Ox aorta glycoprotein	Ala-Phe-Gly-ASN-Gly [41]
Pineapple stem bromelain	Ala-Arg-Val-Pro-Arg-Asn-ASN-Glu-Ser-Ser-Met [42]
α-Amylase (Aspergillus oryzae)	Ser-ASN [43]
Glycopeptide from Metridium dianthus connective tissue	Gly-ASN-Thr [44]

* Carbohydrate may be on either of the two asparagine residues.

ASN represents those asparagine residues that are glycosylated.

the site of glycosylation. A specific site close to the active site on the enzyme may bind the β-hydroxy-amino acid residue or, alternatively, the β-hydroxy-amino acid may interrupt the formation of α-helix in the apoprotein (Perutz, 1962) and allow access to the L-asparagine residue.

A possible mechanism has been proposed*, however, involving a hydrogen-bonded interaction between the L-asparagine residue and the L-threonine and L-serine residue, based on the interpretation of the results of acid-base titration of a glycopeptide containing this sequence. The experimental titration curve of a glycopeptide containing the amino acids L-aspartic acid, L-leucine, L-threonine and L-serine, together with small amounts of other amino acids is shown in Fig. 1.6 (Johansen, Marshall & Neuberger, 1961). In addition to the titration of the peptide α-carboxyl and α-amino groups, titration was observed at higher values of pH, and this was suggested to be a group with a pKa of about 9.5 beginning to titrate. When a glycopeptide containing L-aspartic acid as the only amino acid (Montgomery, Lee & Wu, 1965) and the synthetic GlcNAc-Asn (Fletcher, 1965) were titrated, no extra titration was observed until a pH of about 2.5 units higher was reached. It was postulated that the titration observed at high pH was due to the imido group constituting the linkage (marked with an arrow in Plate 1.1), but the acidity of the group was greater

* Neuberger & Marshall (1969).

Fig. 1.6. The titration curve of a glycopeptide, containing several amino acid residues, prepared from hen ovalbumin.

From Johansen, Marshall & Neuberger (1961).

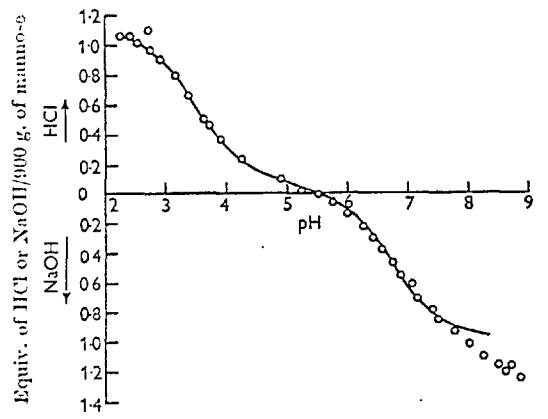
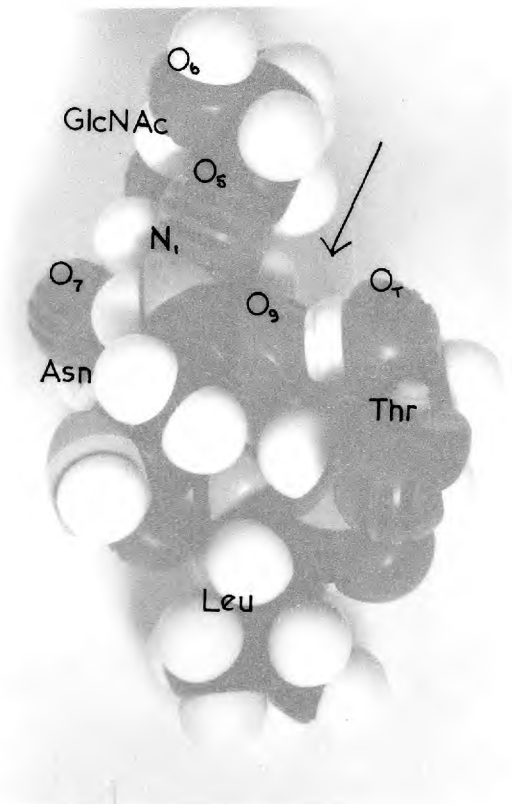


Fig. 1. Titration curve of a sample of the glycopeptide. The points represent some of the experimentally determined results with appropriate corrections for titration of the solvent. The continuous line indicates the theoretical values obtained by assuming there are 1·05 equiv. each of ionizing groups with pK values of 3·5 and 6·7 respectively.

Plate 1.1. A space-filling model representing a glycopeptide in which a hydrogen bond is incorporated.



An N-acetyl-D-glucosaminyl residue (marked GlcNAc) is linked by an N-acylglycosylamine type of structure to the β-amide group of an L-asparaginyl residue (marked Asn), which is incorporated in a peptide, Asn-Leu-Thr. The 3-hydroxyl group forms a hydrogen bond (marked with an arrow) to the oxygen atom of the β-amide group of the L-asparaginyl residue. The following atoms are marked: The ring oxygen = O_5; the oxygen atom of the linking amide group = O_9; the nitrogen atom of the linking amide group = N_1; the oxygen atom of the 2-acetamido group = O_7; the oxygen atom of the C_6 hydroxyl group = O_6 and oxygen atom of the 3-hydroxyl group of the L-threonine residue = O_τ. The model is based on that proposed by Neuberger & Marshall (1969).

Plate 1.1. A space-filling model representing a glycopeptide in which a hydrogen bond is incorporated.

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Table 1.5 Sequences of peptides containing glycosylated serine and threonine residues.

Heavy chain disease protein, Zuc.	Pro-Gly-Gly-SER-Ser-Glu-Pro-Lys [1]
Ox submaxillary glycoprotein	Gly-SER [2] Ser-SER Ser-THR-Gly-SER
Chondroitin-4-sulphate	Gly-Gly-SER-Gly [3]
α-Amylase (Aspergillus oryzae)	SER-Glu-Asp-Gly-(Ala,Thr) [4]
Rabbit γ G immunoglobulin	Ser-Lys-Pro-THR-Cys-Pro-Pro-Pro-Glu-Leu [5]
"Anti-freeze" glycoprotein	THR-Ala-Ala-THR-Ala-Ala-THR [6]
Ox encephalitogen	Val-Thr-Pro-Arg-THR-Pro-Pro-Pro-Ser-Gln-Gly-Lys [7]

SER/THR represent those serine or threonine residues that are glycosylated.

Sequences of peptides containing glycosylated hydroxylysine residues.

Guinea-pig skin tropocollagen	Gly-Met-HYL-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu- Asp-Gly-Ala-Lys-Gly-Asn-Thr-Gly-Pro-Ala-Gly- Pro-Lys-Gly-Glu-Hyp-Gly-Ser-Hyp-Gly-Glx-Asx [8,9]
Carp swim bladder collagen	Gly-Ile-HYL-Gly-His-Arg [10]
Human skin collagen	Gly-Phe-HYL-Gly-Ile-Arg [10] Gly-Pro-HYL 10

HYL represents those hydroxylysine residues that are glycosylated.

in the glycopeptide containing several amino acids.

It was suggested that a hydrogen-bond formed between the proton of the β-hydroxyl group of the L-threonine residue to the oxygen atom of the linkage imide group would facilitate the dissociation of the N-H imide proton. A model of this structure may be built (Plate 1.1) using a distance between the two hydrogen-bonded atoms of 2.7 Å (Pauling, 1948). The side chain of the intermediate amino acid with a non-polar side chain (L-leucine) is directed away from the carbohydrate moiety.

If a hydrogen-bonded structure of the type shown in Plate 1.1 is present in one of the conformations adopted by the apoprotein, it might provide the basis for the specificity towards the transferase.

The specificity may be elaborated further by an examination of the crystal-structure of ribonuclease A or S (Wyckoff et al., 1970). The L-asparagine residue No. 34 corresponds with the glycosylated L-asparagine residue in ribonuclease B. Out of the 10 L-asparagine residues, No. 34 is not the most exposed, but is the only one with a β-hydroxy amino acid in the position next but one towards the C-terminus.

Sequence specificities for other alterations of residues in polymers.

Only a few sequences in the neighbourhood of glycosylated L-serine or L-threonine residues have been established (Table 1.5). Encephalitogen is not naturally

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3. Ishihara & Schmid (1967a)
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5. Hester (1969)
6. Hashemeyer, Cynkin, Han & Trindle (1966)
7. Iwanago et al. (1968)
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11. Rosevear & Smith (1961)
12. Clamp & Putnam (1964)
13. Nolan & Smith (1962)
14. Hill et al. (1966)
15. Smyth & Utsumi (1967)
16. Duquesne, Monsigny & Montreuil (1965)
17. Howell, Hood & Sanders (1967)
18. Melchers (1969)
19. Sox & Hood (1970)
20. Shimizu et al. (1971a)
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22. Spik & Montreuil (1968)
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24. Rawitch, Liao & Pierce (1968)
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26. Papkoff, Sairam & Li (1971)
27. Cunningham, Clouse & Ford (1963)
28. Lee & Montgomery (1962)
29. Johansen, Marshall & Neuberger (1961)
30. Monsigny, Adam-Chosson & Montreuil (1968)
31. Montgomery & Wu (1963)
32. Tanaka (1961)
33. De Lange (1970)
34. Williams (1968)
35. Elleman & Williams (1970)
36. Augustyniak & Martin (1968)
37. Shainkin & Perlmann (1971)
38. Plummer & Hirs (1964)
39. Jackson & Hirs (1970)
40. Catley, Moore & Stein (1969)
41. Radhakrishnamurthy & Berenson (1966)
42. Yasuda, Takahashi & Murachi (1970)
43. Anai, Irenaka & Matsushima (1966)
44. Katzman & Oronsky (1971)

References for Table 1.5

1. Frangione & Milstein (1969)
2. Ozeki & Yosizawa (1971)
3. Katsura & Davidson (1971)
4. Tsugita & Akabori (1959)
5. Smyth & Utsumi (1967)
6. De Vries, Vandenheede & Feeney (1971)
7. Hagopian, Westall, Whitehead & Eylar (1971)
8. Butler & Cunningham (1966)
9. Butler (1970)
10. Morgan, Jacobs, Segrest & Cunningham (1970)

glycosylated, but may function as a sugar acceptor in the presence of UDP-N-acetyl-D-galactosamine and an appropriate transferase (Macpian & Eylar, 1968). An L-proline triplet occurs near to the glycosylated L-threonine residues here, and also in rabbit γG immunoglobulin. On the other hand, the submaxillary mucins do not have many, if any, sequences of this type. Other modifications that occur to amino acid residues subsequent to incorporation in a protein chain seem to require that the amino acid undergoing change lies in a restricted type of sequence. Hydroxylation of proline in collagen appears to require the sequence: X-Pro-Gly where X may be any amino acid except glycine (McGee, Rhoads & Udenfriend, 1971). There are indications that the specificity requirements for the L-proline hydroxylation involve longer sections of the protein chain (Kivirikko et al., 1971). There also appears to be specificity for methylation and acetylation of L-lysyl residues in histones (De Lange, Smith & Donner, 1970) and for the formation of betaine residues (in cytochromes ; De Lange, Glazer & Smith, 1970), each of which is followed by an unaltered L-lysyl residue.

It is of interest also that glycosylation of hydroxy methyl cytosine residues in certain bacteriophage DNA appears to have specific sequence requirements in that those residues that lie in the sequence PupH*pHpHpPu (where H* is the glycosylated residue) are

more frequently glycosylated (Lunt & Newton, 1965).

Section 5. The present work.

The acid-base titration behaviour of glycopeptide material, prepared from hen ovalbumin, containing the sequence ASN-Leu-Thr was reinvestigated and interpreted on the basis of all the titrating groups found to be present from analytical data. In addition, the ovalbumin from a related species, the Embden goose, was isolated and analysed, and glycopeptides of a similar nature prepared by enzymic digestion.

CHAPTER 2.

THE ACID-BASE TITRATION OF GLYCOPEPTIDES.

EXPERIMENTAL.

Some of the analytical methods, such as those used for the determination of amino acids, sugars and amino groups, are described in Chapter 6.

Preparation of hen ovalbumin.

Two hundred fresh, non-fertilised, eggs from a number of hens were purchased from Appleby Farm, Ashford, Kent. The ovalbumin was prepared by ammonium sulphate fractionation of the egg-white (Sørensen & Høyrup, 1915-1917), followed by five recrystallisations from half-saturated solutions of ammonium sulphate. The crystals were examined under the photomicroscope at 160 magnification.

Disc-gel electrophoresis.

The preparation was examined by polyacrylamide disc-gel electrophoresis (Davis, 1964) on gels of 9.4% (w/v) acrylamide and 0.25% (w/v) N,N'-methylenebis-acrylamide at pH 8.5. Crystalline ovalbumin was separated by centrifugation, and 1mg was dissolved in running buffer (1ml) together with sucrose (100mg). An aliquot (50µl) of this solution was loaded on the top of each gel.

Electrophoresis was also performed on gels containing 0.1% (w/v) sodium dodecyl sulphate, as described by Marshall & Zamecnik (1969), except that the gels were run at room temperature and, before electrophoresis, the protein solutions (1mg/ml) were left overnight at

+4°C in the running buffer together with 0.1% (v/v) mercaptoethanol and 0.1% (w/v) sodium dodecyl sulphate. The mobility of each protein band was expressed relative to the distance moved by bromophenol blue. The gels were stained with Amido Schwarz and destained with 7% acetic acid.

Proteolytic digestion.

The crystalline material was dissolved in water, dialysed extensively against water, and then denatured by pouring into 5 volumes of methanol. The precipitate was filtered off and dried over anhydrous calcium chloride in vacuo (yield 114g). Proteolytic enzymes were bought from Armour Laboratories, Eastbourne.

Denatured ovalbumin (100g) was suspended in water (2 l), covered with a layer of toluene, and equilibrated at 37°C. The pH was brought to 2.8 with 4M-HCl, and 1.6g crystalline pepsin added. Further amounts of pepsin were added after 26 hr (1.6g), 48 hr (0.8g) and 110 hr (0.8g). The pH was regulated at pH 2.8 by means of a pH stat (Radiometer Ltd., Copenhagen) by addition of 4M-HCl from a burette operated by a magnetic valve.

The progress of digestion was monitored by measuring the amount of acid added, and by the increase in colour, after reaction with ninhydrin, of small aliquots withdrawn from the digestion mixture. After 120 hr, the pH was raised to 7.8 with 4M-NaOH, and trypsin (0.27g) and chymotrypsin (0.27g) were added. The pH was kept constant with 4M-NaOH during the initial period of digestion with trypsin and chymotrypsin, and then with

4M-HCl at the later stages (after 182 hr of total digestion time) when the pH began to rise above 7.8. The digestion was stopped at 225 hr and the pH lowered to 5.5.

Purification of glycopeptides.

The digest was concentrated to 200ml by freeze-drying, small amounts of insoluble material were removed by centrifugation, and small aliquots assayed for D-mannose. n-Propanol (1800ml) was added. The mixture was stirred, and stood at +4°C for 3 hr. The precipitate was collected by centrifugation at +4°C and taken up in 100ml water. Small aliquots were removed and analysed for D-mannose and total nitrogen. Ninety percent of the D-mannose present in the digest (1.6g) appeared to have been precipitated, and the molar ratio of nitrogen to D-mannose had been reduced to a value of 42, in the precipitate, from the calculated value of 102, in the original protein.

A portion (0.5ml) of the redissolved precipitate was placed on a column (79 x 2.5cm) of Sephadex G-25 (fine grade, Pharmacia Ltd., Uppsala, Sweden), equilibrated with 0.1M-acetic acid at +4°C, and was eluted with 0.1M-acetic acid at 14.4ml per hr at +4°C. Fractions of 11ml were collected and assayed for D-mannose, nitrogen and extinction at 275nm. The D-mannose-containing fractions (7.4mg D-mannose were) collected and freeze-dried.

Further concentration of the rest of the original solution (100ml), containing n-propanol-precipitated material, produced a solution that was too viscous for

gel-chromatography. Glacial acetic acid (0.6ml) was added to the solution containing the rest of the digest, and the material was placed on a large column of Sephadex G-25 (75 x 6cm), equilibrated with 0.1M-acetic acid as before, and eluted at a flow rate of 50ml/hr. The fractions containing hexose were collected, concentrated to about 25ml, and the concentrated material was again subjected to gel-chromatography on the same column, which had previously been washed extensively with 0.1M-acetic acid. The fractions containing hexose were again concentrated and the material was subjected to gel-chromatography for a third time.

The fractions containing high concentrations of glycopeptide, obtained by the third cycle of gel-chromatography (Fractions 104-107; see Fig. 2.2b), were retained separately. Fractions 90-103 were pooled and dried in the frozen state, and the residue was taken up in water (10ml). Fractions 108-120 were also pooled and concentrated.

<u>High-voltage paper electrophoresis</u>.

Small amounts (containing about 250µg of hexose) of glycopeptide material, that had been separated by gel-chromatography as described above, were subjected to paper electrophoresis at pH 2.0 for 2 hr at 32V/cm. The electrophoretic strips were dipped either through the ninhydrin reagent or the periodate/permanganate reagent (see Chapter 6).

<u>Analysis of glycopeptide material</u>.

The fractions obtained by gel-chromatography, and pooled as described above, were analysed for

D-glucosamine and amino acids, which were released by acid hydrolysis in 4M and 6M-HCl (see Chapter 6). The values for threonine and serine were corrected for losses of 3.6 and 7.7% respectively, occurring during acid hydrolysis. Each fraction was also assayed for its content of nitrogen and hexose and for the colour produced by reaction with ninhydrin.

Acid-base titration.

Glycopeptide material, containing about 40 μmoles of bound aspartic acid, was precipitated from 3ml portions of each of Fractions 104, 105, 106 and 107 (see Fig. 2.2b) by the addition of 57ml ethanol at room temperature, and the suspensions were shaken, and centrifuged. The pellet was then redissolved in 0.5ml water, and glycopeptide material was re-precipitated by the addition of ethanol (9.5ml). The precipitate was isolated, dried over $CaCl_2$ *in vacuo* at room temperature, and taken up in 4ml of freshly-deionised water. Solid KCl, recrystallised from aqueous ethanol, was added in quantities such that the solution was 1M in KCl when made up to 5ml by addition of water. A small amount of the solution was subjected to acid hydrolysis (6M-HCl; 110°C; 20 hr) and the amino acids released were analysed.

This solution (3.6ml) was titrated in a water-jacketed titration cell at a controlled temperature. The surface of the solution was flushed with nitrogen saturated with water-vapour, and the solution was stirred magnetically during titration. The pH was measured with a Radiometer (Copenhagen) pH meter, which was standardised against 0.05M-potassium hydrogen phthalate buffer (Fisons, Ltd.) and 0.05M-sodium borate buffer (British Drug Houses) at various

temperatures:-

T°C	pH of phthalate buffer	pH of borate buffer
2	4.01	10.6
20	4.00	10.00
25	4.01	9.96
40	4.03	9.84
60	4.10	9.68

The two buffers agreed to within ± 0.02 pH units.

Titrant (0.1M, 0.4M, 1.0M or 4.0M, -KOH or-HCl (prepared by dilution of British Drug Houses Volucon solutions with freshly deionised water) was added from a 0.5ml micrometer syringe (Burroughs Wellcome & Co., London). Sufficient volumes of titrant were added to change the pH about 0.1 unit each time, and the pH was read 30 seconds after the addition of titrant. Blank titrations were performed on 3.6ml 1M-KCl and corrections were made for volume differences that occurred during titration.

Titrations were performed with KOH solutions up to the alkaline end-point, and then back titrations were performed with the HCl solutions down to about pH 2. Samples from Fraction 106 and Fraction 104 were also titrated initially with acid down to about pH 2, at temperatures of 40°C and 60°C respectively.

One of the samples of glycopeptide material (Fraction 105; see Fig. 2.2b and Table 2.3), which had been titrated at 2°C, was reisolated and recovered by placing on a column of Dowex 50-X8 (20-50 mesh; H^+ form;

55 x 3cm) and eluting with water at +4°C. The carbohydrate-containing fractions (eluting at a peak after 120ml) were neutralised with a measured volume of 0.4M-KOH (about 2ml) concentrated on the rotary evaporator to about 3ml, and made 1M in KCl in a volume of 5ml as before. A sample of this (3.6ml) was titrated and a portion was analysed for amino acids as described above. Another of the samples (Fraction 104), which had previously been titrated at 60°C, was reisolated and retitrated in the same way.

RESULTS.

Hen ovalbumin preparation.

Under the light microscope, the hen ovalbumin preparation appeared as small, cigar shaped needles which grouped themselves to form rosettes (Plate 2.1).

Polyacrylamide disc-gel electrophoresis without the inclusion of detergent gave one broad band with a mobility relative to that of bromophenol blue of 0.54 and a narrow band with a relative mobility of 0.49 (see Plate 2.2). Electrophoretic heterogeneity of ovalbumin was suggested by Linderstrøm-Lang & Ottesen (1949), and has been shown by Perlmann (1955), to be due to the attachment of two, one or no phosphate groups to L-seryl residues in the protein. The protein component containing two phosphate groups might be expected to give rise to the faster-running band under the conditions used here.

Plate 2.1. Crystals of hen ovalbumin viewed under a magnification of 160.

The crystals separated from solutions, which were half-saturated in ammonium sulphate, at pH 4.6.

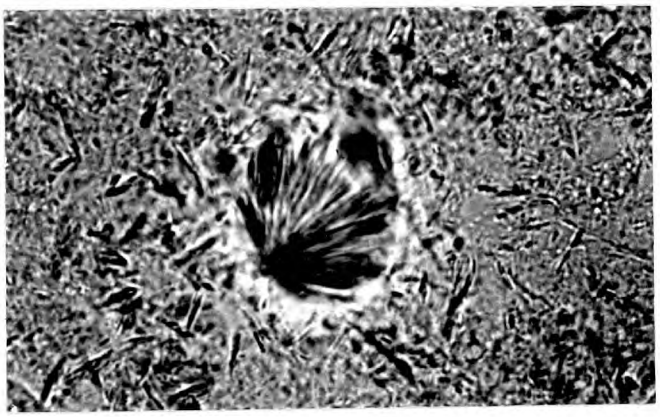
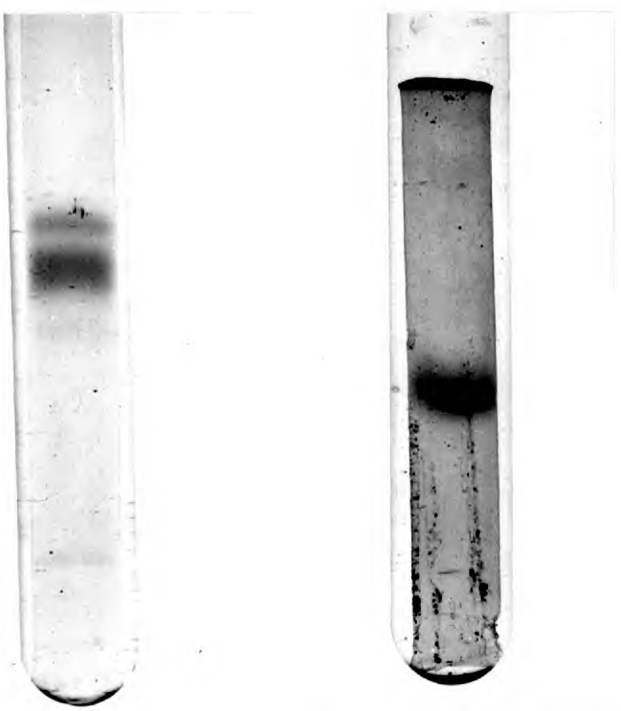


Plate 2.2. Electrophoresis of hen ovalbumin on polyacrylamide gels.

-ve A B



+ve

Gel A was run under the conditions of Davis (1964). The pH was 8.5.

Gel B was run in 0.1% sodium dodecyl sulphate as described by Marshall & Zamecnik (1969). About 50µg of protein was placed on the top of each gel. Gels were cut at the band given by bromophenol blue.

Electrophoretic heterogeneity in de-phosphorylated ovalbumin was observed by Lush (1964) on starch gels at pH 5, and two genetic variants have been separated and distinguished by the presence of either an L-asparaginyl or an L-aspartyl residue in the same relative position in peptides of otherwise identical sequence (Wiseman, Fothergill & Fothergill, 1971).

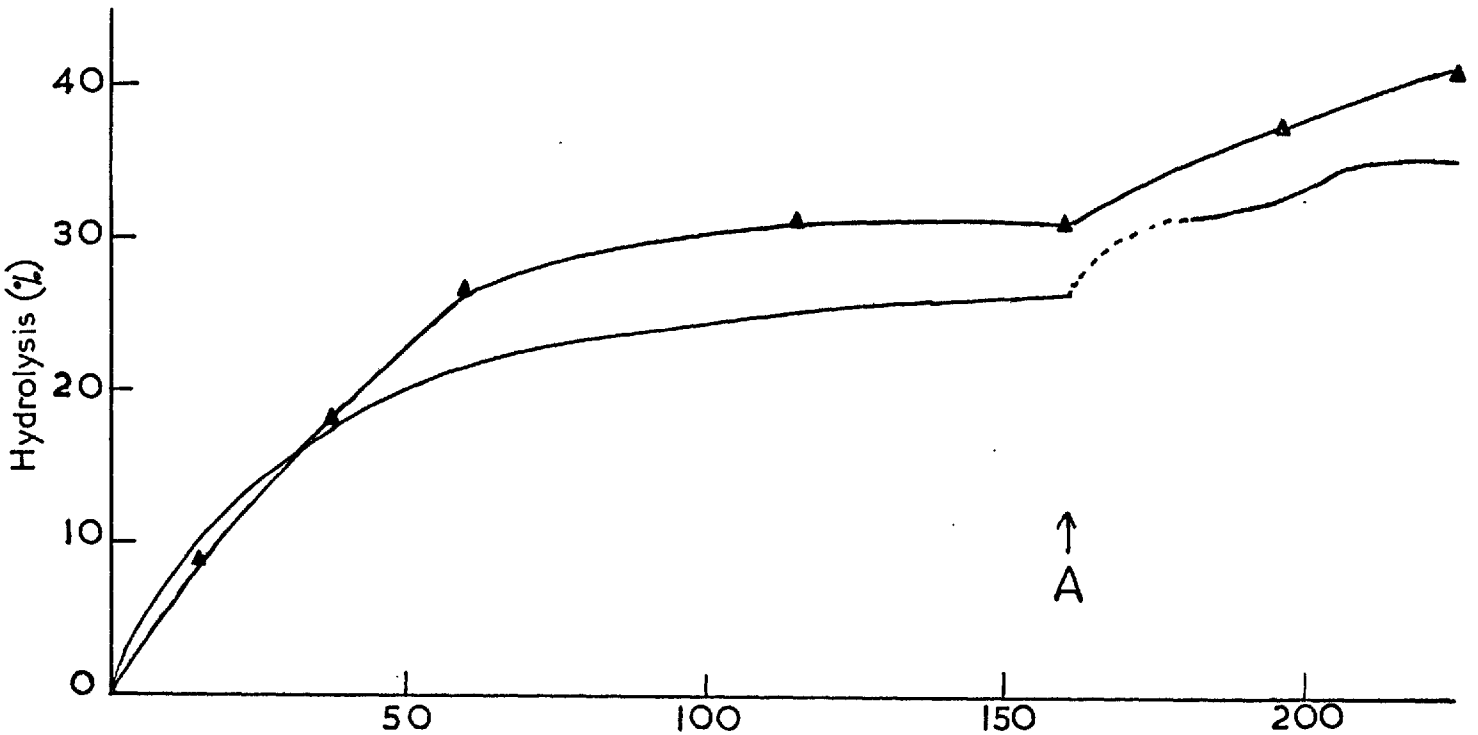
Polyacrylamide disc-gel electrophoresis in 0.1% (w/v) sodium dodecyl sulphate gave one band with a mobility relative to that of bromophenol blue of 0.53 (Plate 2.2).

Proteolytic digestion.

The increase in colour after reaction with ninhydrin (using glycine as standard), and uptake of acid or alkali that occurred during digestion of ovalbumin at constant pH, are shown in Fig. 2.1. The values shown were calculated on the assumption that the free amino group formed from each peptide bond split gave the same molar colour yield with ninhydrin as glycine, while the uptake of acid or alkali is expressed as the number of moles of acid or alkali per total number of peptide bonds (387). From the ninhydrin values, approximately 30% of the total number of peptide bonds may be calculated to have been split with pepsin, and a further 10% with trypsin and chymotrypsin. A 40% splitting of peptide bonds has been observed with ovalbumin (Fletcher, Marks, Marshall & Neuberger, 1963) after digestion with the same combination of enzymes.

The number of moles of acid taken up during the first part of the reaction with pepsin (up to 40 hr) was

Fig. 2.1. Rate and extent of hydrolysis of hen ovalbumin by pepsin, trypsin and chymotrypsin.



Digestion was commenced at pH 2.8 with pepsin

At the time marked A, pH was raised to 7.8 and digestion was continued with trypsin and chymotrypsin.

▲——▲ Number of free amino groups, measured by reaction with ninhydrin, as % of total number of peptide bonds (387)

——— Number of moles of H^+ taken up as % of total number of peptide bonds (387)

----- Number of moles of OH^- taken up as % of total number of peptide bonds (387)

equivalent to the number of moles of free amino group released as measured by the ninhydrin reaction, but dropped below this value as the reaction proceeded. At pH 7.8, alkali was added to keep the pH constant during the initial digestion by trypsin and chymotrypsin, and acid was added at the later stages of the reaction. This is discussed below.

Purification and analysis.

Ninety percent n-propanol precipitated a high yield (90%) of carbohydrate-containing material as (measured by hexose analysis) from the digest, leaving some free peptide and amino acids in the supernatant. The molar ratio of total nitrogen to D-mannose in the precipitate was 42, compared to a value of 102 in the original protein.

Gel-chromatography on a small column of Sephadex G-25 of a small proportion (0.5%) of the enzymic digest gave good separation of carbohydrate-containing material from free amino acids and peptides, as judged by the D-mannose and total nitrogen estimation (Fig. 2.2a). The most concentrated carbohydrate-containing fractions had a ratio of 9.3 moles of nitrogen per 5 moles of D-mannose. The u.v. absorption measurements made on the fractions showed that the aromatic peptides and amino acids were more retarded on Sephadex than other peptides or amino acids.

The same nitrogen to mannose ratio (9:5) was achieved only by three successive chromatographic separations on a large Sephadex G-25 column when the

Fig. 2.2a. Gel chromatography of a small proportion of the material obtained by enzymic digestion of hen ovalbumin.

Column of Sephadex G-25 (79 x 2.5 cm) at 4°C. Eluted by 0.1M-acetic acid.
Digest obtained from 0.5g hen ovalbumin was chromatographed.
Flow rate = 14.4ml per hr.

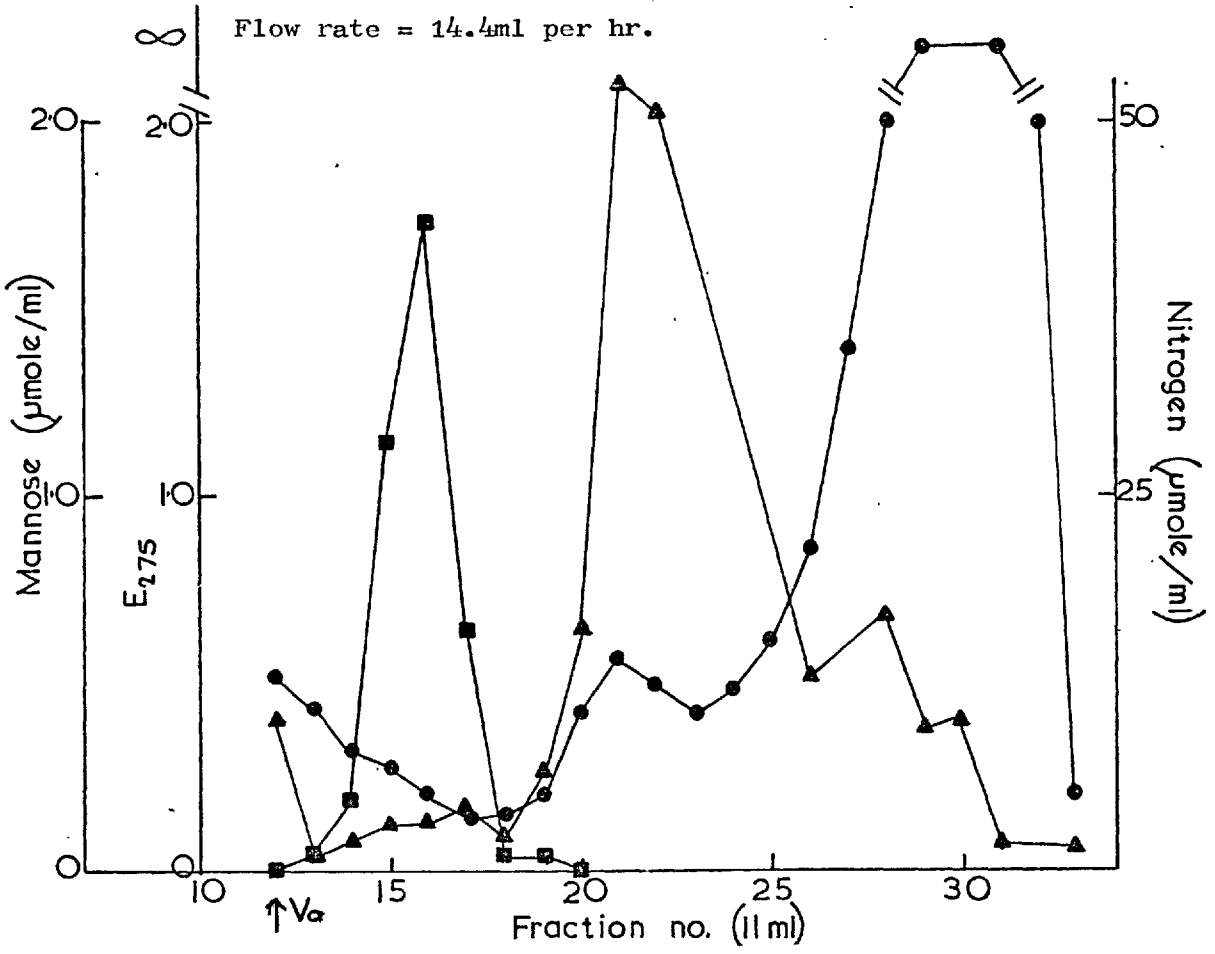


Fig.2.2b. Gel chromatography of the total material obtained by enzymic digestion of hen ovalbumin.

Column of Sephadex G-25 (76 x 6 cm) at 4°C. Eluted by 0.1M-acetic acid.
The elution profile of the third re-cycle (see text) is shown.
Digest obtained from 99.5g hen ovalbumin was chromatographed.
Flow rate = 50ml per hr

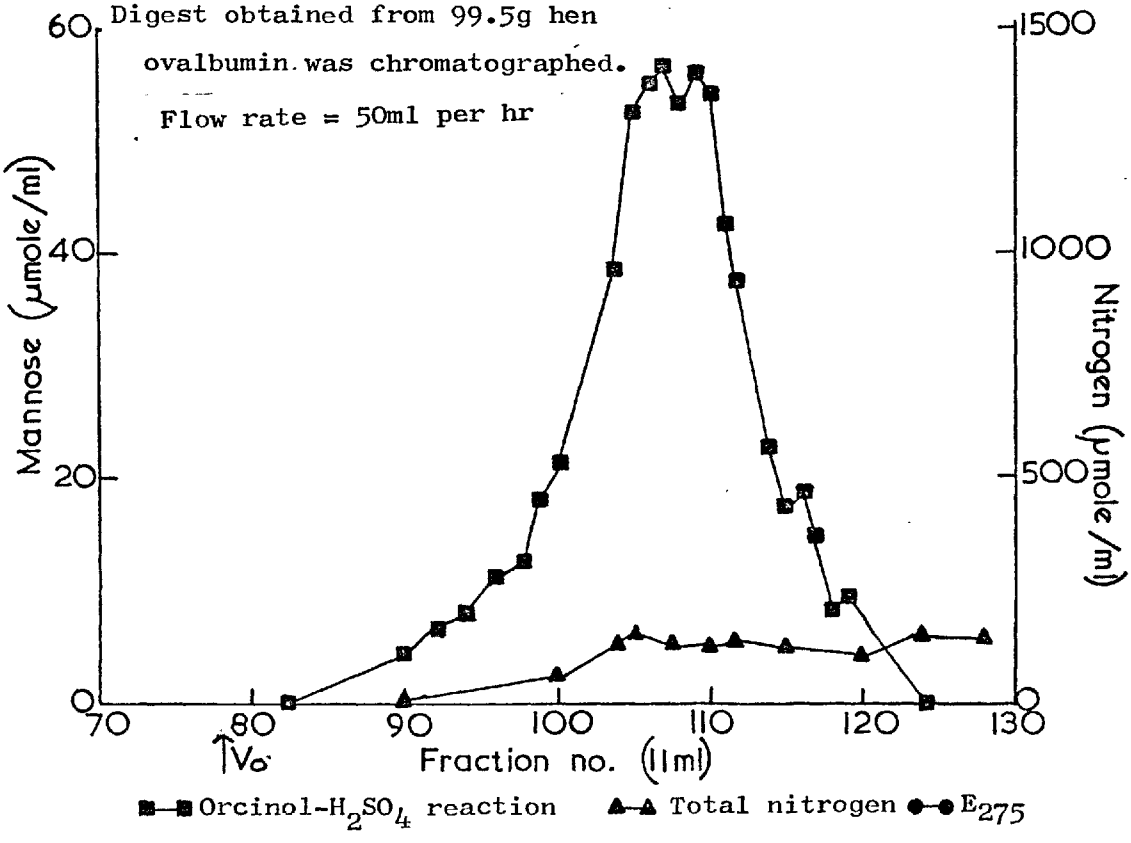


Table 2.1a. Analysis of fractions (see Fig. 2.2b) containing glycopeptide material obtained by col-chromatography.

Residue	Moles per mole Asp. Fraction No.					
	90-103	104	105	106	107	108-120
Glu	0.14	0.16	0.31	0.27	0.32	0.50
Lys	0.07	0.04	0.07	0.07	0.05	0.06
Tyr	0.07	0.06	0.07	0.08	0.07	0.05
Asp	1.00	1.00	1.00	1.00	1.00	1.00
Leu	0.96	0.95	0.87	0.87	0.81	0.77
Thr	0.85	0.81	0.78	0.77	0.72	0.69
Ser	1.02	0.93	0.95	0.89	0.86	1.04
Val	0.16	0.25	0.40	0.41	0.41	0.56
Gly	0.14	0.15	0.39	0.30	0.42	0.77
Ala	0.16	0.15	0.39	0.15	0.42	0.81
Glc N	4.13	3.42	3.14	3.28	2.28	2.38
Man	3.24	4.98	4.55	4.25	3.62	3.21
Total N (found)	9.94	8.75	8.55	n.d.	7.72	n.d.
Total N (calc.)	9.75	8.56	9.48	9.27	8.36	9.45
Ninhydrin value	0.62	0.62	0.60	0.61	0.63	0.63
% Asp in (Asn_2 Ser_3 Glu_4 Gly_5 Ala_6 Val_6)	0%	0%	8%	6%	12%	19%

rest of the digest was applied. The high viscosity of the concentrated solutions limited the minimum volume which could be applied to the column.

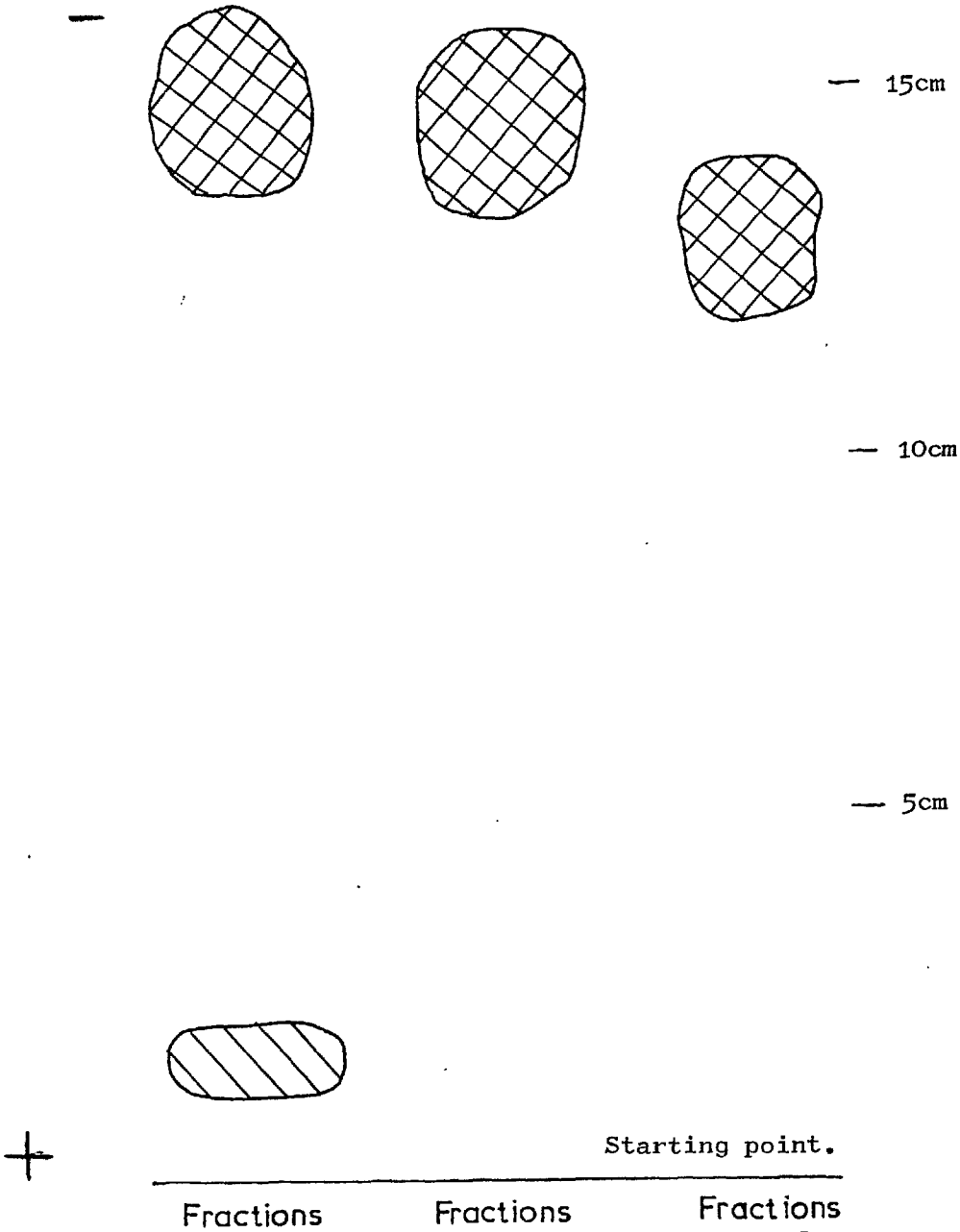
Some of the fractions obtained were examined by paper electrophoresis (Fig. 2.3). Fractions 90-107 gave one broad band which stained yellow with the periodate/permanganate reagent, while the ninhydrin reagent produced a brown colour initially, changing to a purple colour after a few days. A brown colour has been noted previously with this reagent when detecting the free amino group of glycosylated L-asparagine residues on paper (Marshall & Neuberger, 1972).

Combined portions from fractions 104-107 gave only one peak, on the Locarte autoanalyser, eluting 29 min after loading the sample, in the course of elution with the pH 3.15 (0.2M-Na^+) citrate buffer (see Chapter 6). A molar colour yield of 0.53 with respect to norleucine was calculated; the number of moles of glycopeptide loaded was calculated on the basis of the yield of aspartic acid measured after acid hydrolysis, on the assumption that hydrolysis yielded one mole of aspartic acid per mole of glycopeptide. The colour yield of GlcNAc-Asn on the Locarte autoanalyser was 0.407; the Technicon autoanalyser gave much lower values (see Chapter 4). Assay with the ninhydrin reagent gave a molar colour yield of about 0.6 with respect to that of glycine.

Examination of the analyses of fractions shown in (Table 2.1a) shows that the glycopeptide had fractionated

Fig. 2.3. Paper electrophoresis of glycopeptides prepared from hen ovalbumin.

Glycopeptide material (containing about 250µg mannose) was subjected to electrophoresis at pH 2 for 2 hr at 32 V/cm.Fractions were obtained by gel chromatography as shown in Fig. 2.2b.



Spots shaded ⧻ developed with both the periodate / permanganate and the ninhydrin reagents.

Spots shaded ⫽ developed only with the ninhydrin reagent.

Table 2.1b. Analysis of carbohydrate-containing material from the pilot gel-chromatographic purification of digested ovalbumin, and composition in terms of the known amino acid sequence in the vicinity of the carbohydrate moiety.

Analysis		Composition	
Residue	Moles per mole aspartic acid	Glycopeptide	Mole Fraction
Glu	0.05	Glu-Glu-Lys-Tyr-ASN	0.07
Lys	0.07		
Tyr	0.07	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)Ser	0.07
Asp	1.00		
Leu	0.95	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.12
Thr	0.72		
Ser	0.79	ASN-Leu-Thr-Ser-Val	0.25
Val	0.42		
Gly	0.18	ASN-Leu-Thr-Ser	0.30
Ala	0.17		
GlcN	3.12	ASN	0.19
Man	4.71		

Composition of carbohydrate-containing material from Fractions 90-103 (Fig.2.2b)

Glycopeptide	Mole fraction
Glu-Glu-Lys-Tyr-ASN	0.07
ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)Ser	0.16
ASN-Leu-Thr-Ser	0.69
ASN	0.08

ASN represents the glycosylated asparagine residue.

according to the size of its heterogeneous carbohydrate moiety (Cunningham, 1968), and according to the number of amino acid residues in the peptide portion. It may be seen that a mixture of glycopeptides containing different lengths of peptide was obtained. Less than one mole, per mole of aspartic acid, of each of the other amino acids listed was released by acid hydrolysis. This was presumably due to the use of enzymes of differing specificities.

The amino acid sequence in the vicinity of the carbohydrate moiety is known (Table 1.4) to be as follows:-

carbohydrate
|
Glu-Glu-Lys-Tyr-ASN-Leu-Thr-Ser-Val-Leu

The compositions of the fractions of glycopeptide material obtained are expressed as mole fractions of glycopeptides, each of which contains a different length of peptide chain (Table 2.1b). The compositions were deduced from the amino acid analyses and from the known sequence, extended at the C-terminus by glycine, L-alanine and L-serine.

High voltage paper electrophoresis of the material containing hexose which was obtained in the fractions that were more retarded by chromatography on Sephadex G-25 (Fractions 108-120) separated a component with slow mobility. This component stained faintly blue with ninhydrin and did not contain carbohydrate (Fig. 2.3). The component was eluted from some previously washed electrophoretic paper, and the faster moving material that contained carbohydrate was also

Table 2.2. Analysis of glycopeptide and peptide after paper electrophoretic purification of Fractions 108-120 (see Fig. 2.3).

Glycopeptide (Band 2) 6% of total no. of moles of Asp.		Peptide (Band 1) 1% of total no. of moles of Asp.	
Asp	1.00	Asp	1.00
Leu	0.96	Ser	1.46
Thr	0.84	Glu	2.05
Ser	0.91	Gly	2.74
Val	0.25	Ala	3.19
Gly	0.15	Val	2.82
Ala	0.15	Leu	0.03
GlcN	2.52	Thr	0.02
Man	4.13	Probable composition:- $Asx_2\ Ser_3\ Glx_4\ Gly_5\ Ala_6\ Val_6$	

Analysis of Fractions 104 and 105 after titration, and reisolation from Dowex 50.

Fraction 104		Fraction 105	
Asp	1.00	Asp	1.00
Leu	0.95	Leu	0.96
Thr	0.88	Thr	0.85
Ser	0.90	Ser	0.89
Val	0.19	Val	0.08
Gly	0.07	Gly	0.06
Ala	0.08	Ala	0.08
Glu	0.08	Glu	0.07
Lys	0.04	Lys	0.02
Tyr	0.08	Tyr	0.08
Man	4.93	Man	4.85
GlcN	n.d.	GlcN	n.d.

eluted. The sugar and amino acid analyses of the two bands are presented in Table 2.2.

The low mobility of this peptide on paper electrophoresis at pH 2 might suggest that it was a fairly large peptide, and the fact that its elution volume from the Sephadex column was similar to that of the glycopeptide might suggest that it had a molecular weight of about 2,000, considering that peptides are retarded to a greater extent on G-25 than carbohydrates of the same molecular weight (see Chapter 3). Its composition is described in Table 2.2. The peptide did not yield leucine or threonine on acid hydrolysis, and inspection of the analyses of the earlier fractions (90-107) showed that only trace amounts ($<13\%$) of the aspartic acid could be contained in this peptide in these fractions. The amount of peptide present was deduced from the amounts of glutamic acid found in excess of those expected from the known sequence.

It is reasonable to believe that the contributions made by this peptide to the experimental titration curve were small, but they were nevertheless taken into account when interpreting the data. The peptide was separated from some of the fractions on Dowex 50-X8 (20-50 mesh; H^+ form) by elution with water (Rosevear & Smith, 1961). The carbohydrate-containing peak emerged after 120ml, and was collected. The analysis of glycopeptide material obtained by this treatment suggested that free peptide had been removed. The titration curve of this material was not significantly different from curves obtained on glycopeptide material which had not been subjected to Dowex 50 chromatography (Figs. 2.8 and 2.9).

Analysis of the titration curves of glycopeptides.

The experimental titration curves, corrected for the titration of equal volumes of 1M-KCl, are shown in Figs. 2.4 to 2.9. Each titration curve is calculated as the number of moles of titrant added per mole of aspartic acid released by acid hydrolysis. The experimental curves were analysed by fitting to them theoretical curves constructed from the following equation:-

$$pK = pH + \log \frac{[Acid]}{[Salt]} \qquad \text{(Henderson, 1908)}$$

A composite curve was then constructed by summing the individual, theoretically derived, curves. The amounts of titrating groups and their pKa values are tabulated (Tables 2.3 to 2.8).

It may be seen from Figs. 2.4 to 2.9 that the curves are complex, but two major groups, titrating with apparent pKa values of 3.15 (20°C) and 6.62 (20°C), may be identified. These pKa values may be assigned to the several α-carboxyl groups of the glycopeptides containing a number of amino acid residues on the C-terminal side of the glycosylated L-asparagine residue, and to the α-amino groups of these glycopeptides, respectively. The behaviour of these titrating groups at different temperatures are in keeping with this assignment. As the temperature is increased, the pKa of the group titrating at 6.62 (20°C) changes to a more marked extent than the group of pKa 3.30 (20°C). These changes cause the overall shape of the curve to become increasingly flat.

Table 2.3. The composition of Fraction 105 in terms of the known peptide sequence in the vicinity of the carbohydrate moiety.

	Glycopeptide	Mole fraction
1.	Glu-Glu-Lys-Tyr-ASN	0.07
2.	ASN	0.07
3.	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.15
4.	ASN-Leu-Thr-Ser-Val	0.09
5.	ASN-Leu-Thr-Ser	0.54
6.	$(Asn_2\ Ser_3\ Glu_4\ Gly_5\ Ala_6\ Val_6)$	0.04

Assignments of the pK_a values observed at 2°C (see Fig 2.4) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[(a)]	Experimental		Calculated		
	Moles	pK_a(2°C)	Group	Moles	pK_a(25°C)
2	?	2.10	α -COOH	0.07	2.10
3,4,5)			(α -COOH	0.78	3.10
1)	0.95	3.30	(α -COOH	0.07	2.80
6)			(α -COOH	0.04	3.00
1,6	0.29	4.25	γ -COOH	0.31	4.40
3,4,5	0.95	6.98	α -NH_2	0.78	6.60 (20°C)
1)	0.08	8.00	(α -NH_2	0.07	8.00
6)			(α -NH_2	0.04	8.00
2	0.12	8.75	α -NH_2	0.08	8.60
1	0.06	10.20	-OH	0.07	10.00
1	0.07	10.75	-NH_2	0.07	10.40

(a) The numbers in this column refer to the number of the glycopeptide given at the top of this table.

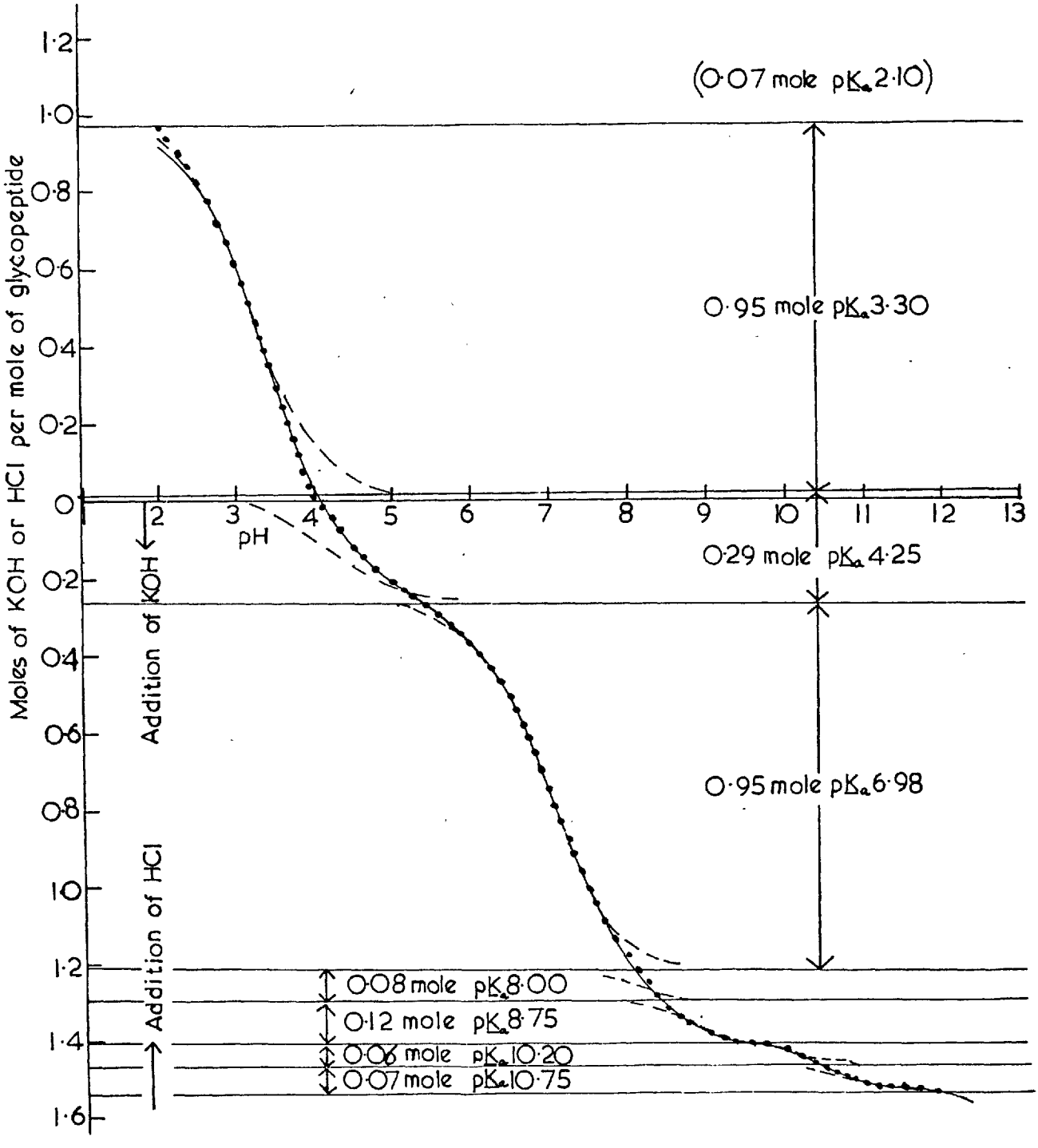
Fig. 2.4. Titration of glycopeptide material at 2°C.

Glycopeptide material from Fraction 105 (see Fig. 2.2b) in 1M-KCl.

Titration commenced at pH 4.1 by addition of KOH to pH 12.5.

Back titration from pH 12.5 to pH 2.0 was coincident with forward titration (down to pH 4.1).

The amounts and pK_a values of titrating groups are compared to the amino acid analysis in Table 2.3.



——— Experimental curve - forward titration to pH 12.5 and back titration to pH 2.0

– – – – Theoretical curves constructed for mole fractions of groups titrating at certain pK_a values

. . . . Composite theoretical curve constructed by addition of the titration expected from individual groups

Table 2.4. The composition of Fraction 107 in terms of known peptide sequence in the vicinity of the carbohydrate moiety.

	Glycopeptide	Mole fraction
1.	Glu-Glu-Lys-Tyr-ASN	0.07
2.	ASN	0.09
3.	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.10
4.	ASN-Leu-Thr-Ser	0.62
5.	$(Asn_2\ Ser_3\ Glu_4\ Gly_5\ Ala_6\ Val_6)$	0.06

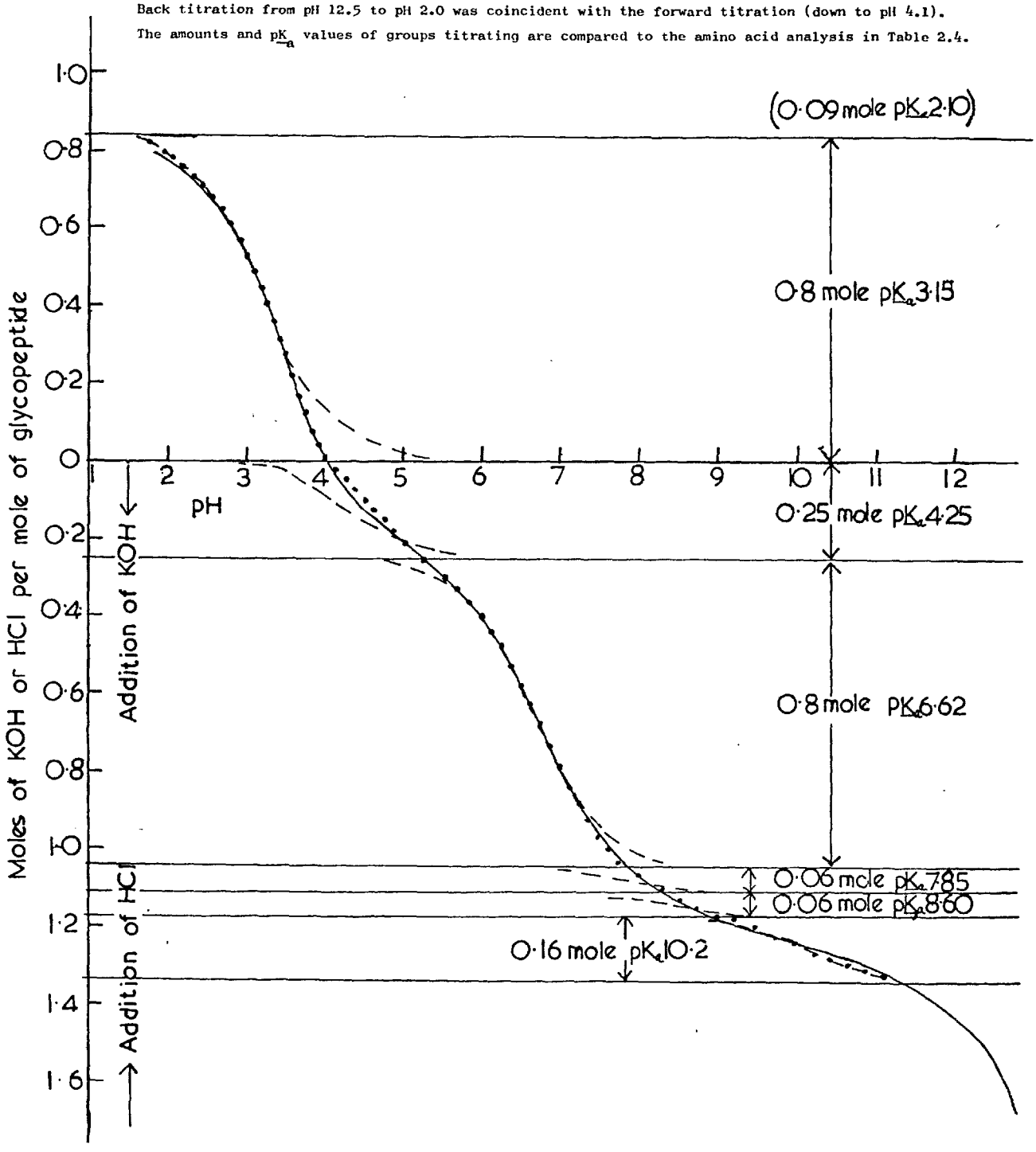
Assignments of the pK_a values observed at 20°C (see Fig. 2.5) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[a]	Experimental		Calculated		
	Moles	pK_a(20°C)	Group	Moles	pK_a(25°C)
2	?	2.10	α-COOH	0.09	2.10
3,4			α-COOH	0.75	3.10
5	0.80	3.15	α-COOH	0.06	3.00
1			α-COOH	0.07	2.80
1,5	0.25	4.25	γ-COOH	0.32	4.40
3,4	0.80	6.62	α-NH_2	0.72	6.60 (20°C)
1			α-NH_2	0.07	8.00
5	0.06	7.85	α-NH_2	0.06	8.00
2	0.06	8.60	α-NH_2	0.06	8.60
1	0.16	10.2	-OH	0.07	10.00
1			α-NH_2	0.07	10.40

(a) The numbers in this column refer to the number of the glycopeptide given at the top of this table.

Fig. 2.5. Titration of glycopeptide material at 20°C.

Glycopeptide material from Fraction 107 (see Fig. 2.2b) in 1M-KCl.
Forward titration from pH 4.1 to pH 12.5 by addition of KOH.
Back titration from pH 12.5 to pH 2.0 was coincident with the forward titration (down to pH 4.1).
The amounts and pK_a values of groups titrating are compared to the amino acid analysis in Table 2.4.



——— Experimental curve - forward titration to pH 12.5 and back titration to pH 2.0

– – – – Theoretical curves constructed for mole fractions of groups titrating at certain pK_a values

. . . . Composite theoretical curve constructed by addition of the titration expected from individual groups

Table 2.5. The composition of Fraction 106 in terms of the known peptide sequence in the vicinity of the carbohydrate moiety.

	Glycopeptide	Mole fraction
1.	Glu-Glu-Lys-Tyr-ASN	0.07
2.	ASN	0.14
3.	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.25
4.	ASN-Leu-Thr-Ser-Val	0.11
5.	ASN-Leu-Thr-Ser	0.36
6.	Asn_2 Ser_3 Glu_4 Gly_5 Ala_6 Val_6	0.03

Assignments of the pKa values observed at 40°C (see Fig. 2.6) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[a]	Experimental		Calculated		
	Moles	pK (40°C)	Group	Moles	pK (25°C)
2	?	2.10	α -COOH	0.14	2.10
3,4,5)			(α -COOH	0.72	3.10
1)	0.80	3.15	(α -COOH	0.07	2.80
6)			(α -COOH	0.03	3.00
1,6	0.26	4.10	γ -COOH	0.27	4.40
3,4,5	0.80	6.25	α $-NH_2$	0.72	6.60 (20°C)
1)	0.06	7.40	(α $-NH_2$	0.07	8.00
6)			(α $-NH_2$	0.03	8.00
2	0.14	8.10	α $-NH_2$	0.14	8.60
1)	0.10	9.90	(-OH	0.07	10.00
1)			(NH_2	0.07	10.40

(a) The numbers in this column refer to the number of the glycopeptide given at the top of this table.

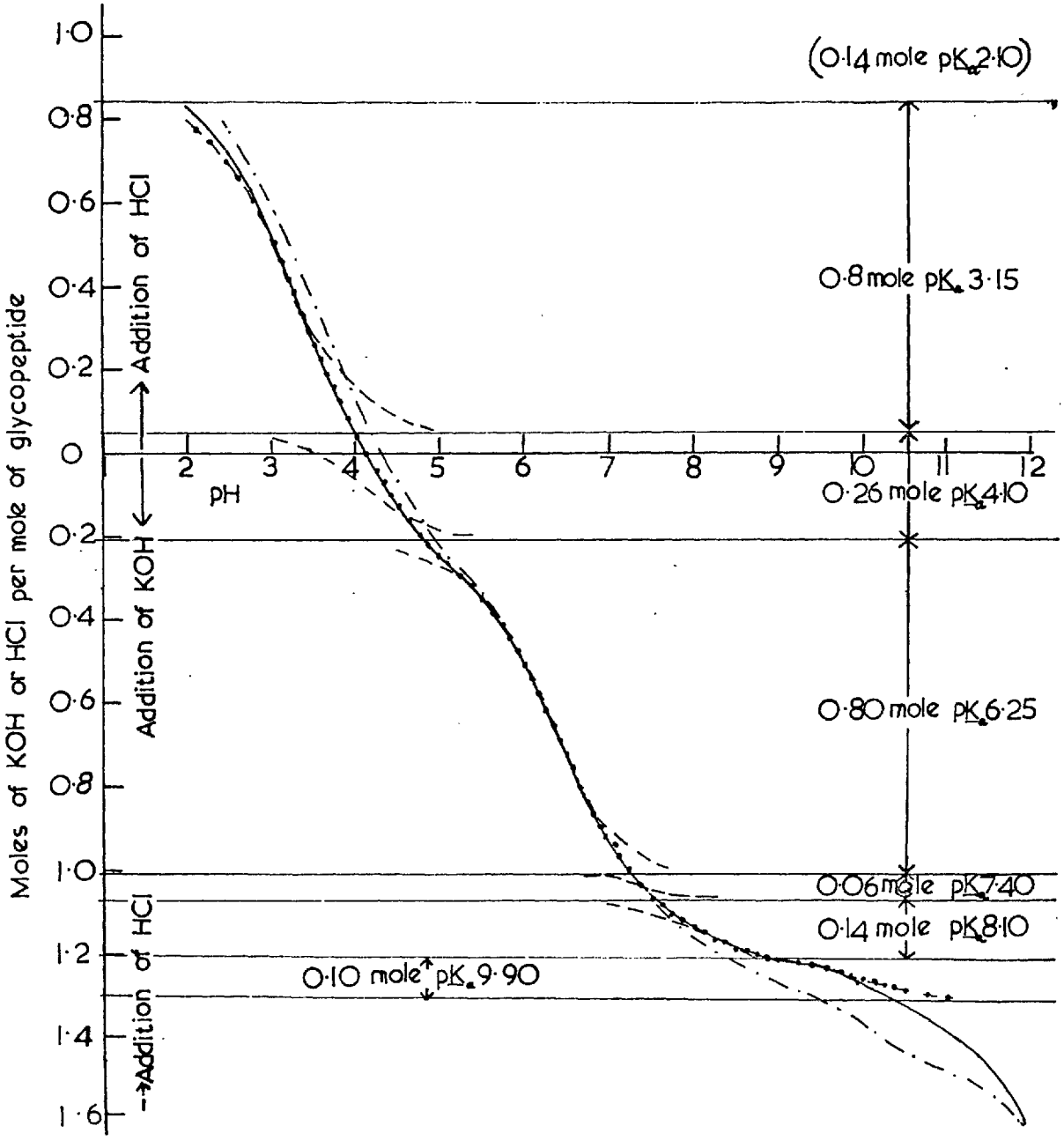
Fig. 2.6. Titration of glycopeptide material at 40°C.

Glycopeptide material from Fraction 104 (see Fig. 2.2b) in 1M-KCl.

Forward titration from pH 4.2 to pH 12.0 by addition of KOH, and on another sample from pH 4.2 to pH 2.2 by addition of HCl.

Back titration from pH 12.0 to pH 2.5 by addition of HCl.

The amounts and pK_a values of groups titrating are compared to the amino acid analysis in Table 2.5.



——— Experimental curve - forward titration

—·—·— Experimental curve - back titration from high pH

— — — Theoretical curves constructed for mole fractions of groups titrating at certain pK_a values

. . . . . Composite theoretical curve constructed by addition of titration expected from individual groups

Table 2.6. The composition of Fraction 104 in terms of the known peptide sequence in the vicinity of the carbohydrate moiety.

	Glycopeptide	Mole fraction
1.	Glu-Glu-Lys-Tyr-ASN	0.06
2.	ASN	0.13
3.	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)-Ser	0.12
4.	ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.03
5.	ASN-Leu-Thr-Ser-Val	0.10
6.	ASN-Leu-Thr-Ser	0.56

Assignments of the pK_a values observed at 60°C (see Fig. 2.7) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[(a)]	Experimental		Calculated		
	Moles	pK_a(60°C)	Group	Moles	pK_a(25°C)
2	?	2.10	α -COOH	0.13	2.10
1)	0.87	3.26	(α -COOH	0.06	2.80
3,4,5,6)			{ α -COOH	0.81	3.10
1	0.16	4.25	γ -COOH	0.16	4.40
3,4,5,6	0.81	5.92	α -NH_2	0.81	6.60 (20°C)
2)	0.17	7.78	(α -NH_2	0.15	8.60
1)			(α -NH_2	0.06	8.00
1	0.06	9.65	-OH	0.06	10.00
1	0.05	9.30	-NH_2	0.06	10.40

(a) The numbers in this column refer to the number of the glycopeptide given at the top of the table.

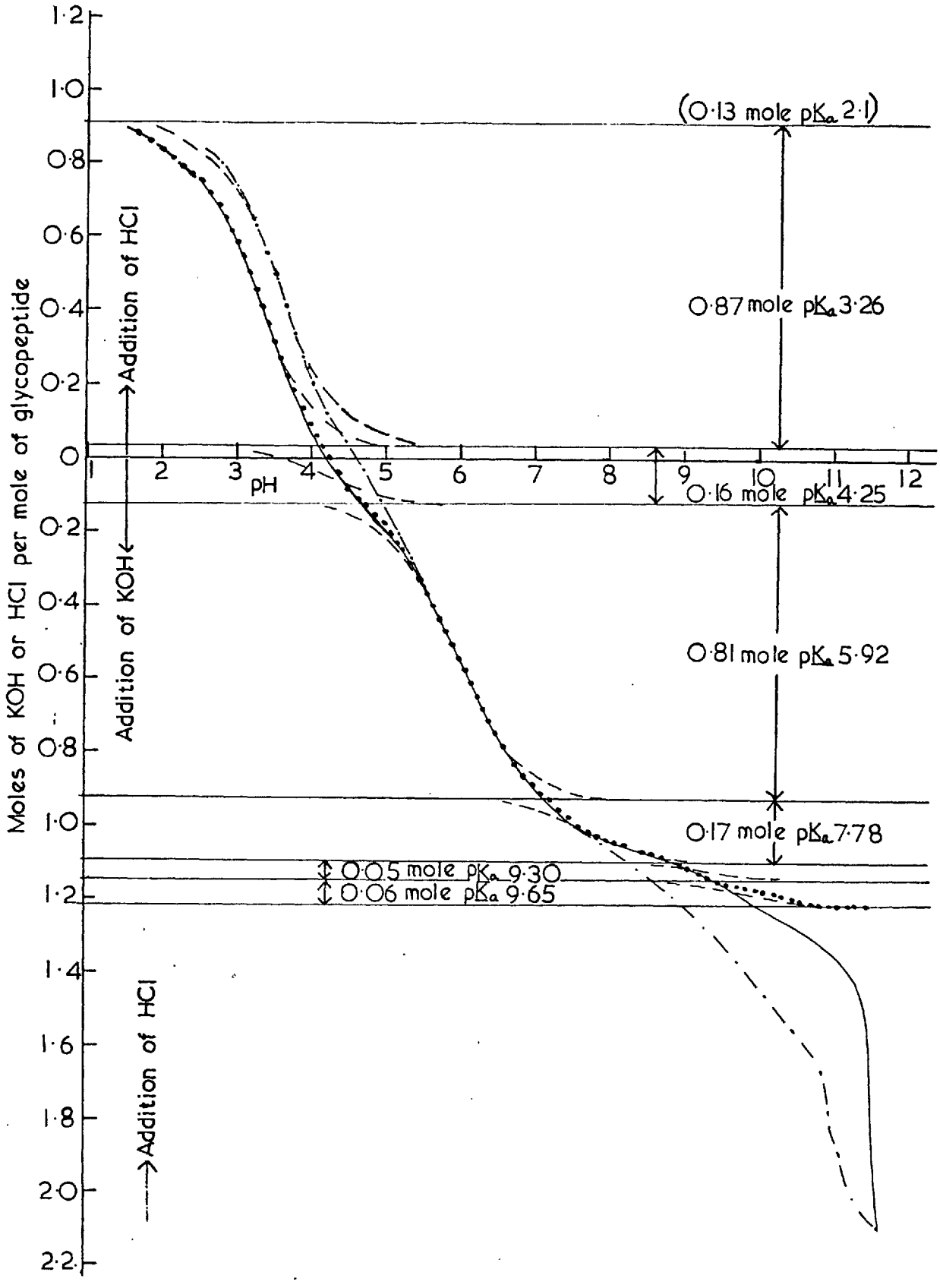
Fig. 2.7. Titration of glycopeptide material at 60°C.

Glycopeptide material from Fraction 104 (see Fig. 2.2b) in 1M-KCl.

Forward titration from pH 4.2 to pH 11.7 by addition of KOH, and on another sample from pH 4.2 to pH 2.4 by addition of HCl.

Back titration from pH 11.7 to pH 2.4 by addition of HCl.

The amounts and pK_a values of groups titrating are compared to the amino acid analysis in Table 2.6.



——— Experimental curve - forward titration

———·——— Experimental curve - back titration from high pH

— — — Theoretical curves constructed from mole fractions of groups titrating at certain pK_a values

. . . . . Composite theoretical curve constructed by addition of titration expected from individual groups

Table 2.7. The composition of Fraction 105 previously titrated at 2°C after treatment with Dowex 50x8 (20-50 mesh)(H^+ form), in terms of the known peptide sequence in the vicinity of the carbohydrate moiety.

Glycopeptide	Mole fraction
1. Glu-Glu-Lys-Tyr-ASN	0.04
2. ASN	0.10
3. ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.08
4. ASN-Leu-Thr-Ser	0.74
5. ASN-Leu	0.04

Assignments of the pK_a values observed at 40°C (see Fig. 2.8) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[a]	Experimental		Calculated		
	Moles	pK_α (40°C)	Group	Moles	pK_α (25°C)
2	?	2.10	α-COOH	0.10	2.10
1)	0.95	3.10	(α-COOH	0.04	2.80
3,4,5)			(α-COOH	0.84	3.10
1	0.07	4.50	-COOH	0.08	4.40
3,4,5	0.87	6.30	α-NH_2	0.84	6.60 (20°C)
1)	0.12	8.25	(α-NH_2	0.04	8.00
2)			(α-NH_2	0.10	8.60
1	0.10	9.90	(-OH	0.04	10.00
1			(-NH_2	0.04	10.40

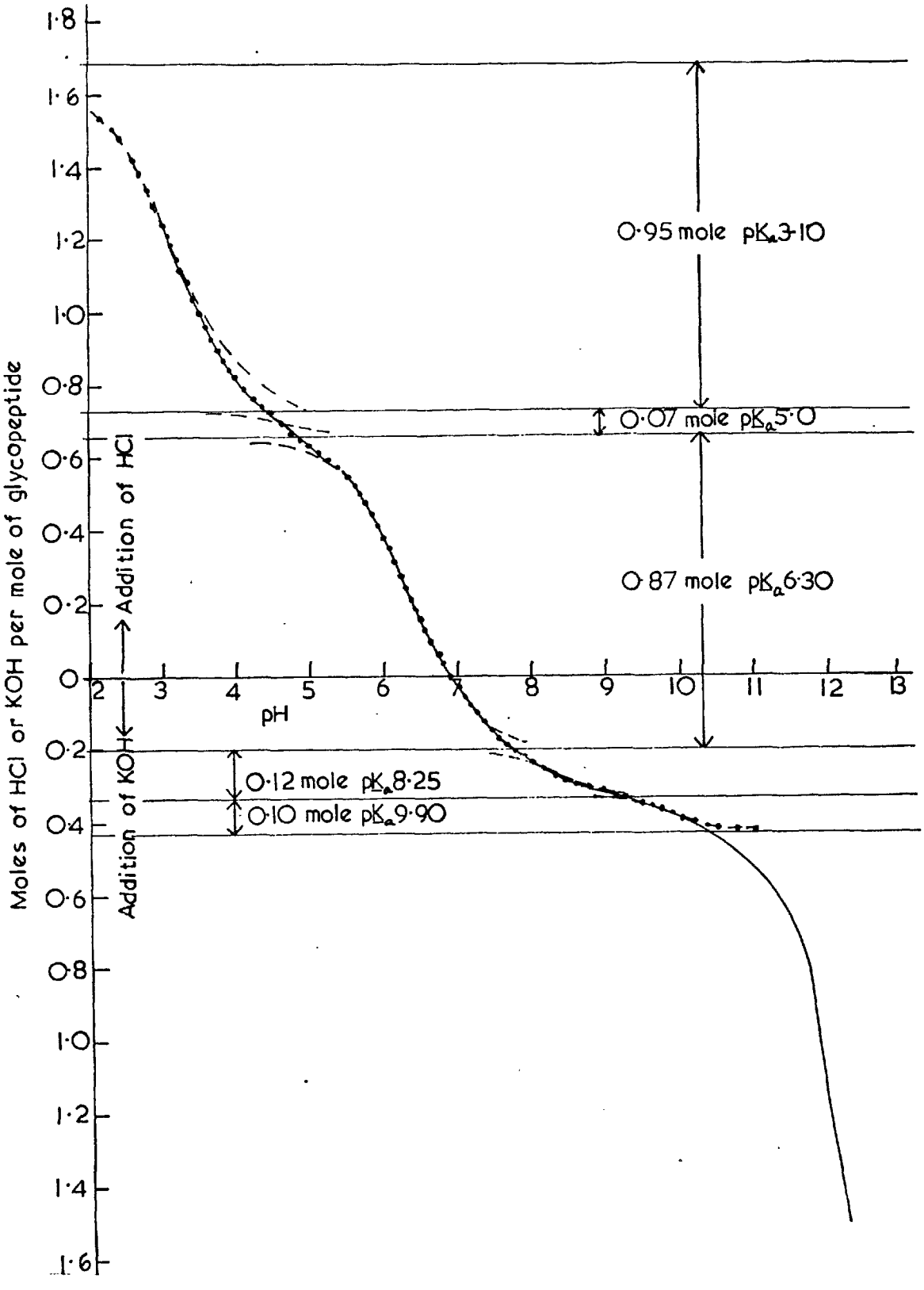
(a) The numbers in this column refer to the number of the glycopeptide given at the top of this table.

Fig. 2.8. Titration of glycopeptide material at 40°C after treatment with Dowex 50 (H^+ form).

Glycopeptide material was isolated from glycopeptide material previously titrated at 2°C (see Fig. 2.4) by elution from Dowex 50 (H^+ form) with water. The glycopeptide material was brought to pH 7.0, concentrated, and brought to a concentration of 1M in KCl.

Forward titration from pH 6.9 to pH 12.3 by addition of KOH, and on another sample from pH 6.9 to pH 3.0 by addition of HCl.

The amounts and pK_a values of groups titrating are compared to the amino acid analysis in Table 2.7.



——— Experimental curve - forward titration

– – – – Theoretical curves constructed for mole fractions of groups titrating at certain pK_a values

. . . . Composite theoretical curves constructed by addition of titration expected for individual groups

Table 2.8. The composition of Fraction 104, previously titrated at 60°C, after treatment with Dowex 50-x8 (20-40 mesh)(H^+ form), in terms of the known peptide sequence in the vicinity of the carbohydrate moiety.

Glycopeptide	Mole fraction
1. Glu-Glu-Lys-Tyr-ASN	0.04
2. ASN	0.06
3. ASN-Leu-Thr-Ser-Val-Leu-(Gly,Ala)	0.08
4. ASN-Leu-Thr-Ser-Val	0.11
5. ASN-Leu-Thr-Ser	0.71

Assignments of the pK_a values observed at 25°C (see Fig. 2.9) to groups within the glycopeptide. The assignments were made by comparisons of the values found with those established for similar groups in other peptides and amino acids.

Sequence[a]	Experimental		Group	Calculated	
	Moles	pK_α (25°C)		Moles	pK_α (25°C)
2	?	2.10	α-COOH	0.06	2.10
3,4,5)	0.95	3.45	(α-COOH	0.90	3.10
1)			(α-COOH	0.04	2.80
1	0.08	4.50	γ-COOH	0.08	4.40
3,4,5	0.87	6.55	α-NH_2	0.90	6.60 (20°C)
1)	0.08	8.00	(α-NH_2	0.04	8.00
2)			(α-NH_2	0.06	8.60
1	0.10	9.90	(-OH	0.04	10.00
1			(-NH_2	0.04	10.40

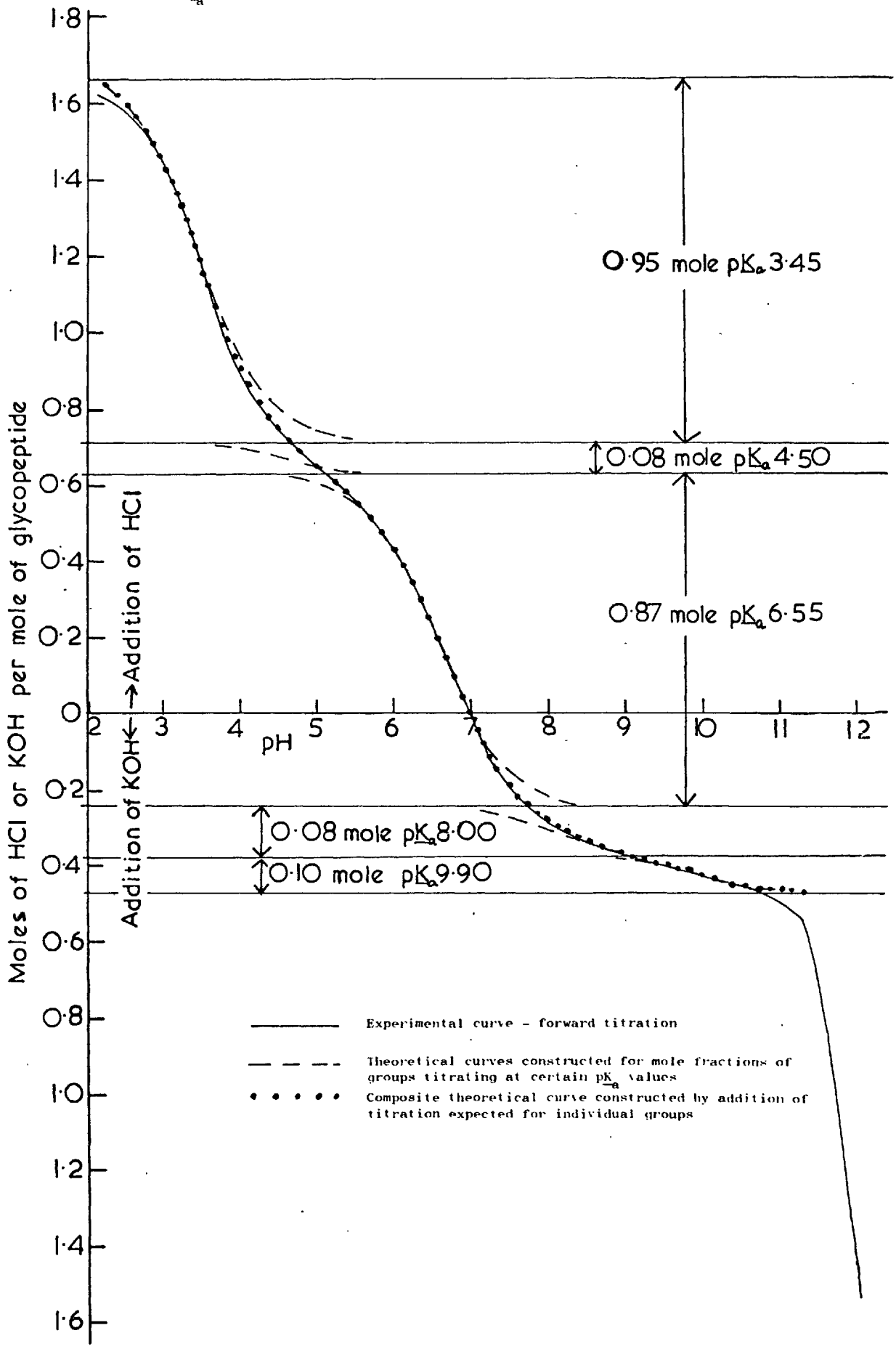
(a) The numbers in this column refer to the number of the glycopeptide given at the top of this table.

Fig. 2.9. Titration of glycopeptide material at 25°C after treatment with Dowex 50 (H^+ form).

Glycopeptide material was isolated from glycopeptide material previously titrated at 60°C (see Fig. 2.7) by elution from Dowex 50 (H^+ form) with water. The glycopeptide material was brought to pH 7, concentrated, and brought to a concentration of 1M in KCl.

Forward titration from pH 7.0 to pH 12.2 by addition of KOH, and on another sample from pH 7.0 to pH 2.0 by addition of HCl.

The amounts and pK_a values of groups titrating are compared to the amino acid analysis in Table 2.8.



The pKa values and amounts of titrating groups expected from the composition of glycopeptides of varying length in each fraction were deduced from the pKa values known for various glycopeptides and peptides (Tables 2.3-2.8). The apparent values at 26°C of pKa of titrating groups of the glycopeptide containing L-asparagine as the only amino acid are 2.3 and 8.2 (Montgomery, Lee & Wu, 1965), and of GlcNAc-Asn at 25°C are 2.1 and 8.6 (Marshall & Neuberger, 1964). The pKa value of the α-COOH of the glycosylated L-asparaginyl residue the α-amino group of which is bound to L-tyrosine might be expected to be about 2.8 (25°C) (Greenstein & Winitz, 1961). The pKa of the α-amino group of the glycopeptide containing the L-glutamyl residue at its N-terminus might be expected to be about 8.0 (25°C), while the pKa values of the β-carboxyl groups of the L-glutamyl residues would be about 4.4. Values for pKa (25°C) of 10.0 and 10.4 were assigned to the side chains of L-tyrosyl and L-lysyl residues respectively.

There appeared to be small contributions from the ionising side chains of some of the residues in the trace amounts of the contaminating free peptide whose composition was Asx_2, Ser_3, Glx_4, Gly_5, Ala_6, Val_6, (Tables 2.3-2.5). The data suggest that part, but not all, of the aspartyl and glutamyl residues arising on acid hydrolysis of the peptide were originally present as non-amidated residues.

The results described in Tables 2.3-2.8 show that all titration observed up to high values of pH (pH 12 at 2°C; pH 10 at 60°C) may be accounted for by

consideration of the contributions of glycopeptides of varying length.

At high values of pH, further titration commenced, which appeared to be due to more than one titrating group per mole of aspartic acid (see Figs. 2.6-2.9), but no accurate assignment was possible owing to the large correcting factor for the ionisation of water which had to be taken into account.

It was also observed that back titrations at 40°C and 60°C from high pH did not coincide with the forward titrations, suggesting that some irreversible change had taken place (Figs. 2.6 and 2.7). The difference at the alkaline end of the titration curve suggested that the same number of groups were titrating here, but at lower values of pH, while the difference at the acid end suggested that groups here were titrating at higher values of pH. The back titrations performed at 20°C and 2°C were coincident with the forward titration, showing, as expected, that the changes observed were temperature dependent. Glycopeptide material that had been titrated at 60°C and subjected to chromatography on Dowex 50 retitrated normally (and reversibly) in the alkaline regions at 25°C, but continued to titrate at higher values of pH in the acid region (Fig. 2.9).

The observed values of p$\underline{K}$a for the α-amino and α-carboxyl groups of the glycopeptide ASN-Leu-Thr-Ser-(etc) plotted against $^{1}/T^{\circ}K$ are shown in Fig. 2.10. From the expression for the van't Hoff isochore:-

$$\frac{\text{In } \underline{K}}{T} = \frac{H}{RT^2}$$

Fig. 2.10. The pK_a values, measured at various temperatures, of the α-amino and α-carboxyl groups of a glycopeptide.

The composition of the glycopeptide was ASN-Leu-Thr-, where ASN represents the glycosylated L-asparagine residue.

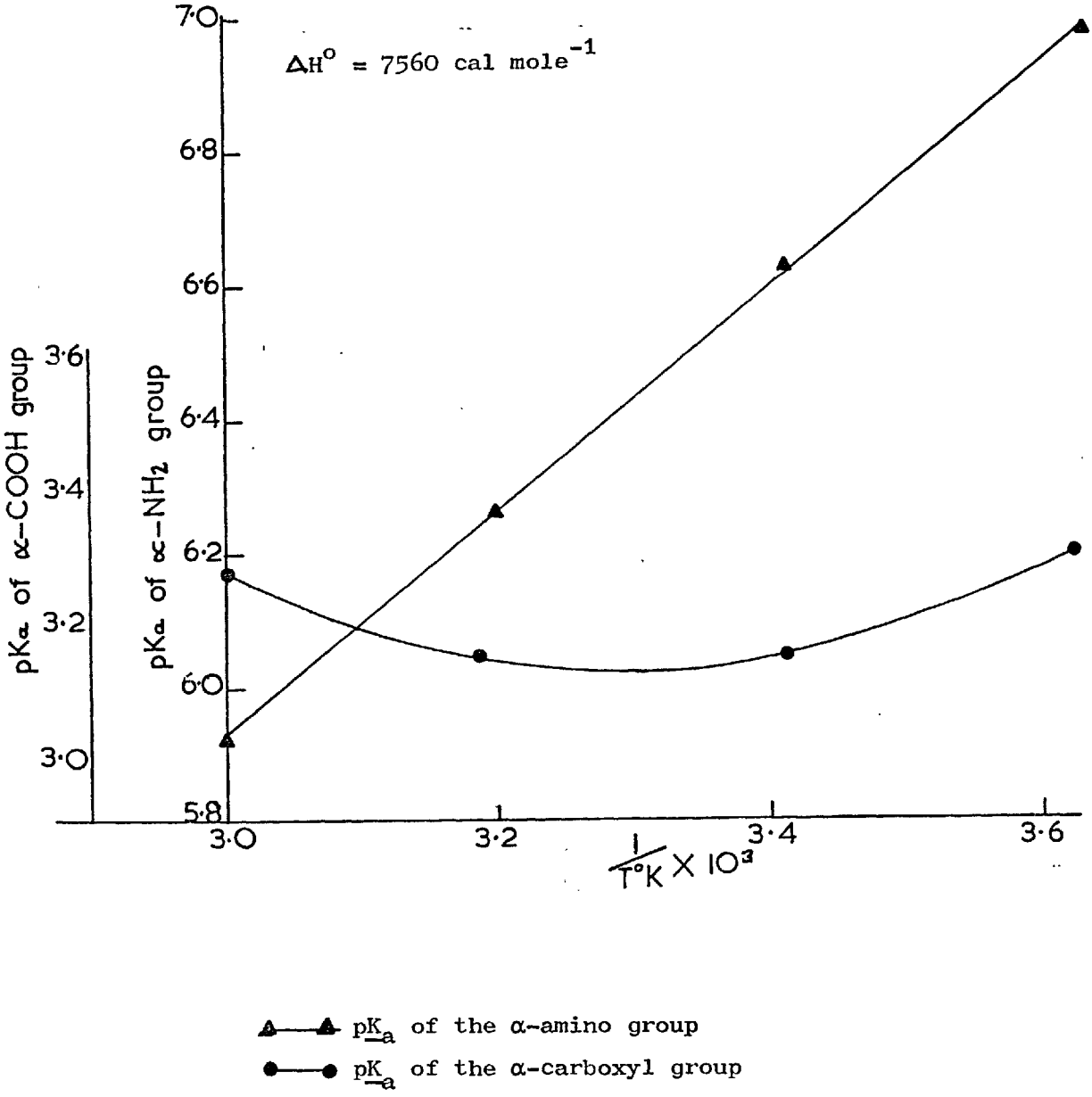


Table 2.9. Dissociation constants and some thermodynamic parameters for some amino acids, their derivatives and peptides.

Compound.	pK_a of α-amino group	Reference.
L-Asparaginyl-glycine	7.21 (18°C)	1
L-Asparagine	8.80 (25°C)	2
Glycyl-L-asparagine	8.40 (25°C)	2
GlcNAc-Asn	8.60 (25°C)	3
L-Aspartate diethyl ester	6.40 (25°C)	4
L-Aspartate diamide	7.00	5

	pK_a (25°C)	ΔG°	ΔH°	ΔS°	
Glycine	9.78	13,340	10,810	-8.5	6
L-Alanine	9.87	13,460	10,980	-8.3	7
Glycyl-glycine	8.17	11,158	10,620	-1.8	2
Glycyl-L-alanine	8.25	11,270	11,100	-0.6	2
ASN-Leu-Thr-Ser(etc)	6.54	8,930	7,560	-4.6	8

1. Leach & Lindley (1954)
2. Greenstein & Winitz (1961)
3. Marshall & Neuberger (1964)
4. Edsall & Blanchard (1933)
5. Chambers & Carpenter (1955)
6. Owen (1934)
7. Smith, Taylor & Smith (1937)
8. Present results

Values of ΔH° and ΔG° are in units of cal $mole^{-1}$

Values of ΔS° are in units of cal K^{-1} $mole^{-1}$

(1 cal = 4.184 J)

where K is the ionisation constant for the group, T is the temperature in ^{o}K, and R is a constant of 1.987 cal deg^{-1} mol^{-1}, ΔH, the enthalpy change upon ionisation, may be calculated. A linear plot was obtained for the α-amino group ionisation, suggesting that the change in the constant-pressure heat capacity (ΔCp) upon ionisation was very small. Other thermodynamic parameters were calculated for the α-amino ionisation from the relationships:-

$$G^{o}(25^{o}C) = 2.303\ RT\ pKa\ (25^{o}C)$$

$$\Delta G^{o}(25^{o}C) = \Delta H^{o} - T\Delta S^{o}(25^{o}C)$$

and are compared with the parameters for similar ionisations in peptides and amino acids in Table 2.9.

DISCUSSION.

The enzymic digestion of the protein.

An evaluation of the rate of enzymic hydrolysis of ovalbumin may be gained by measurements of the increase of ninhydrin colour. However, this may only be a crude approximation as a measure of the percentage of peptide bonds split, as small peptides have been found to have colour yields between 0.6 and 2.0 relative to glycine or leucine (Moore & Stein, 1948; Yanari, 1956).

During digestion of the protein by pepsin, protons will be taken up quantitatively by the carboxyl group released, only when the pKa of that group is well above the pH at which the reaction is taking place, viz:-

$$R\text{-}NH\text{-}CO\text{-}R^1 \xrightarrow[+H^+]{H_2O} RNH_3^+ + R'COOH$$

Consequently, measurement of acid uptake will not give an accurate assessment of the number of peptide bonds split on a mole to mole basis.

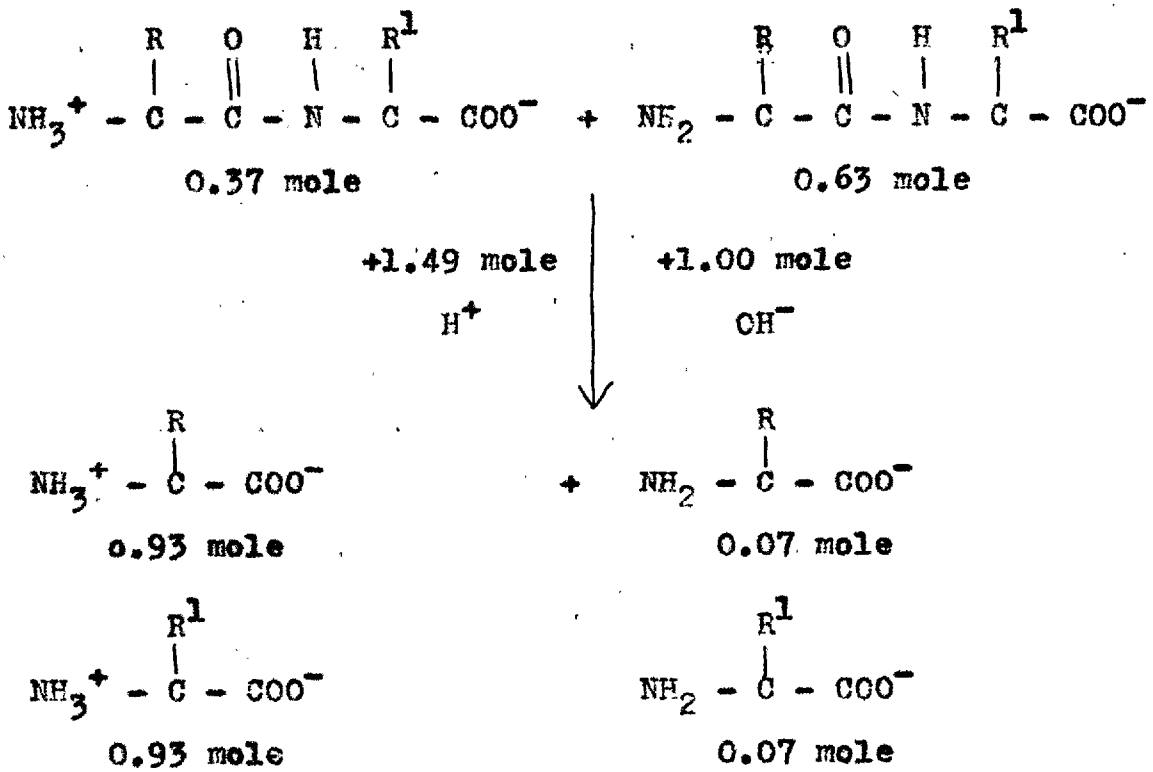
The quantity of hydrogen ions (n_{H^+}) required during the splitting of peptide bonds is given by a relationship proposed by Jacobsen et al., (1957):-

$$n_{H+} = B \frac{1}{1 + 10^{pH-pKa}}$$

where B is the total quantity of carboxyl group produced, and pKa is the pKa value of that carboxyl group. Thus, assuming that the correct number of peptide bonds split after 150 hr is given by the ninhydrin estimation, an average pKa value of 3.7 may be calculated for the carboxyl groups released in this experiment. This value is similar to that determined experimentally after the peptic hydrolysis of haemoglobin (Bohak, 1970).

Hydrolysis of ovalbumin by chymotrypsin and trypsin at the constant pH of 7.8 required an uptake of alkali for the first 24 hr, followed by uptake of acid in the later stages of the digestion. The uptake of acid might have occurred as a result of slow hydrolyses of N-terminal amino acid residues. To illustrate the problem, the hydrolysis of the dipeptide Phe-Arg will be considered. The α-amino group of this dipeptide has a pKa value of 7.57 (at 25°C), while the pKa values of α-amino groups of free phenylalanine and arginine are 9.13 and 9.09

respectively. The percentages of free and charged groups before and after hydrolytic cleavage of one mole of this dipeptide at pH 7.8 may be calculated:-



The uptake of base required to neutralise the carboxyl group formed (1.00 mole) will be more than counter-balanced by the uptake of acid by the free amino groups (1.49 mole), and a net uptake of 0.49 mole of acid per mole of peptide completely hydrolysed will be observed. When the pKa values of the amino groups of the products are not very far removed from those of the reactant peptides, little uptake of acid will be required for their neutralisation. A net uptake of base will therefore be observed.

Purification of the glycopeptide.

Precipitation of the digest with n-propanol appeared to give no better purification than precipitation with ethanol (Fletcher, Marks, Marshall & Neuberger, 1963).

Concentration of the digest to a volume which was sufficiently small to place on the G-25 column produced a very viscous solution, and it was found that repeated gel-chromatography was necessary to achieve separation of glycopeptide material from free peptides and amino acids. The state of purification of the glycopeptide material was judged by the ratio of the number of nitrogen atoms to the number of moles of hexose in the product.

Acid-base titration of the glycopeptides.

Analysis of the titration curves (Tables 2.3-2.8) showed that all the titration up to pH 11.5 (at 20°C) could be accounted for by a consideration of all the ionising groups present in the mixtures of glycopeptides of different length. The hydrogen of the amido group of the N-acylglycosylamine linkage, therefore, does not titrate in this region as had been suggested earlier (Neuberger & Marshall, 1969) and the postulated hydrogen-bonded structure (discussed on page 78) cannot be identified by this technique. It is possible that the titration observed above pH 9.0 in earlier studies (Johansen, Marshall & Neuberger, 1961; see Fig. 1.6) may have been due to the ionisations of glycopeptides of varying length.

Additional titration was observed at pH's above 11.5 (20°C), 11.0 (25°C), 10.4 (40°C) and 9.8 (60°C), but the titrations at high pH at 40°C and 60°C were accompanied by irreversible changes. Fletcher (1965) demonstrated the presence of a group ionising at high pH in a glycopeptide that was prepared from hen ovalbumin. This glycopeptide contained L-aspartic acid as the only amino acid. Spectrophotometric titration indicated a pKa of 11.9 at 26°C, but potentiometric titration at 20°C gave a lower value of 11.25. It was

Table 2.10. The pK_a values of some weak acids.

Compound	pK_a	T°C	Reference
Ethanol	16.2		Ballinger & Long (1966)
Acetamide	15.1		Branch & Clayton (1928)
α-Methyl-D-glucoside	13.71	16.5-19	Michaelis & Rona (1913)
D-Sorbitol	13.6	17.5	Michaelis & Rona (1913)
	14.14	0	Thamsen (1952)
	13.57	18	Thamsen (1952)
D-Mannitol	13.47	17.5	Michaelis & Rona (1913)
	14.09	0	Thamsen (1952)
	13.50	18	Thamsen (1952)
D-Glucose	12.18	17.5-18	Michaelis & Rona (1913)
	12.92	0	Thamsen (1952)
	12.45	18	Thamsen (1952)
	12.09,13.85	25	Urban & Shaffer (1931)
	12.34,14.10	17-18.5	Urban & Shaffer (1931)
D-Mannose	11.97	17-18.5	Michaelis & Rona (1913)
Raffinose	12.74	18.5	Michaelis & Rona (1913)
N-Acetyl-D-glucosamine	11.76	20	Fletcher (1965)
	11.65	26	Fletcher (1965)

found difficult in the present studies to obtain an accurate assessment of the number or pKa values of groups titrating at this high pH because of the large correction that had to be made for the titration of water. However, from the titration performed at 25°C, which was essentially reversible, more than one group would appear to be involved. The amido hydrogens are possible candidates, although the pKa value of acetamide has been stated to be as high as 15.1 at 25°C (Branch & Clayton, 1928). The pKa values for non-reducing carbohydrate hydroxyl-groups have been reported in the region of 13 to 14 (see Table 2.10). Derevitskaya, Smirnova& Rogovin (1961) studied the electrometric titration of various carbohydrate derivatives in ethylenediamine and deduced that all hydroxyl groups can ionise on addition of sufficiently strong base.

The ionisation of hydroxyl groups appears to have a large temperature dependence, similar to that of water (pKw (20°C) = 14.17; pKw (60°C) = 13.02) (Harned & Owen, 1950). The pKa value of mannitol, for example, decreases by 0.59 units upon increasing the temperature from 0°C to 18°C (Table 2.10). In the present studies, a marked drop in the value of pH at which titration of the glycopeptide at alkaline pH commenced was observed, as the temperature was increased. Some further considerations of this ionisation using chiroptical methods will be described in Chapter 5.

The pKa value of the α-amino group of the glycosylated L-asparagine residue which is linked to peptide by its α-carboxyl group (6.54 at 25°C) is lower than

many other recorded p*K*a values for α-amino groups of peptides and free amino acids (Table 2.9), and is about the same as that of the diethyl ester of aspartic acid (Edsall & Blanchard, 1933).

In Table 2.9, the molar enthalpy (ΔH^{o}) and molar entropy (ΔS^{o}) changes occurring on ionisation of the α-amino group of the glycopeptide are compared to values reported elsewhere for the ionisations of α-amino groups of other peptides and amino acids. ΔH^{o} values determined for the ionisations of the amino groups of most amino acids and peptides are in the range 10,000 to 13,000cal $mole^{-1}$ (Cohn & Edsall, 1943), whereas the ΔH^{o} value determined in the present studies for the ionisation of the α-amino group of the glycopeptide is much lower (7,500cal $mole^{-1}$).

The ΔS^{o} values determined for the ionisations of the α-amino groups of amino acids are in the region -8.0 to -9.0 cal deg^{-1} $mole^{-1}$, whereas those for peptides are less negative (about -2.0 cal deg^{-1} $mole^{-1}$). The ΔS^{o} value determined for the glycopeptide in the present studies (-4.6 cal deg^{-1} $mole^{-1}$) is between the values that have been measured for free amino acids and peptides, and is not, therefore, abnormal.

The low values of ΔH^{o} and of p*K*a may be caused by electron-withdrawing induction or electrostatic interaction exerted on the α-amino group by the β-amido group. It is possible that similar induction effects or electrostatic interactions are responsible for the fact that the p*K*a value of the α-amino group of Asn-Gly (7.21) is lower than that of the α-amino group of Gly-Gly (Table 2.9).

The p*K*a of the α-amino group of the glycopeptide was measured in a solution of ionic strength (I) of 1.0. The p*K*a measured under these conditions might have approximately the same value at zero ionic strength. A formula proposed by Neuberger (1937) relates pKa^{o} (p*K*a at zero ionic strength) with pKa^{I} (p*K*a at ionic strength I) for the type of ionisation considered here:

$$pKa^{I} = pKa^{o} - \frac{0.5 \quad I/2}{1 + \quad I/2} + K_{R} \quad I/2$$

(where K_R is constant). A value for K_R of 0.4 was determined experimentally for the ionisation of the amino group of glycylglycine (Neuberger 1937). When this value is inserted into the above expression, the p*K*a value at ionic strength 1.0 may be calculated to be only 0.007 pH units lower than the value at zero ionic strength.

The values of p*K*a of the carboxyl groups of the glycopeptide were more difficult to measure precisely, but it was clear that they varied much less with temperature.

The irreversible changes observed at high pH and raised temperature in solutions of these glycopeptides should, in future, be taken into account when interpreting the titration curves of glycopeptides and possibly of some glycoproteins. Some of the changes which linkages of the GlcNAc-Asn type undergo in alkali have been investigated (see the following Chapter).

CHAPTER 3.

REACTIONS IN ALKALI AND IN ALKALINE BOROHYDRIDE OF SOME N-ACYLGLYCOSYLAMINES AND OF HEN OVALBUMIN GLYCOPEPTIDES.

Systematic studies of the effects of alkali on linkages involving N-acetyl-D-glucosamine and L-asparagine have not been previously reported, although the rates of production of ammonia from ovalbumin glycopeptides (Marks, Marshall & Neuberger, 1963) and from Glc-Asn (Marks & Neuberger, 1961) have been determined (see also page 42). There are a number of reasons for an interest in the stability of GlcNAc-Asn to alkaline conditions. Firstly, there have been reports that GlcNAc-Asn undergoes rearrangements when it is subjected to alkali under relatively mild conditions (Micheel, Tanaka & Romer, (1964). It was also demonstrated in Chapter 2 that the ovalbumin glycopeptide undergoes some irreversible changes at about pH 12 at temperatures in excess of 40°C, and it seemed reasonable to examine the nature of the changes that the carbohydrate-peptide linkage undergoes in alkali. In some unreported experiments by R.D. Marshall some years ago, it was found that there were very small yields of aspartic acid formed when GlcNAc-Asn was treated with 0.1M NaOH at 100°C, although these conditions were known to result in the production of ammonia from egg albumin glycopeptide. The fate of the L-aspartyl moiety was unknown.

It was also highly desirable to obtain oligosaccharides and reduced oligosaccharides from glycopeptides in which sugars originally present in non-reducing terminal

positions are still intact. Some of the most useful procedures for doing this, in the case of carbohydrate moieties attached through L-seryl or L-threonyl residues, have involved treatment under relatively mild alkaline conditions, as used by Morgan & Watkins and their colleagues in studies on the blood group substances (see page 63). Related procedures, in which rather more concentrated alkali has been employed, have also been used for studies of glycoproteins of similar types, and in these sodium and potassium borohydride has usually been incorporated into the reaction medium. Possibly the most useful conditions for splitting linkages in which L-serine and L-threonine are involved and obtaining reduced oligosaccharides are those described by Carlson (1968), in which relatively high temperatures (45-50°C) and concentrations of borohydride (1M) in about 0.05M alkali are used, but other conditions may also be useful in some cases.

Alkaline reductive cleavage has not been extensively applied previously to GlcNAc-Asn linkages. It was shown earlier (Lee & Montgomery, 1961) that treatment of hen ovalbumin with sodium borohydride at concentrations less than 0.1M did not lead to cleavage of dialysable fragments from the protein.

In the present studies the rates of cleavage in alkali and in alkaline borohydride of N-acylglycosylamine linkages have been measured and some of the products, particularly of those arising from ovalbumin glycopeptide, have been examined.

RESULTS.

PART 1. TREATMENT OF COMPOUNDS WITH ALKALI.

The kinetics of the degradation of GlcNAc-Asn and of Glc-Asn.

GlcNAc-Asn was found to be stable at room temperature in 0.2M-NaOH for 5 days, as judged by measurements made with the Technicon autoanalyser under conditions used earlier for chromatographing the compound (Marshall, 1969). The rates of destruction at 100°C of GlcNAc-Asn in NaOH at concentrations 0.05, 0.2 and 1.0M and of Glc-Asn in 0.2M-NaOH are shown in Figs. 3.1 to 3.6. The amounts remaining follow first-order rate expressions (Fig. 3.3), from which first-order rate constants (k) may be calculated. The rate constant for the decomposition of Glc-Asn in 0.2M-NaOH ($5.97 \times 10^{-3} min^{-1}$) is similar to that of GlcNAc-Asn under the same conditions ($6.44 \times 10^{-3} min^{-1}$), suggesting that the 2-acetamido group of GlcNAc-Asn is not an important factor in determining its rate of reaction in 0.2M-NaOH. The rate of destruction of GlcNAc-Asn and the rate of its conversion to aspartic acid are the same in 0.2M-NaOH, 1M-KCl as they are in 0.2M-NaOH alone (Fig. 3.1). The ionic strength (I) of the medium, therefore, has no effect on the rate or extent of reaction over the range of values of I from 0.2 to 1.2.

The complex nature of the reaction is shown by the fact that, after a time approximately equivalent to ten half-lives for the decomposition of both GlcNAc-Asn and of Glc-Asn, less than one mole of aspartic acid per mole of substrate destroyed is released (Figs. 3.1 and 3.2). It should be emphasized that the inability to cause quantitative

Fig. 3.1. Destruction of GlcNAc-Asn by treatment with 0.2M-NaOH at 100°C and the formation of aspartic acid, both in the presence and absence of 1M-NaCl

The formation of aspartic acid and D-glucosamine when acid hydrolysis (4M-HCl; 3 hr; 100°C) followed the alkaline treatment is also shown.

Concentrations are moles per mole of GlcNAc-Asn at zero time.

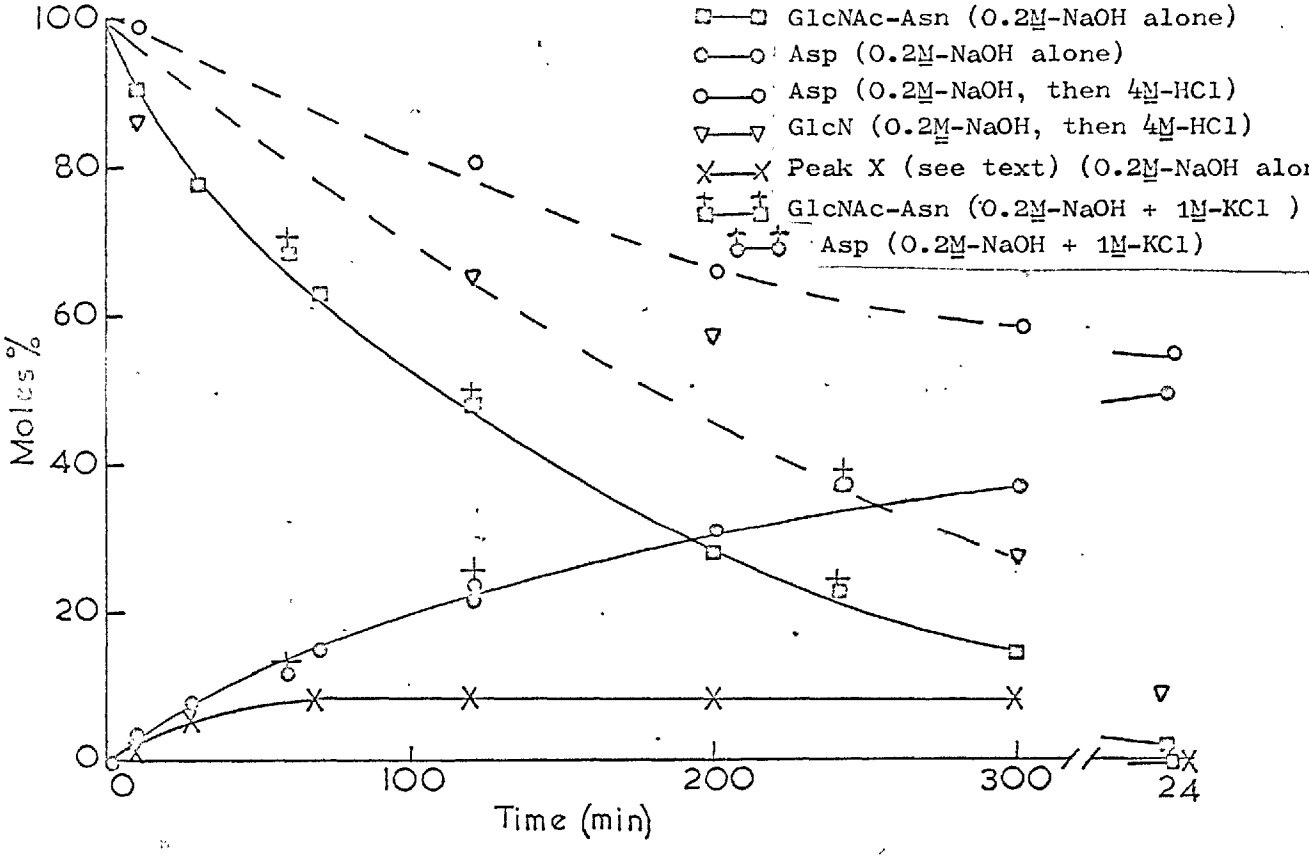


Fig. 3.2. Destruction of Glc-Asn by treatment with 0.2M-NaOH at 100°C and formation of aspartic acid.

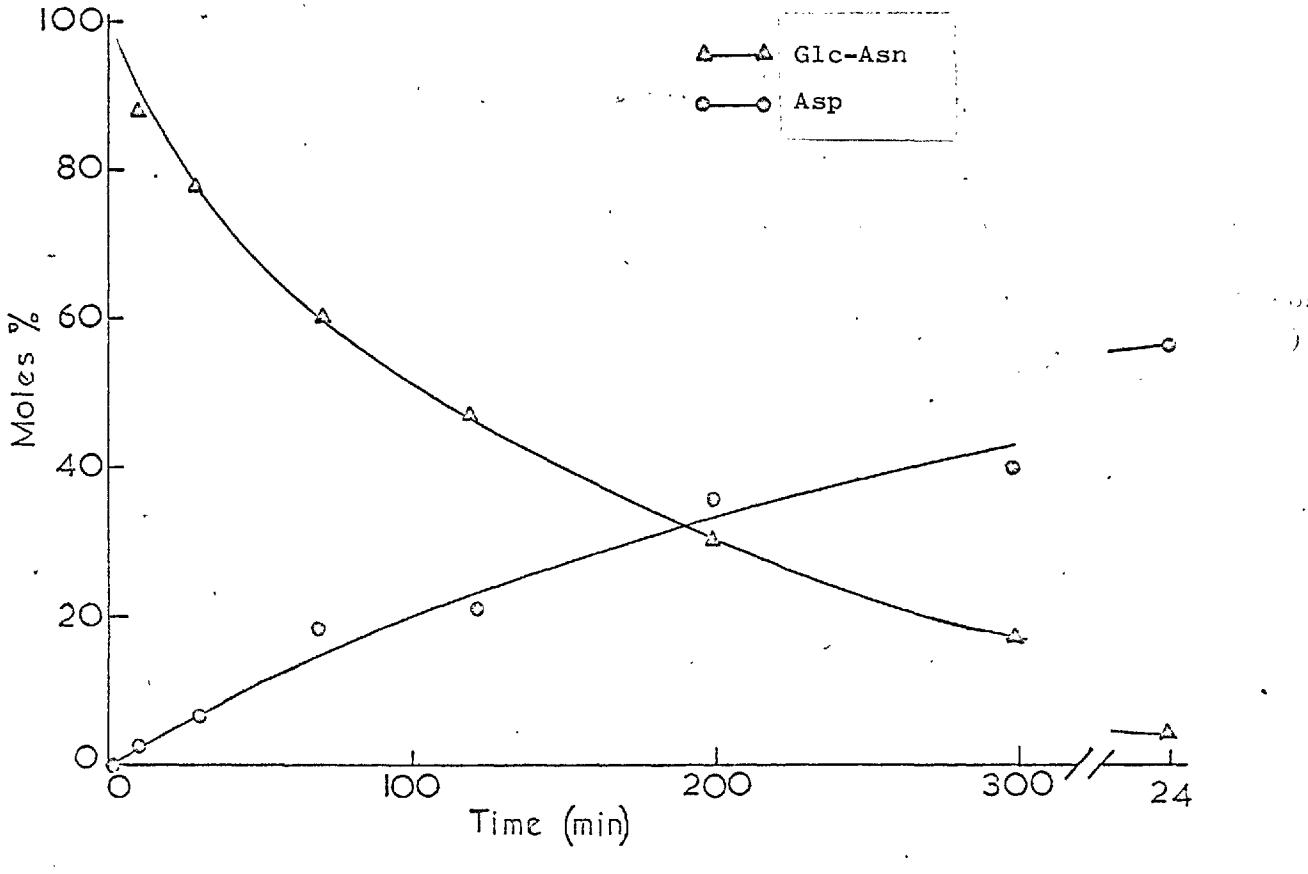


Fig. 3.3. The rates of destruction of GlcNAc-Asn and Glc-Asn in 0.2M-NaOH at 100°C, shown as first-order rate reactions.

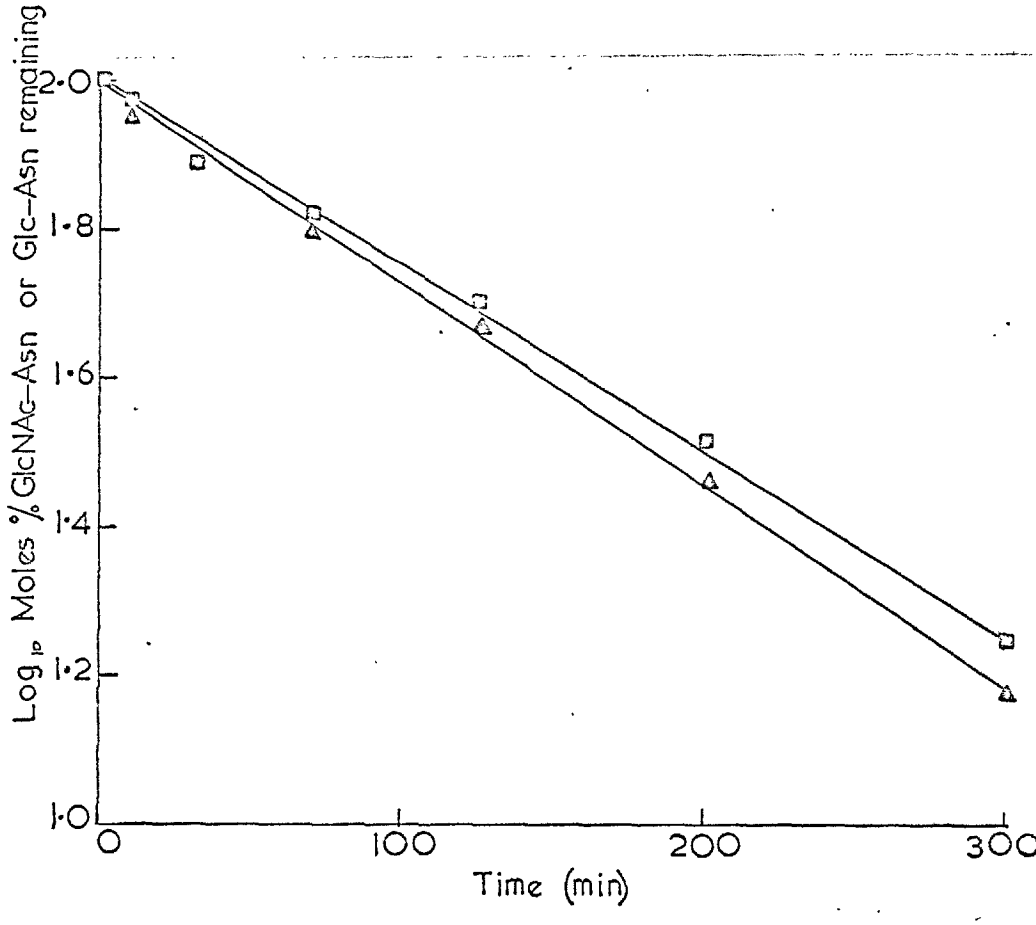


Fig. 3.4. Destruction of GlcNAc-Asn by treatment with 0.05M-NaOH at 100°C and the formation of aspartic acid.

The formation of aspartic acid and D-glucosamine when acid hydrolysis (4M-HCl; 3 hr; 100°C) followed the alkaline treatment is also shown.

Concentrations are moles per mole of GlcNAc-Asn at zero time.

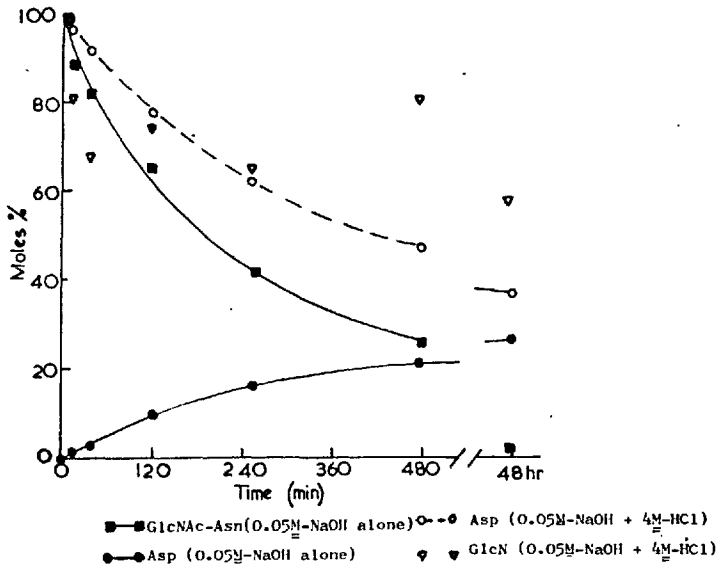


Fig. 3.5. Destruction of GlcNAc-Asn by treatment with 1M-NaOH at 100°C and the formation of aspartic acid.

The formation of aspartic acid and D-glucosamine when acid hydrolysis (4M-HCl; 3 hr; 100°C) followed the alkaline treatment is also shown.

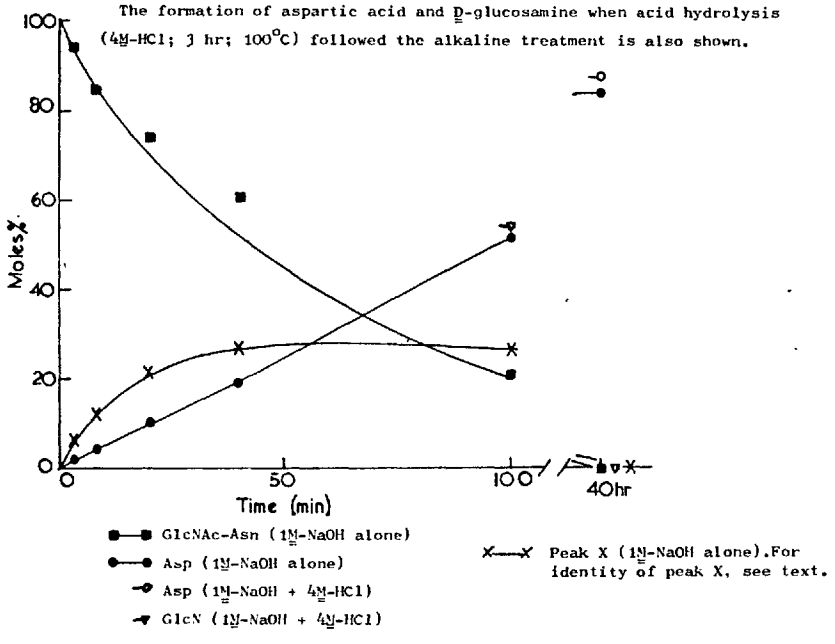


Fig. 3.6. The rates of destruction of GlcNAc-Asn in 0.05M-NaOH, 0.2M-NaOH and 1M-NaOH at 100°C, shown as first-order rate reactions.

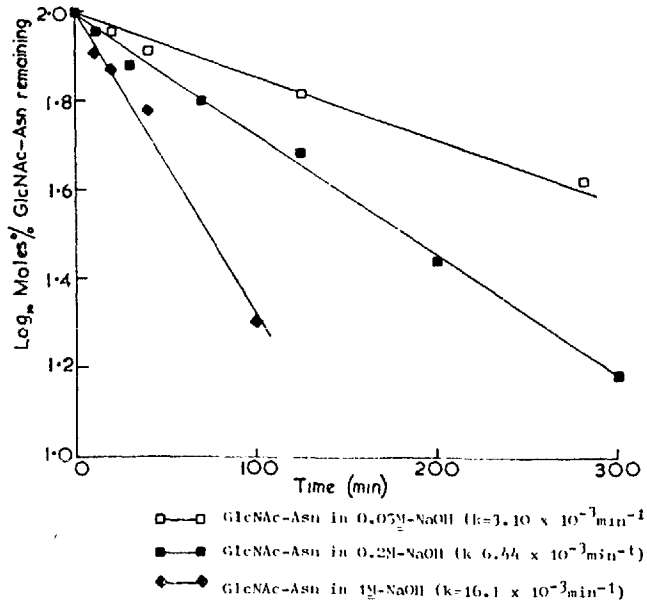


Table 3.1. First-order rate constants at 100°C for the destruction of GlcNAc-Asn (6mM) for Glc-Asn (6mM) and the formation of aspartic acid under various conditions of alkali.

Substrate	Conditions of alkali	$k(min^{-1})$ [a]	$t_{\frac{1}{2}}$ min [a]	Mole fraction of Asp after ten half-lives	$k_1 + k_3 (min^{-1})$ [b]	$\frac{k_1 + k_3}{[OH^-]}$ l. mole^{-1} min^{-1}	$k_2 (min^{-1})$ [b]
1. GlcNAc-Asn	0.05M-NaOH	3.10×10^{-3}	224	0.27	0.34×10^{-3}	1.68×10^{-2}	2.26×10^{-3}
2. GlcNAc-Asn	0.2M-NaOH	6.44×10^{-3}	108	0.51	3.28×10^{-3}	1.64×10^{-2}	3.22×10^{-3}
3. GlcNAc-Asn	1.0M-NaOH	16.1×10^{-3}	43	0.87	14.01×10^{-3}	1.40×10^{-2}	2.10×10^{-3}
4. GlcNAc-Asn	pH 10.2 buffer (0.5M-HCO_3^- + CO_3^{2-})	2.67×10^{-3}	260	[0.23] [d]	0.62×10^{-3}	-	2.05×10^{-3}
5. Glc-Asn	0.2M-NaOH	5.97×10^{-3}	105	0.55	3.28×10^{-3} [c]	1.64×10^{-2} [c]	2.69×10^{-3}

(a) k is the rate constant for the destruction of the compound listed, and $t_{\frac{1}{2}}$ is the corresponding half-life. The results from which these values are devised are shown in Fig. 3.6.

(b) The rate constants k_1, k_2 and k_3 are defined on page 141.

(c) The rate constant for this compound does not, of course, include a term for k_3.

(d) This is the amount of aspartic acid produced per mole of GlcNAc-Asn destroyed. It was measured after only 260 min of reaction time.

release of aspartic acid from GlcNAc-Asn is not due, at least largely, to trapping at this stage of the amino acid as a component of 4-N-(2-amino-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcN-Asn). The maximum yields of aspartic acid released by complete destruction of GlcNAc-Asn increased with the concentration of alkali used (Table 3.1, column 5, Figs. 3.1, 3.4 and 3.5). The maximum yield of aspartic acid from GlcNAc-Asn in 0.2M-NaOH (51%) was similar to that from Glc-Asn (55%), again suggesting that the 2-acetamido group of GlcNAc-Asn is not specifically involved in any reaction resulting in loss of aspartic acid.

The production of aspartic acid from Glc-Asn may be considered in terms of an equation of the form

$$Asp = 1 - e^{-kt}$$

where Asp represents the concentration of aspartic acid at time t of alkaline treatment, and k is the rate of destruction of Glc-Asn (see for example Frost & Pearson, 1961). The rectilinear results obtained (Fig. 3.7a), and the fact that production of aspartic acid is not quantitative, are consistent with the degradation of Glc-Asn by competitive pathways, at least one of which yields aspartic acid.

Similar analyses of the data obtained for GlcNAc-Asn give rise to straight lines when the NaOH concentrations were 0.05 or 0.2M (Figs. 3.7b and c), but not in 1.0M-NaOH (Fig. 3.7d). The type of behaviour shown by aspartic acid, when formed from GlcNAc-Asn in 1M-NaOH, is consistent with that of a product which is produced, at least in part, by consecutive reactions, viz:-

Fig. 3.7. Relationship between the amounts of aspartic acid released and the rates of destruction of substrates.

(a) Glc-Asn in 0.2M-NaOH

(b) GlcNAc-Asn in 0.05M-NaOH

(c) GlcNAc-Asn in 0.2M-NaOH

(d) GlcNAc-Asn in 1.0M-NaOH

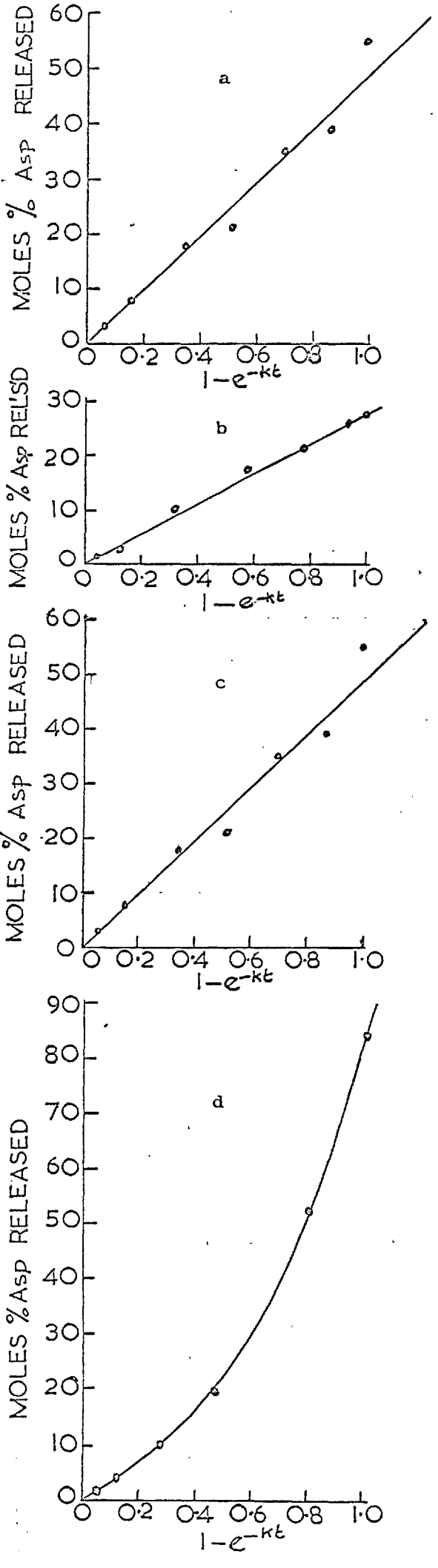


Table 3.2. Elution times of various ninhydrin-positive compounds from the Locarte autoanalyser.

Buffer programme:-

Loading buffer:-	pH 3.15 (0.2$\underline{M}$-Na^+) citrate with 30% (v/v) methanol.	
Running buffers:-	pH 3.15 (0.2$\underline{M}$-Na^+) citrate	- 120 min
	pH 4.25 (0.2$\underline{M}$-Na^+) citrate	- 90 min
	pH 6.65 (1.0$\underline{M}$-Na^+) citrate	- 140 min
	pH 0.2$\underline{M}$-NaOH	- 40 min
	pH 3.65 (0.2$\underline{M}$-Na^+) citrate	- 120 min

Compound.	Elution time (see Chapter 6).
Hen ovalbumin glycopeptide II	29 min
Glc-Asn	46 min
GlcNAc-Asn	48 min
Asp	55 min
Asn	70 min
Homoserine	76 min
2-Amino-n-butyric acid	129 min
Valine	153 min
GlcN	172 min
Glucosaminitol	195 min
Mannosaminitol	202 min
Norleucine	229 min
4'-Fluoro-β-phenylalanine	259 min
Peak X	277 min
2,4-Diamino-n-butyric acid	305 min
Ammonia	314 min

and with pH 5.28 (0.5$\underline{M}$-Na^+) citrate buffer (140 min) instead of the pH 6.65 buffer:-

Ammonia	356 min
Homoserine lactone	414 min

GlcNAc-Asn $\xrightarrow{k3}$ Intermediate $\xrightarrow{k4}$ Aspartic acid

where the rate constants k_3 and k_4 are of similar orders of magnitude.

An unidentified ninhydrin positive substance (called peak X) was found as a reaction product when GlcNAc-Asn was subjected to 1M-NaOH at 100°C, and in reduced amounts when the reaction was performed in 0.2M-NaOH. Only trace amounts (too small to measure) were formed from GlcNAc-Asn in 0.05M-NaOH, and none at all from Glc-Asn in 0.2M-NaOH. Peak X was destroyed by acid hydrolysis (4M-HCl, 100°C, 3 hr) of the alkaline reaction products, but could be identified in partial acid hydrolysates (2M-HCl, 100°C, 20 min) of previously unreacted GlcNAc-Asn.

The peak X eluted from the column of the Locarte autoanalyser with the pH 4.25 buffer after a total elution time of 277 min (Table 3.2). The concentration-time curves of this component are shown in Figs. 3.1 and 3.5, for the construction of which an arbitrary molar colour yield with respect to L-valine of 1.00 was used. The concentration of X was at a maximum after about 75 min of reaction of GlcNAc-Asn in 1M-NaOH, and after about 200 min in 0.2M-NaOH, and was completely destroyed at the end of the reactions. Thus, it behaved in a manner expected for an intermediate that decomposed to form aspartic acid. Although direct evidence is lacking, the conditions that give rise to X might suggest that it is GlcN-Asn.

The competitive and consecutive reactions considered may be summarised thus:-

Glc-Asn $\xrightarrow{k_1}$ Aspartic acid

$\xrightarrow{k_2}$ Products not including aspartic acid

and

GlcNAc-Asn $\xrightarrow{k_1}$ Aspartic acid

$\xrightarrow{k_2}$ Products not including aspartic acid

$\xrightarrow{k_3}$ Intermediate X $\xrightarrow{k_4}$ Aspartic acid

It should be emphasised that, for example, the designation k_1 merely indicates rates of reaction of analogous compounds.

The various values for these rate constants may be calculated from the observed first-order rate constants for substrate decomposition (k), and the mole fractions of aspartic acid produced per mole of substrate destroyed, after ten half-lives, by the relationship:-

$$\frac{k_1 + k_3}{k_2} = \frac{\eta_{Asp}}{1 - \eta_{Asp}}$$

where η_{Asp} is the mole fraction of aspartic acid.

The values obtained are shown in Table 3.1. In the case of GlcNAc-Asn it may be seen that the values of $k_1 + k_3$ increase with hydroxide concentration. If these values are divided in each case by the hydroxide ion

concentrations, there is an approximately constant value for the second-order rate constant (see column 7, Table 3.1), which is closely similar to that found for Glc-Asn under one particular set of conditions, 0.2M-NaOH at 100°C.

The first-order values of k_2 for GlcNAc-Asn were found to be independent of the concentrations of hydroxide ion over the range studied (see last column, Table 3.1). It may be deduced that nucleophilic attack of hydroxide ion is not involved in the rate-determining step of the reaction which does not release aspartic acid. The half-life of this reaction is of the order of 5 hr, but GlcNAc-Asn does not undergo this reaction at all when it is heated at 100°C for 48 hr in water. It seems likely, therefore, that the reaction concerned involves a species of GlcNAc-Asn in which the α-amino group of the L-aspartyl residue is not in a charged form. It is of interest also that the comparable value of k_2 for Glc-Asn is similar to that for GlcNAc-Asn.

Values for k_2 were about the same for the different conditions of alkali, both for GlcNAc-Asn and for Glc-Asn. A mean value of $2.4 \times 10^{-3} min^{-1}$ for the first-order rate constant of this base-independent reaction was calculated.

The rate and extent of reaction of GlcNAc-Asn was also studied in bicarbonate buffer, pH 10.2; after 260 min at 100°C, 0.5 mole GlcNAc-Asn had been destroyed, while 0.12 mole aspartic acid had been released. Values for the various rate parameters calculated from this observation are included in Table 3.1. The release of aspartic acid under these conditions may demonstrate catalysis of

a general-base nature involving the carbonate and bicarbonate anions. The value calculated for k_2 under these conditions was similar to the values found in NaOH solutions, supporting the view that this reaction which leads to loss of aspartic acid is not one subject to base catalysis.

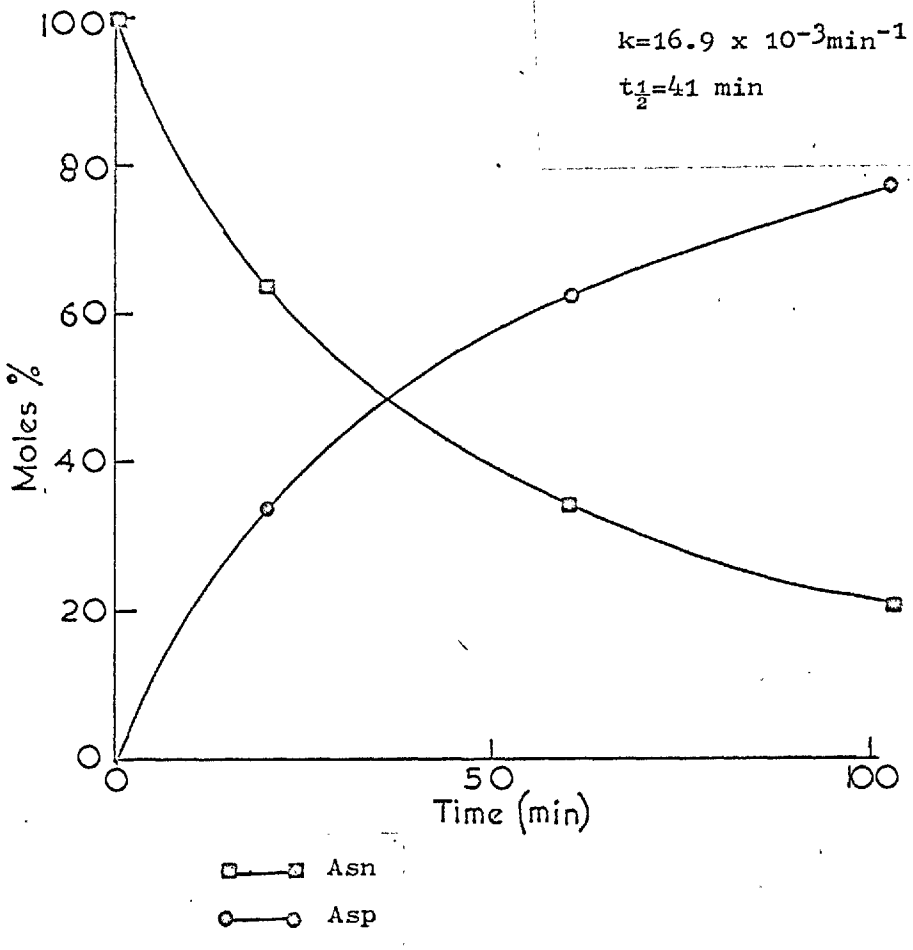
In control experiments it was observed that no loss of aspartic acid occurred when it was heated in 0.2M-NaOH alone, or in the presence of 6mM-N-acetyl-D-glucosamine for 12 hr. This observation, and the fact that the amounts of aspartic acid produced when GlcNAc-Asn and Glc-Asn were heated for prolonged periods in alkali reached a maxima, which thereafter did not change, show that the observed low yields of aspartic acid are not due to further reaction after its initial release.

L-Asparagine decomposed rapidly in 0.2M-NaOH at 100°C, with a first-order rate constant of $1.69 \times 10^{-2} min^{-1}$, about 2.5 times faster than GlcNAc-Asn (Fig. 3.8). The expected amounts of aspartic acid were produced after each period of time when measurements were made.

Acid hydrolysis of samples previously treated with alkali.

Further amounts of aspartic acid were released after acid hydrolyses (4M-HCl, 3 hr, 100°C) of samples removed after the shorter times of alkaline reaction of GlcNAc-Asn (Figs. 3.1, 3.4 and 3.5). The total aspartic acid measured after this treatment was approximately equal to that resulting from unreacted GlcNAc-Asn plus component X, in addition to the free aspartic acid already present. It was assumed from these calculations that the molar

Fig. 3.8. Destruction of L-asparagine and formation of aspartic acid by treatment with 0.2M-NaOH at 100°C



colour yield given by X with ninhydrin, relative to that given by L-valine, was unity.

More D-glucosamine than expected solely from the acid hydrolysis of unreacted GlcNAc-Asn and component X, was found in samples removed from the reaction of GlcNAc-Asn in 0.05M-NaOH and subsequently hydrolysed in acid (4M-HCl, 100°C, 3 hr, Fig. 3.4). Even after 48 hr in alkaline solution, 0.6 mole D-glucosamine per mole of initial substrate was found after acid hydrolysis. Only 0.09 mole D-glucosamine was present after reaction of GlcNAc-Asn in 0.2M-NaOH for 24 hr (Fig. 3.1).

The stability of N-acetyl-D-glucosamine in 0.2M-NaOH was also examined. No D-glucosamine was found after acid hydrolysis of a sample that had previously been heated at 100°C in 0.2M-NaOH for 10 min, showing, not unexpectedly, that it is destroyed very rapidly relative to the rates already determined for GlcNAc-Asn.

2-Acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NH_2) is somewhat more stable to alkali. After 30 min in 0.2M-NaOH at 100°C, it was found that half of the D-glucosamine initially present could be produced by hydrolysis in acid (Fig. 3.9), but after 18 hr under these conditions, no D-glucosamine was formed after acid hydrolysis. GlcNAc-NH_2 would be produced from GlcNAc-Asn if the acyl-nitrogen bond of the linking amido group were split, with the concomitant production of aspartic acid.

It is reasonable to interpret the results in the following way. GlcNAc-Asn decomposes in 0.05M and 0.2M-NaOH at 100°C by at least two pathways. One involves the

Fig. 3.9. The release of D-glucosamine from GlcNAc-NH_2 that had been treated with 0.2M-NaOH at 100°C

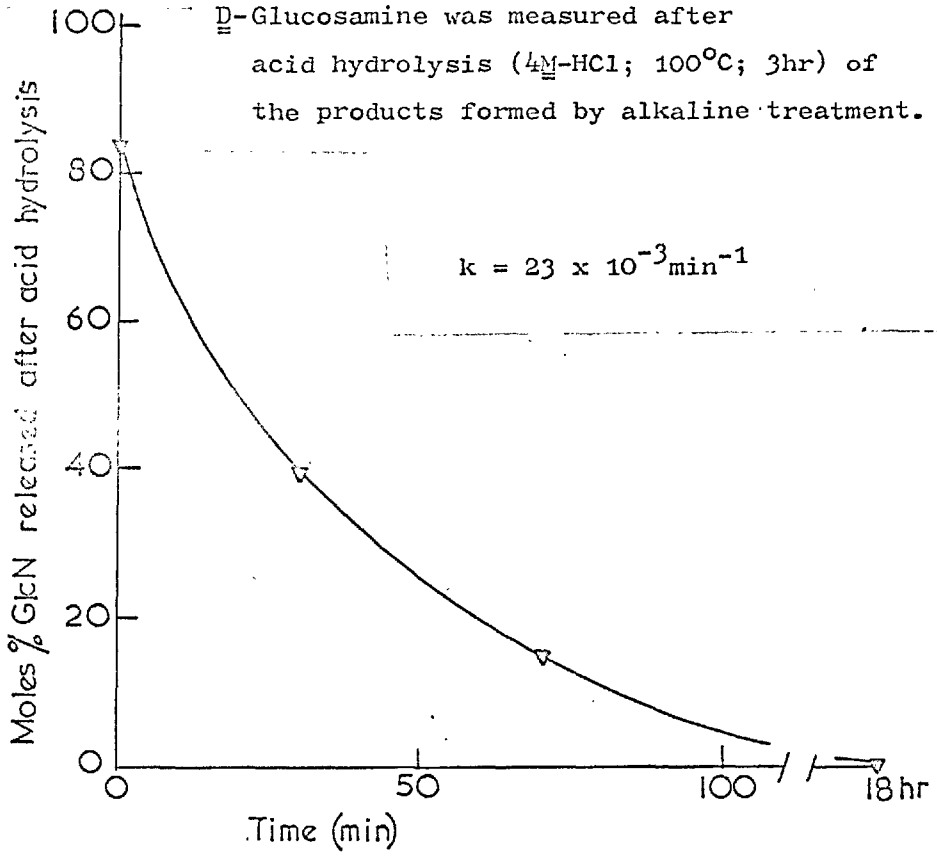
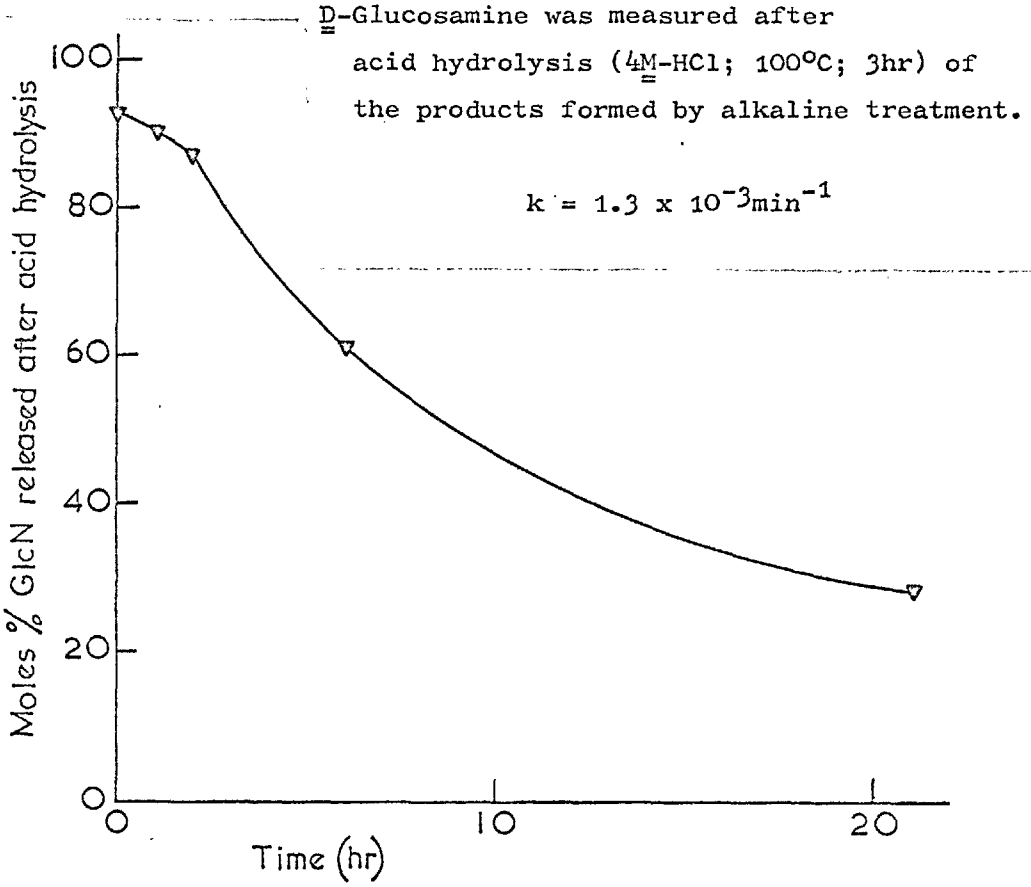


Fig. 3.10. The release of D-glucosamine from GlcNAc-NHAc that had been treated with 0.2M-NaOH at 100°C



cleavage of the acyl-nitrogen bond to form aspartic acid and GlcNAc-NH_2, which is degraded fairly rapidly to form products from which D-glucosamine cannot be released by acid hydrolysis. A second pathway involves conversion of GlcNAc-Asn to products from which D-glucosamine, but not aspartic acid, can be released by acid hydrolysis. At these lower concentrations of sodium hydroxide it would seem that GlcN-Asn is produced as an intermediate only in small quantities, if at all. This deduction is consistent with the data described in Figs. 3.7b and c. In 1M-NaOH this deactylated product probably occurs in significant concentrations.

1-N-Acetyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NHAc) has a half-life in 0.2M-NaOH at 100°C of about 10 hr, as measured by D-glucosamine released after additional acid hydrolysis of samples previously treated with alkali (Fig. 3.10). The first-order rate constant calculated from these results ($k = 1.20 \times 10^{-3} min^{-1}$) is of the same order of magnitude as that for the release of aspartic acid ($k_1 + k_3$) from GlcNAc-Asn under the same conditions ($3.28 \times 10^{-3} min^{-1}$).

Ultra-violet absorption measurements.

The absorption spectra between 220 and 330nm of neutralised (pH 5) solutions of GlcNAc-Asn, after heating in 0.2M-NaOH for various periods of time, are recorded in Fig. 3.11. Ultra-violet absorbing material (λ_{max} 264nm at pH 5.0) was formed, with a minimum molar extinction coefficient of 1300, based on the initial concentration of GlcNAc-Asn. The spectrum changed on lowering the pH from

Fig. 3.11. Ultra-violet spectra of products formed by treating GlcNAc-Asn with 0.2N-NaOH at 100°C for increasing periods of time.

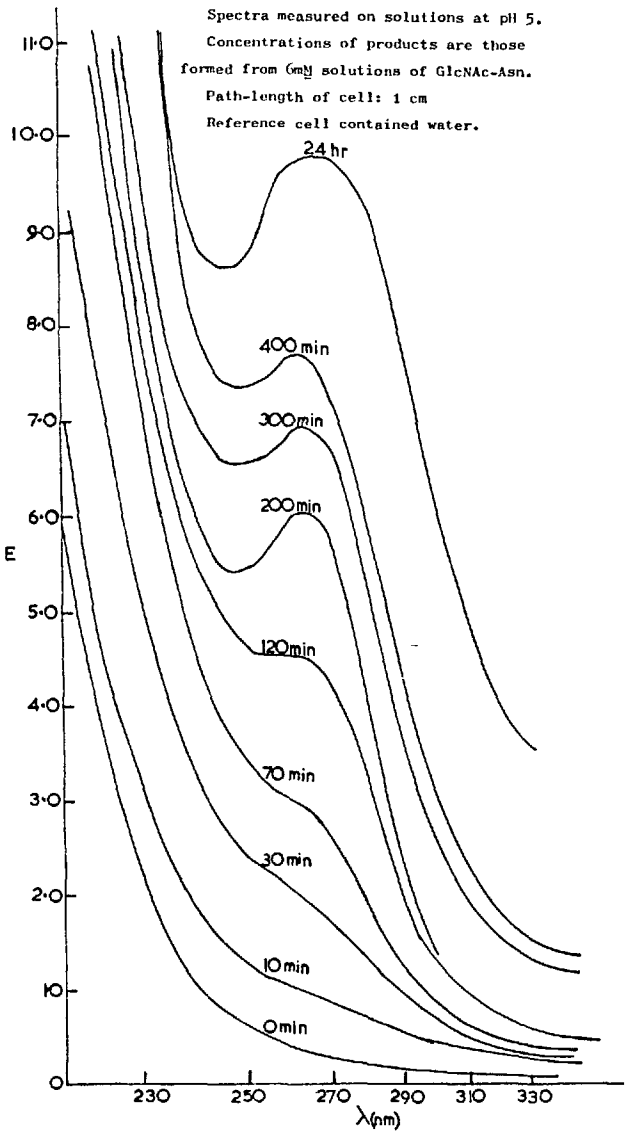
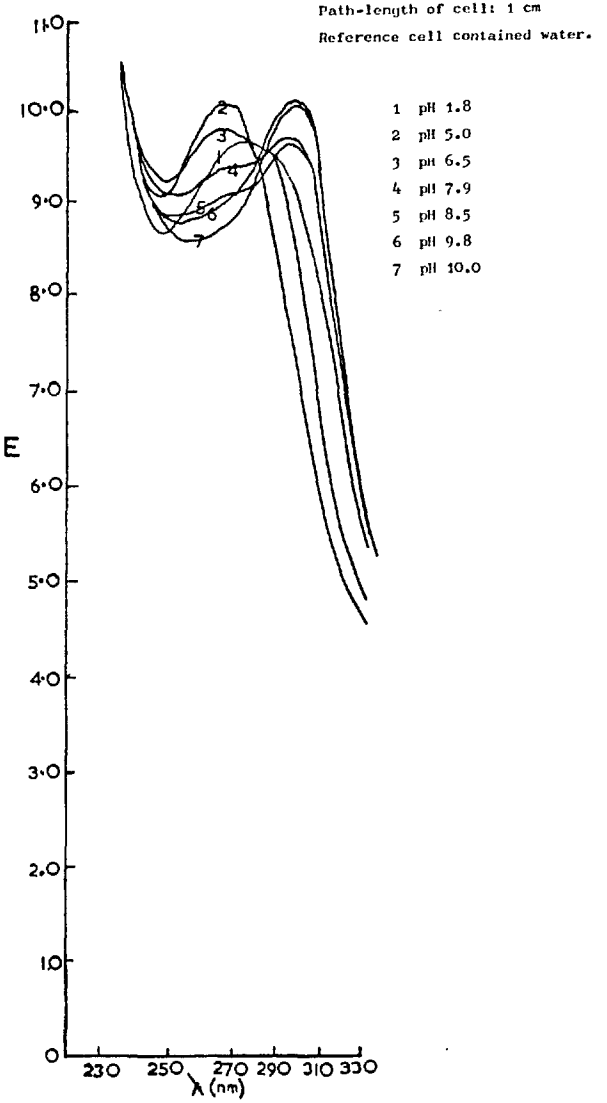


Fig. 3.12. Changes in the ultra-violet spectra of products formed by treating GlcNAc-Asn with 0.2N-NaOH at 100°C for 24 hr; the changes were induced by altering the pH of the products.



above 8 (λ_{max} 295nm) to pH 6 (λ_{max} 264nm) and again on lowering the pH from 5 to 3 (Fig. 3.12), showing that the chromophores are ionisable. The reaction product did not produce a colour upon direct reaction with Ehrlich's reagent.

N-Acetyl-D-glucosamine formed products, the Kuhn chromogens, with an absorption maximum at 230nm when this acetylated amino sugar was heated in 0.2M-NaOH at 100°C for 5 min (Fig. 3.13). These products produced colour with Ehrlich's reagent (E_{585} 0.47). After 10 min heating, an ultra-violet peak at 264nm (pH 5) had replaced that at 230nm (Fig. 3.13), and colour was no longer produced with Ehrlich's reagent. The ultra-violet peak reached its full intensity after 20 min heating and a minimum molar extinction coefficient of 1425 was calculated. Upon lowering the pH from 5 to 2, the intensity decreased (ε 1190) and λ_{max} was shifted to about 250nm (Fig. 3.14).

Ultra-violet absorbing materials (λ_{max} 253nm) were formed also from GlcNAc-NH_2 and GlcNAc-NHAc (Figs. 3.15 and 3.16) in 0.2M-NaOH, at rates which were approximately the same as those at which D-glucosamine, measured after acid hydrolysis, was lost. The absorption maxima shifted to lower wavelengths when the pH was lowered from 5 to 3. Minimum molar extinction coefficients of 1180 were calculated for both of the products from both of these compounds.

It is known that malondialdehyde, which gives a pink colour with 2-thiobarbituric acid (Sasiaw & Waravdekar, 1959), is produced when hexoses are treated with alkali.

Fig. 3.13. Ultra-violet spectra of products formed by treating N-acetyl-D-glucosamine (6mM) with 0.2M-NaOH at 100°C

Spectra of solutions at pH 5
Path-length of cell: 1 cm
Reference cell contained water.

I 5 min heating.
II 20 min heating.

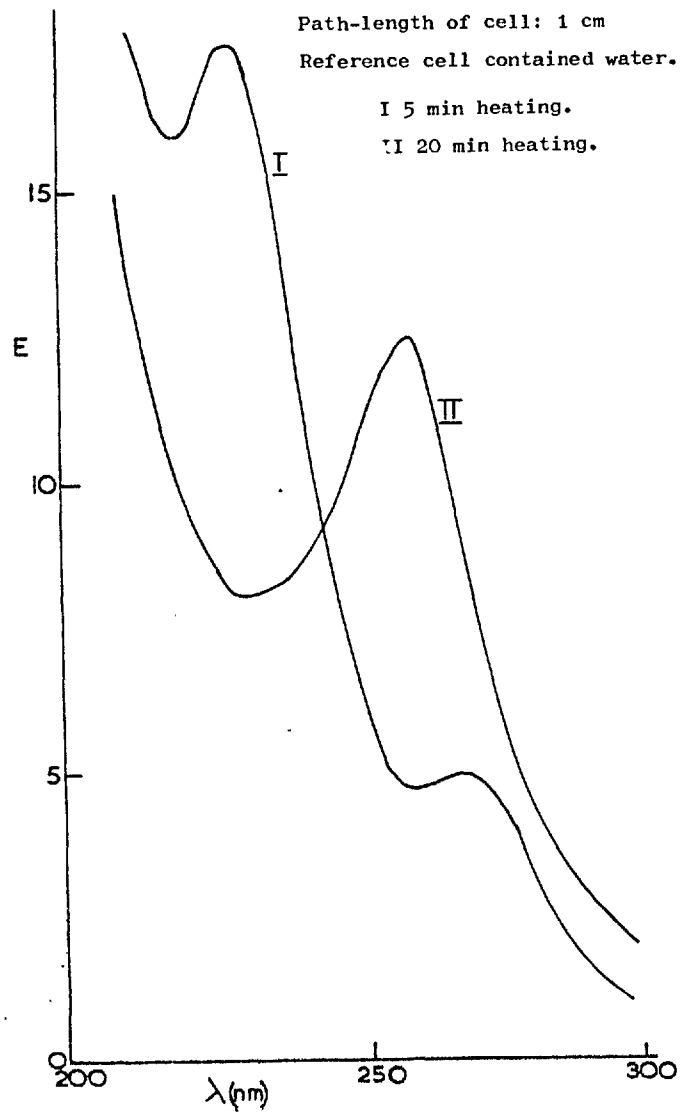
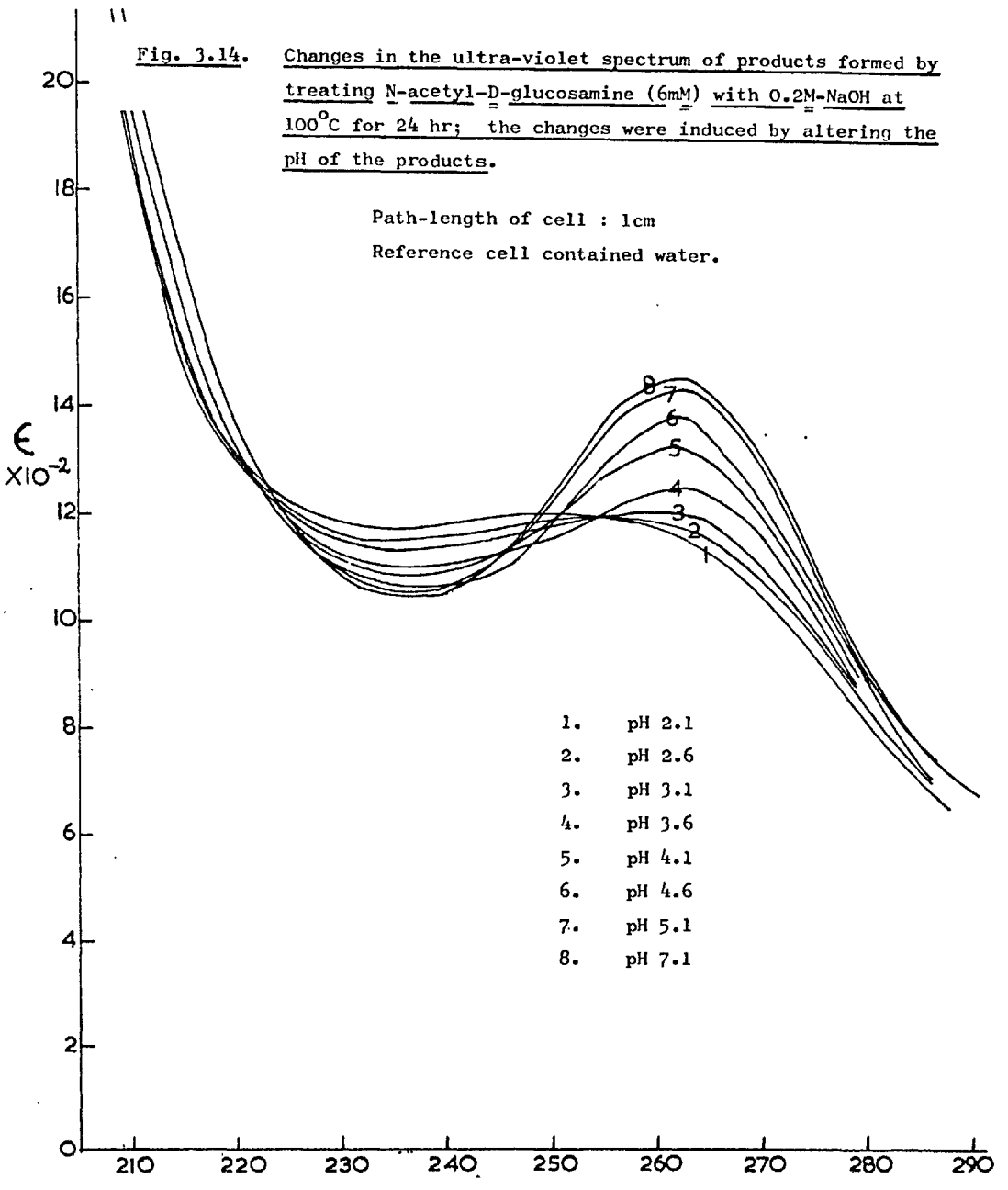


Fig. 3.14. Changes in the ultra-violet spectrum of products formed by treating N-acetyl-D-glucosamine (6mM) with 0.2M-NaOH at 100°C for 24 hr; the changes were induced by altering the pH of the products.

Path-length of cell : 1cm
Reference cell contained water.



1. pH 2.1
2. pH 2.6
3. pH 3.1
4. pH 3.6
5. pH 4.1
6. pH 4.6
7. pH 5.1
8. pH 7.1

Fig. 3.15. Ultra-violet spectra of products formed by treating GlcNAc-NH_2 with 0.2M-NaOH at 100°C for increasing periods of time.

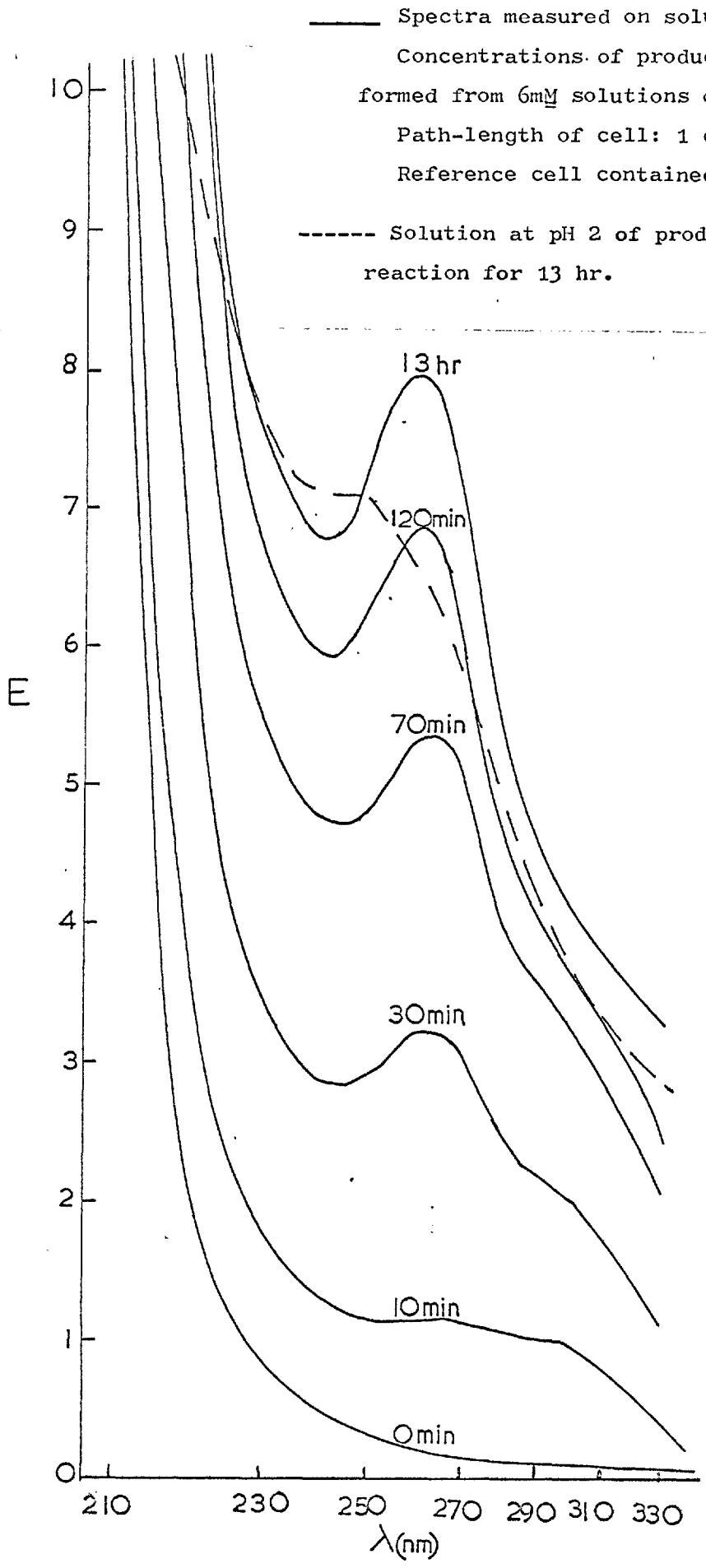
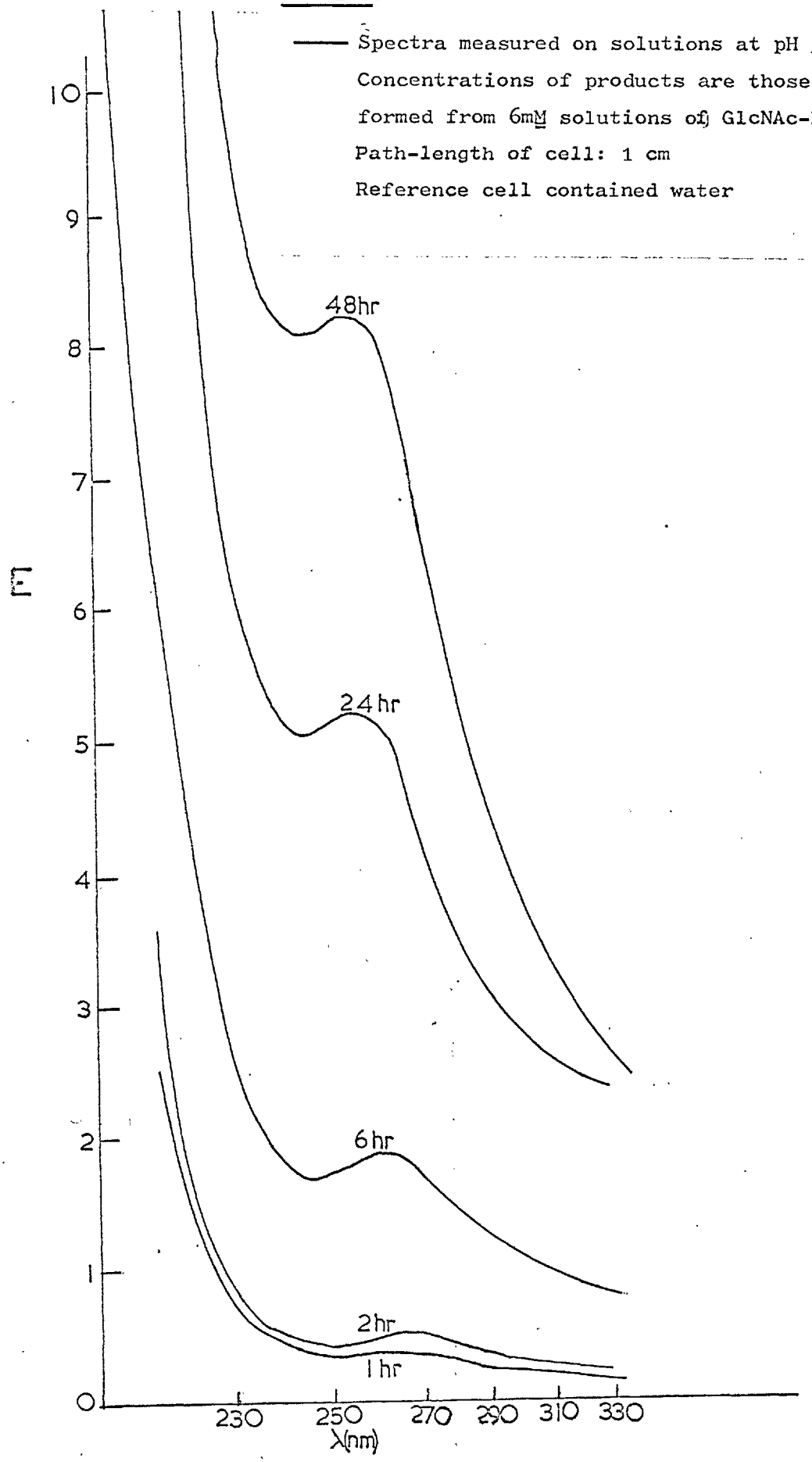


Fig. 3.16. Ultra-violet spectra of products formed by treating GlcNAc-NHAc with 0.2M-NaOH at 100°C for increasing periods of time.



This test for malondialdehyde proved negative for the products obtained when GlcNAc-Asn, N-acetyl-D-glucosamine or GlcNAc-NH_2 were treated with 0.2M-NaOH at 100°C for 24 hr.

The evolution of ammonia from GlcNAc-Asn and N-acetyl-D-glucosamine in 0.2M-NaOH.

About 1.5 residues of ammonia were produced by heating GlcNAc-Asn in 0.2M-NaOH at 100°C for a period of time equivalent to four half-lives (Fig. 3.17). The rate of release of ammonia showed a marked induction period, and the time curve had an inflection point after 80 min. N-Acetyl-D-glucosamine released a maximum amount of 50% of its ammonia after 100 min (Fig. 3.18).

Mutarotation of GlcNAc-NH_2 in 0.2M-NaOH.

The change of rotation with time of GlcNAc-NH_2 (40mg/ml) in 0.2M-NaOH at 25°C is compared to that in water in Fig. 3.19. The initial rotations, and changes in direction, are opposite in sign. N-Acetyl-D-glucosamine mutarotated from a value of $[\alpha]_{365}$ equal to +153 to one of +117° after 14 hr in 0.2M-NaOH.

No loss of D-glucosamine was observed after measurement on a sample hydrolysed in acid of the mutarotated product from GlcNAc-NH_2 in 0.2M-NaOH.

Thin-layer chromatography of carboxylic acids.

The production of relatively large amounts of ammonia and the deduction that the unionised α-amino group of the aspartyl residue was involved in the various reactions in alkali leading to loss of aspartic acid, made it reasonable to consider the possibility that

Fig. 3.17. Ammonia released from GlcNAc-Asn (6mM) by treatment with 0.2M-NaOH at 100°C.
Ammonia was measured by absorption into 2% (w/v) boric acid, and titration.

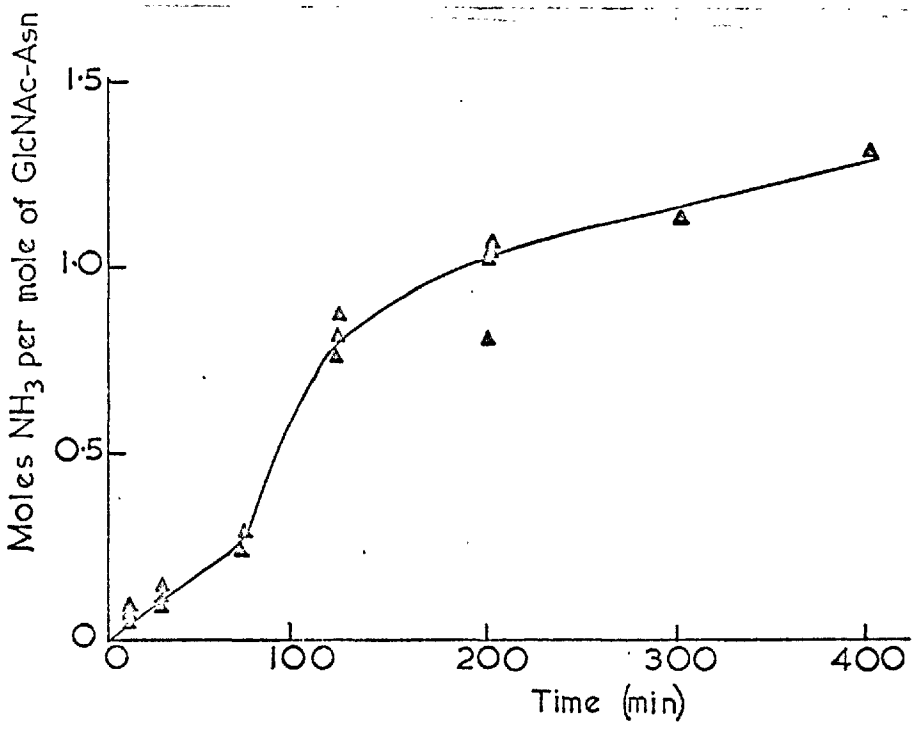


Fig. 3.18. Ammonia released from GlcNAc (6mM) by treatment with 0.2M-NaOH at 100°C
Ammonia was measured by absorption into 2% (w/v) boric acid, and titration.

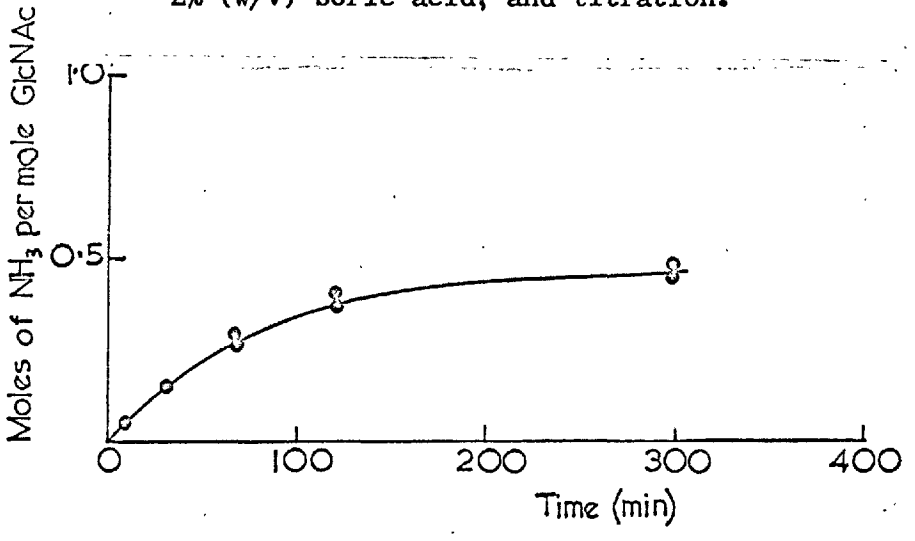


Fig. 3.19. The mutarotation of GlcNAc-NH_2 in water and in 0.2M-NaOH at 25°C

c = 40mg per ml

Path-length of cell = 1dm

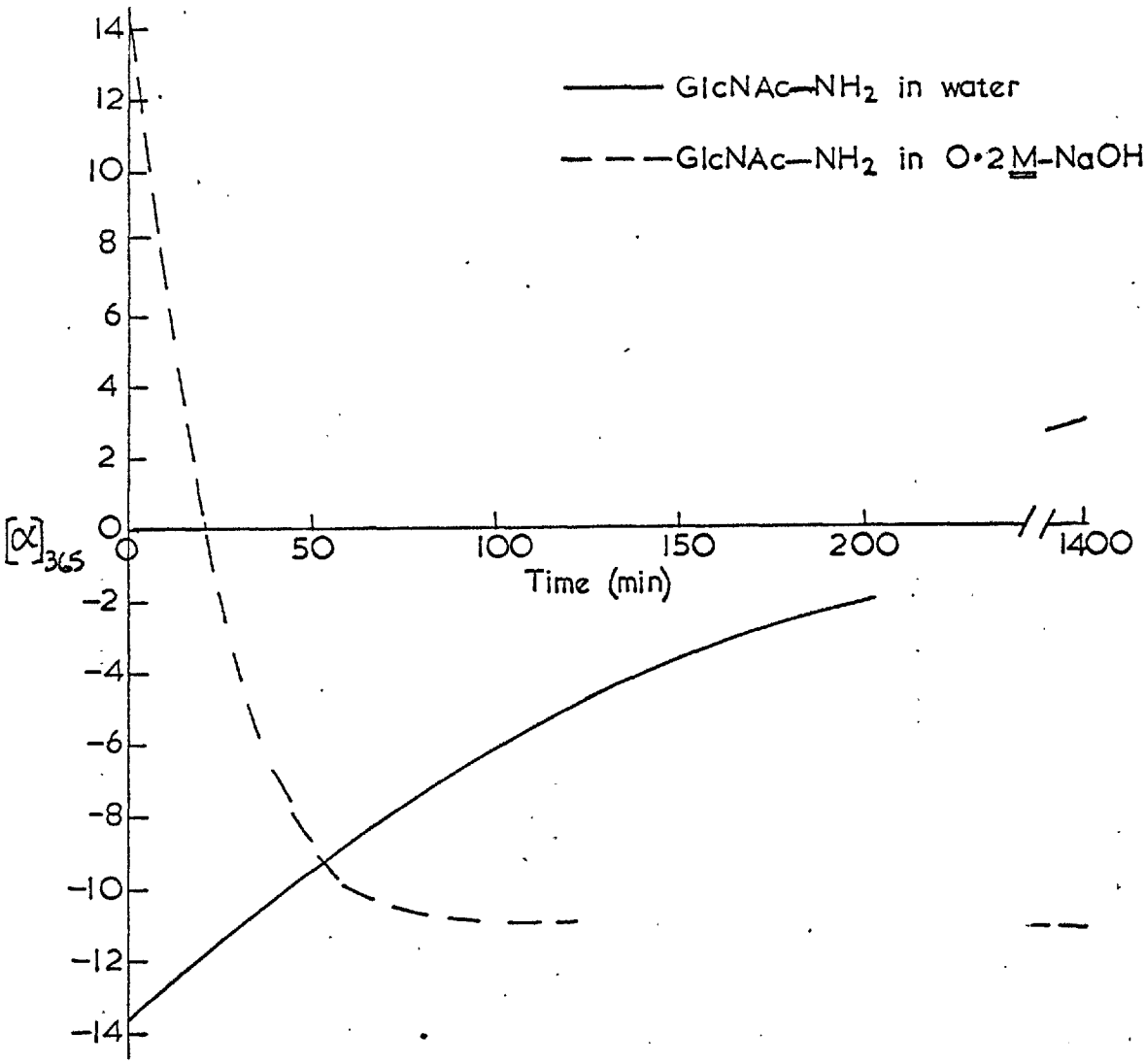


Table 3.3. The reaction rates and yields of products formed when GlcNAc-Asn is reacted with alkaline borohydride.

T^{o}	100	100	100	100	100	82	47
Molarity of NaOH with 1.0 M-$NaBH_4$	0	0.05	0.2	1.0	2.0	0.2	0.05
Moles Asp released per mole of substrate	0.37	0.50	0.64	0.75	0.79	0.45	0.11
First order rate consts.(min^{-1})($x10^{3}$)							
Loss of GlcNAc-Asn	6.49	10.2	20.2	33.4	40.3	32.6	24.1
Formation of Asp	2.40	5.10	12.9	27.2	31.8	1.47	.027
Total time over which reaction studied (min)[a]	220	360	360	360	300	660	48 hr
D-glucosaminitol[b]	0	0.15	0.37	0.43	0.43	0.21	0
D-glucosaminitol after acid hydrolysis[c]	0.40	0.51	0.69	0.56	0.43	0.63	0.16
D-mannosaminitol after acid hydrolysis[c]	0	0	0	0.12	0.16	0	0
Homoserine[b]	0.05	0.09	0.13	0.05	0.02	0.17	0.34
Asparagine[d]	0.14	0.12	0.14	0.08	0.03	0.09	0.02

(a) These times are the maximum times of reaction.

(b) The yields are expressed as mole fractions of GlcNAc-Asn destroyed, found after the times stated [see footnote (a).

(c) The yields are expressed as mole fractions of GlcNAc-Asn destroyed, found after acid hydrolysis (4 M-HCl; 100°; 3 hr) of the substances already formed by alkali treatment.

(d) The yields of asparagine are those found at the maximum.

carboxylic acids might be formed as some of the reaction products.

Three acids were separated by thin-layer chromatography on cellulose by ether - formic acid - water (7 : 2 : 1, by vol), after treatment of GlcNAc-Asn with 0.2M-NaOH at 100°C for 24 hr, followed by elution of the products from Dowex 1 with 0.05M-HCl, and from Dowex 50 with water (Plate 3.1).

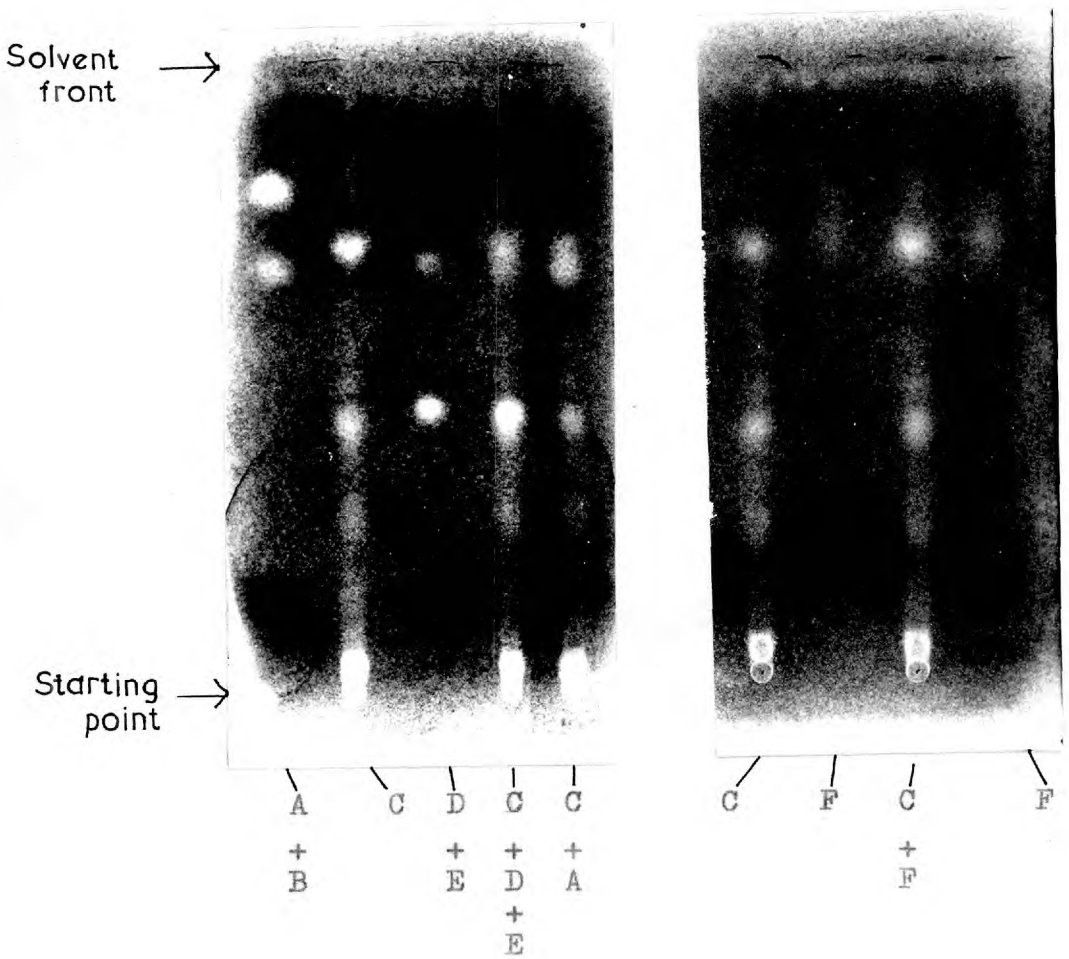
The R_F values of the most mobile component [0.71 in ether-formic acid - water (7 : 2 : 1, by vol) and 0.43 in phenol - water - formic acid (75 : 25 : 1, w/v/v] were the same as those of pyruvic acid. Maleic, fumaric and succinic acids were not present. The slower running component had R_F value of 0.43 in the ether - formic acid - water solvent, and the third component did not move from the baseline.

PART 2. TREATMENT OF COMPOUNDS WITH ALKALINE BOROHYDRIDE.

The kinetics of the degradation of GlcNAc-Asn.

GlcNAc-Asn is destroyed more rapidly when sodium borohydride is added to the reaction media than with alkali alone (Figs. 3.20-3.22). The rates of disappearance of GlcNAc-Asn and of formation of various products were also determined at 100°C in 2M-NaOH which contained 1.0M-$NaBH_4$ (Fig. 3.23). The calculated first-order rate constants for the destruction of GlcNAc-Asn are shown in Table 3.3. The rate observed at 100°C increased threefold in 0.05M-NaOH and 0.2M-NaOH, and twofold in 1.0M-NaOH, when 1M-$NaBH_4$ is included (compare with the results in

Plate 3.1. Thin-layer chromatography of some products formed by treating GlcNAc-Asn with 0.2M-NaOH at 100°C for 24 hr.

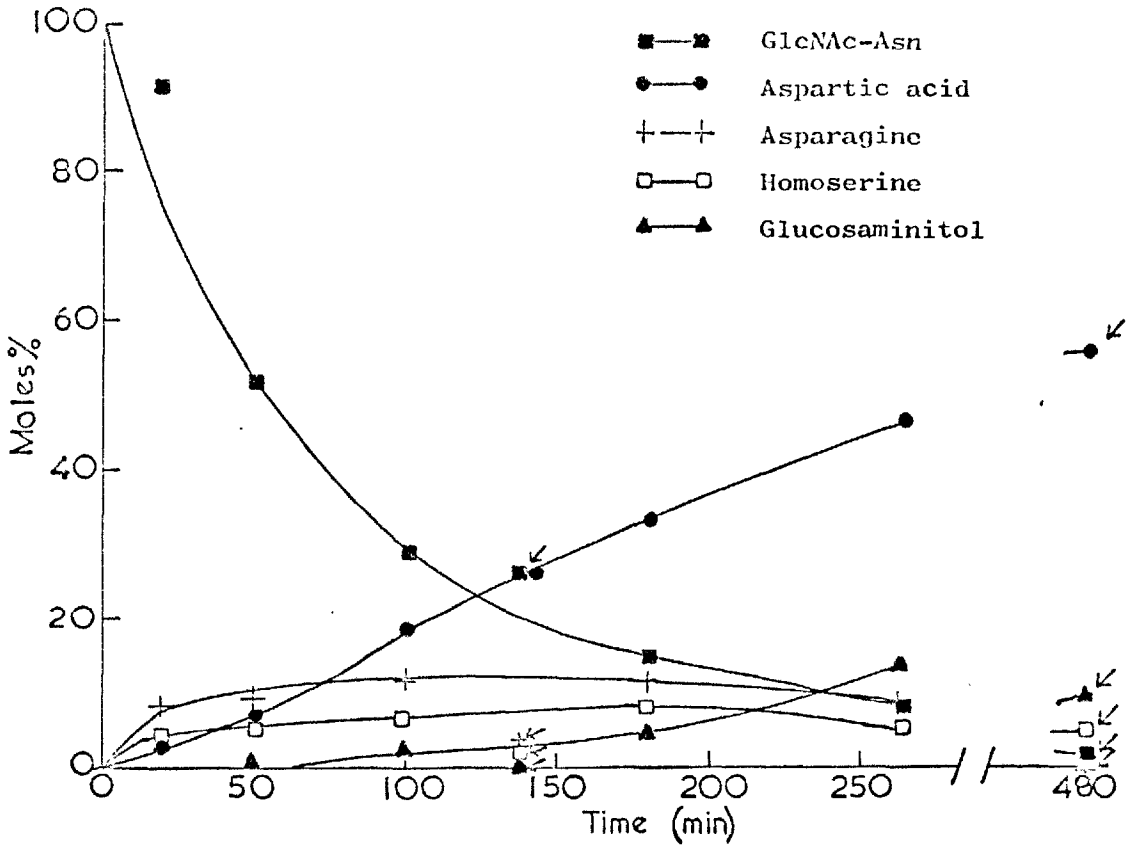


A. Succinic acid (2.5μg; R_f = 0.67)
B. Fumaric acid (2.5μg; R_f = 0.80)
C. Products isolated from GlcNAc-Asn (0.13μmoles) that had been treated with 0.2M-NaOH at 100°C.
D. Malic acid (2.5μg; R_f = 0.43)
E. Maleic acid (R_f = 0.67)
F. Pyruvic acid (2.5μg; R_f = 0.71)

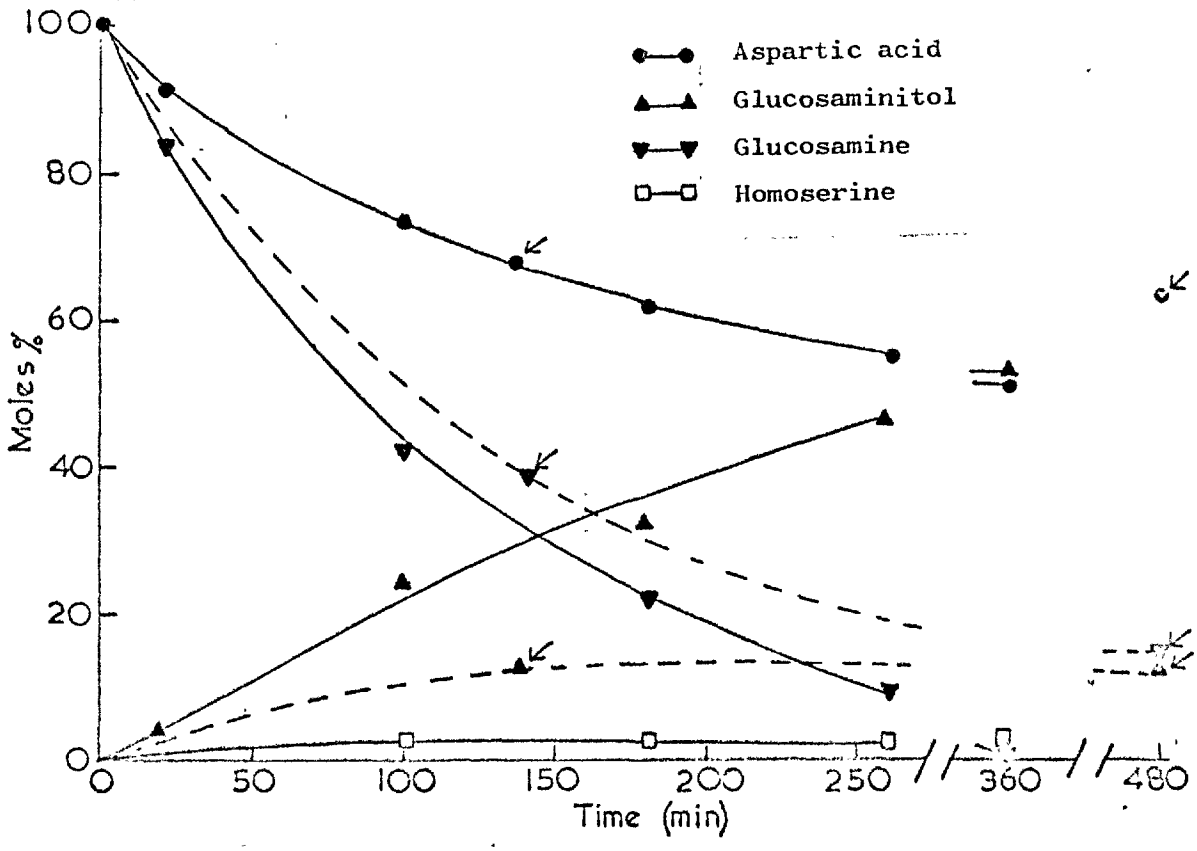
Chromatography was performed on thin layers of cellulose.

Solvent: Ether-formic acid-water (7 : 2 : 1 by vol)

Fig. 3.20. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 0.05M-NaOH and 1M-$NaBH_4$ at 100°C.

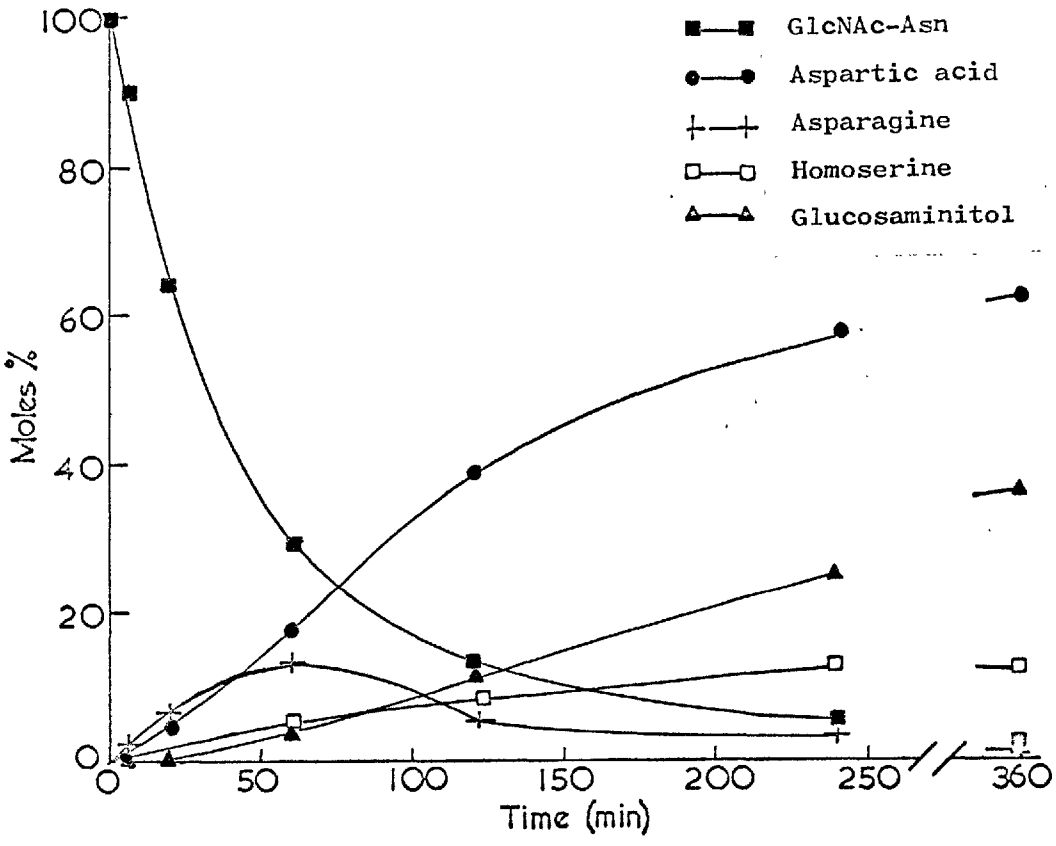


Products formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed the alkaline reduction of GlcNAc-Asn

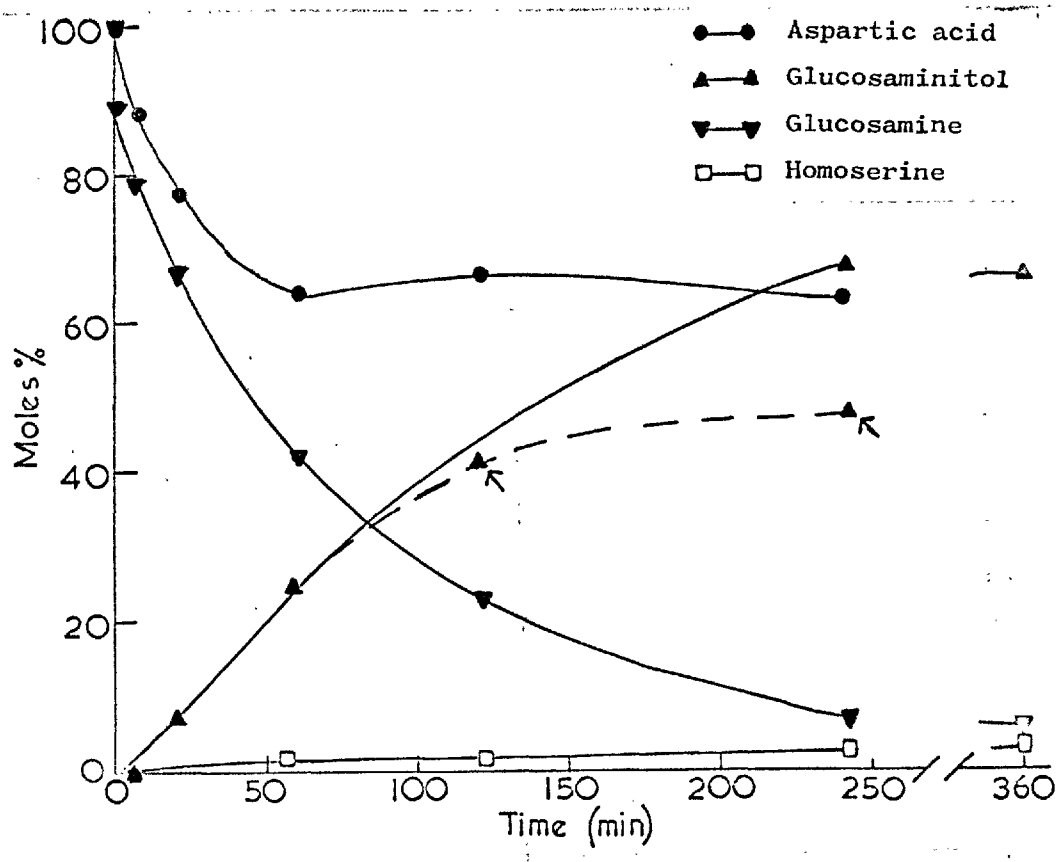


Products marked with ↙ represent those formed when no further quantities of alkaline borohydride were added as the reaction proceeded.

Fig. 3.21. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 0.2M-NaOH and 1M-$NaBH_4$ at 100°C.

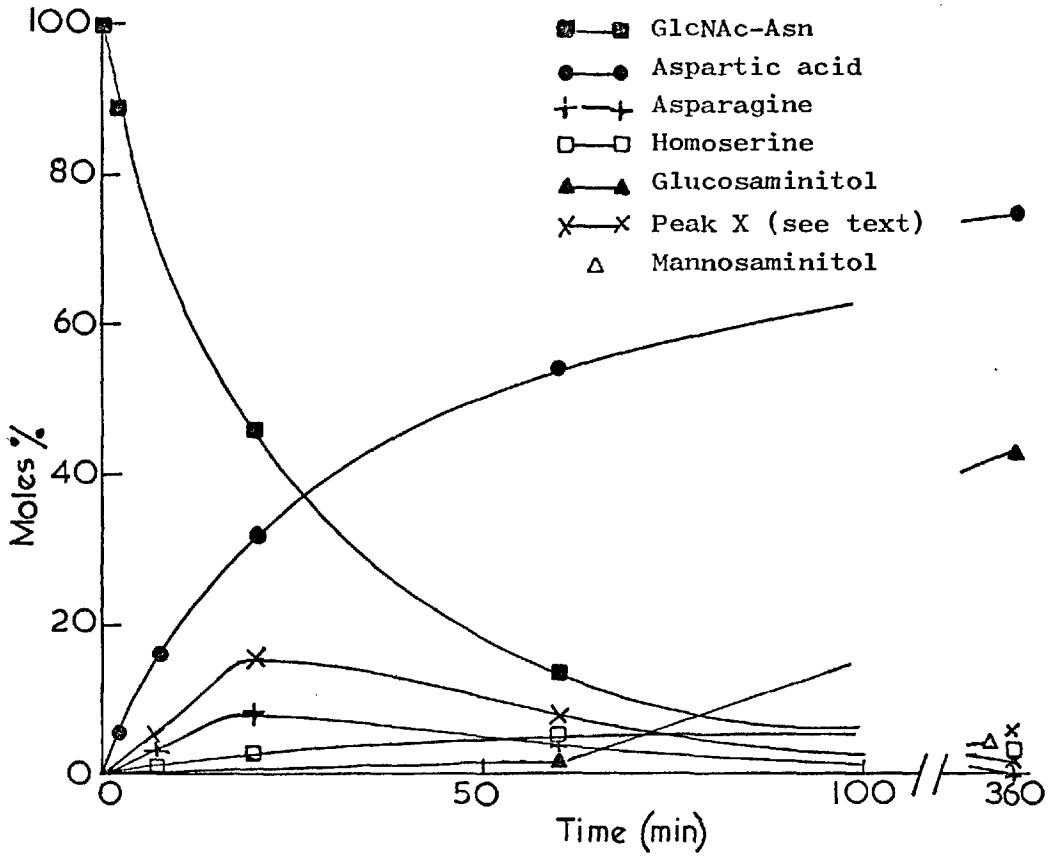


Products formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed the alkaline reduction of GlcNAc-Asn.



Products marked with ↖ represent those formed when no further quantities of alkaline borohydride were added as the reaction proceeded.

Fig 3.22. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 1M-NaOH and 1M-$NaBH_4$ at 100°C.



Products formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed the alkaline reduction of GlcNAc-Asn.

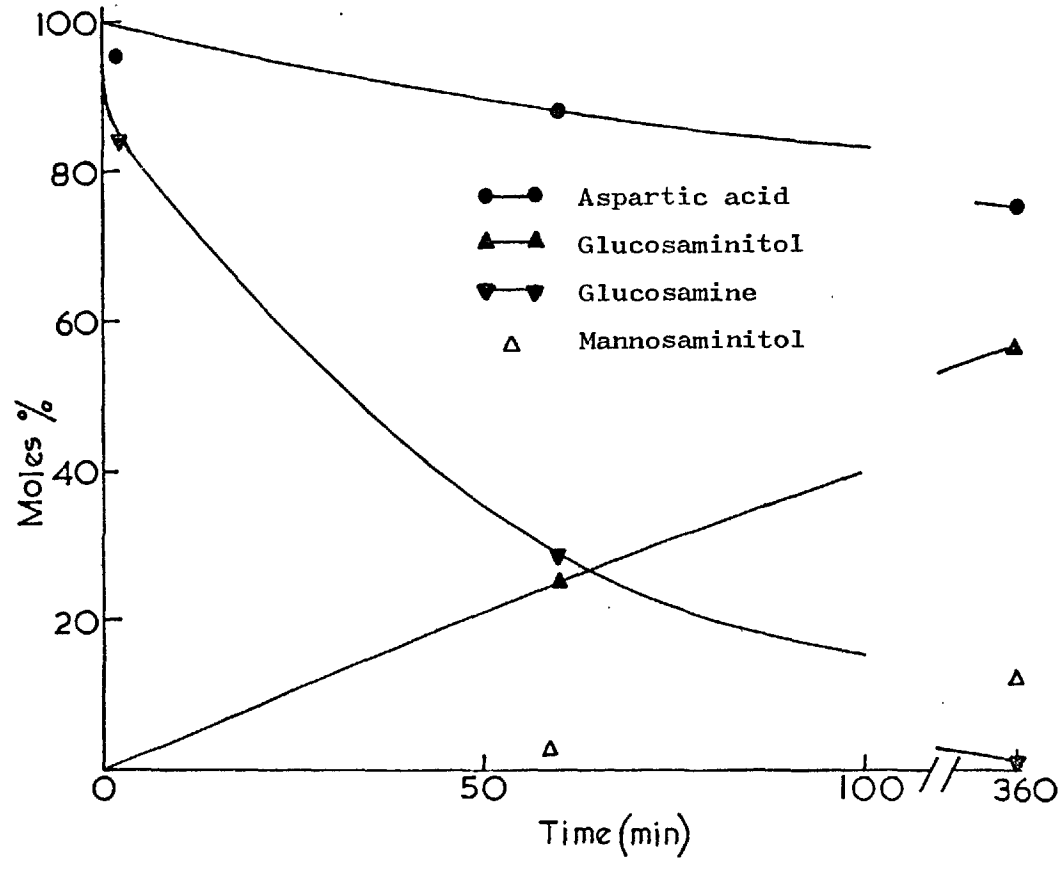
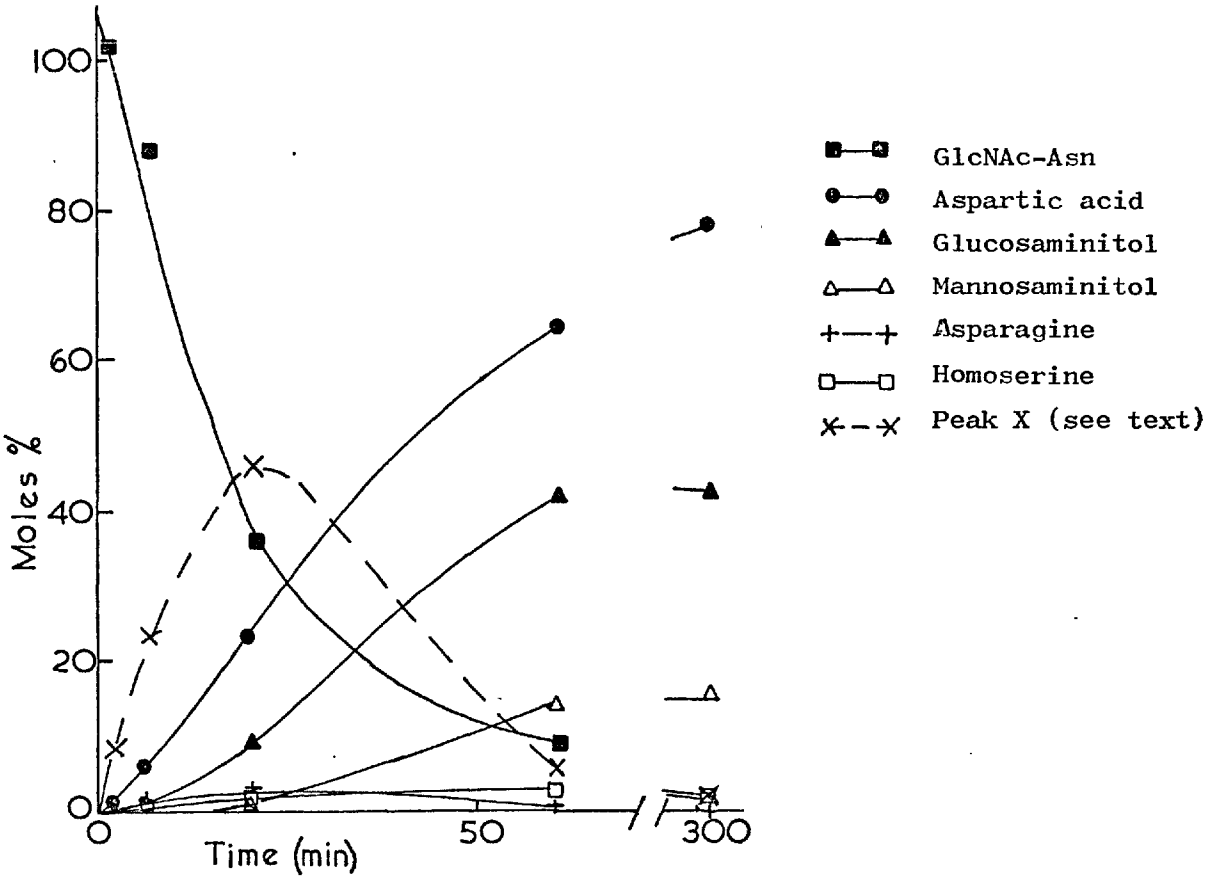


Fig. 3.23. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 2M-NaOH and 1M-$NaBH_4$ at 100°C



Products formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed alkaline reduction of GlcNAc-Asn.

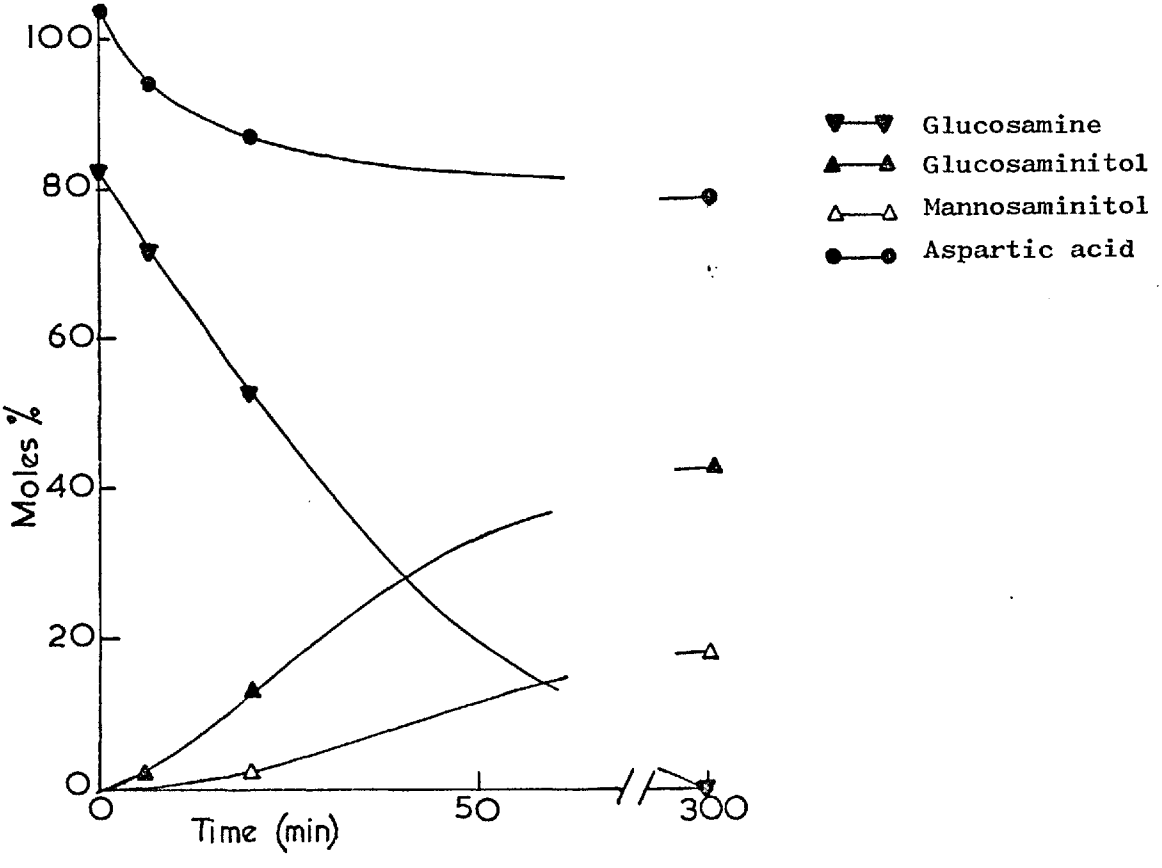


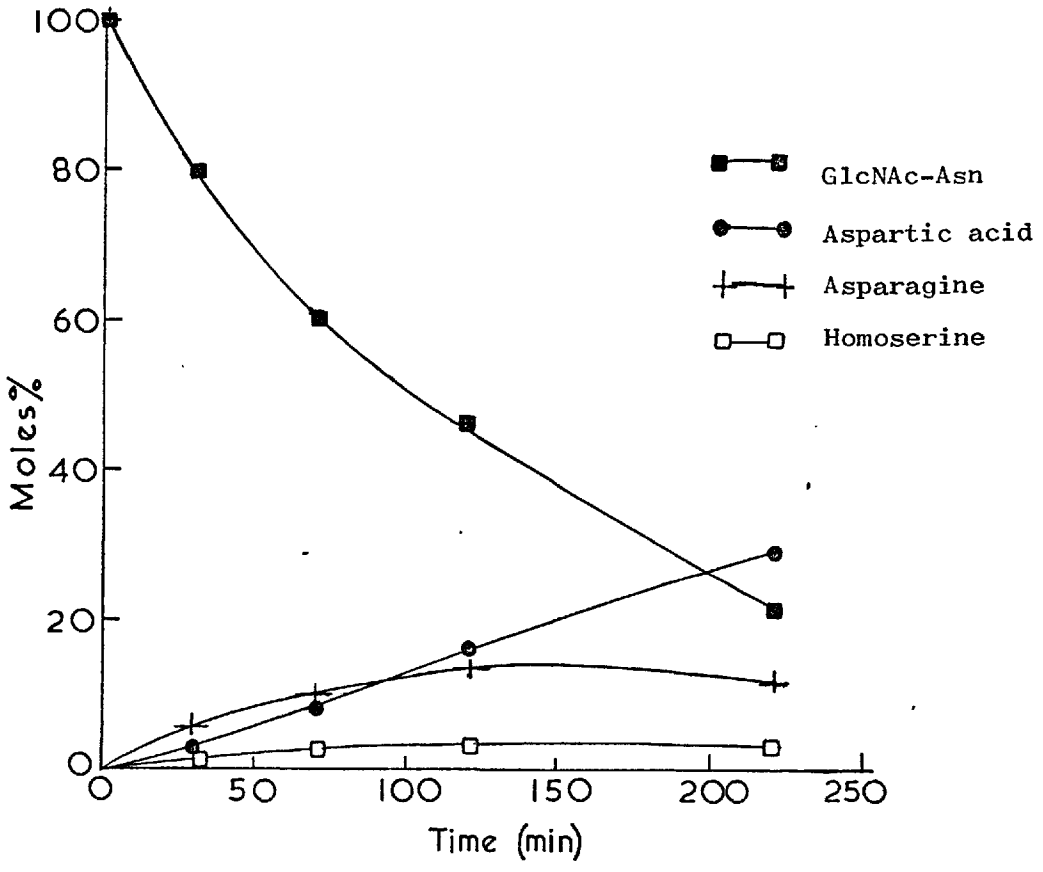
Table 3.1). GlcNAc-Asn is also destroyed in 1M-$NaBH_4$ (pH at 25° = 10.2) alone at a rate equivalent to that observed in 0.2M-NaOH (Fig. 3.24; cf. 3.1). The temperature and concentration of borohydride required to effect this cleavage are much greater than those used earlier by Lee & Montgomery (see page 131).

The reactions were also studied at lower temperatures. At 82°C (Fig. 3.25) in 0.2M-NaOH, 1M-$NaBH_4$ the rate was a sixth of that at 100°C (Table 3.3). A value of 26.7 kcals $mole^{-1}$ was calculated for the Arrhenius activation energy of the reaction, between the two temperatures.

GlcNAc-Asn was also subjected to the conditions that had been applied to cleave carbohydrate-peptide linkages involving L-seryl and L-threonyl residues in pig submaxillary mucin (Carlson, 1968). In 0.05M-NaOH, 1M-$NaBH_4$ at 47°C, the half-life for the decomposition of GlcNAc-Asn was 48 hr (Fig. 3.26). An activation energy of 16.8 kcals $mole^{-1}$ could be calculated for reaction, between this temperature and 100°C. The difference in activation energies found under the two conditions of alkalinity probably reflect the fact that the proportions of GlcNAc-Asn which undergo decomposition by different pathways changes with concentration of alkali.

It was observed also that a substance X, which is probably GlcN-Asn, was produced as an intermediate (Figs. 3.22 and 3.23) in those experiments where relatively high concentrations of alkali were employed (cf. also Figs. 3.1 and 3.5).

Fig. 3.24. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 1M-$NaBH_4$ and no additional alkali at 100°C



Products formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed alkaline reduction of GlcNAc-Asn

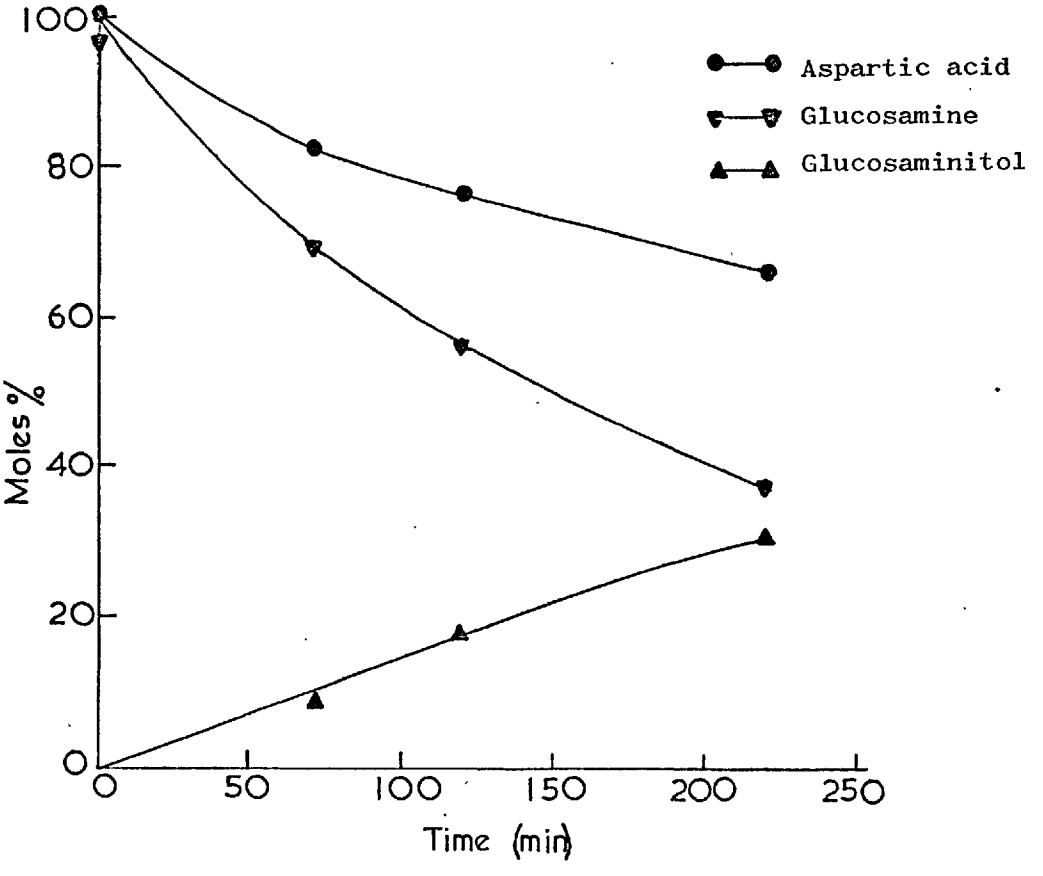
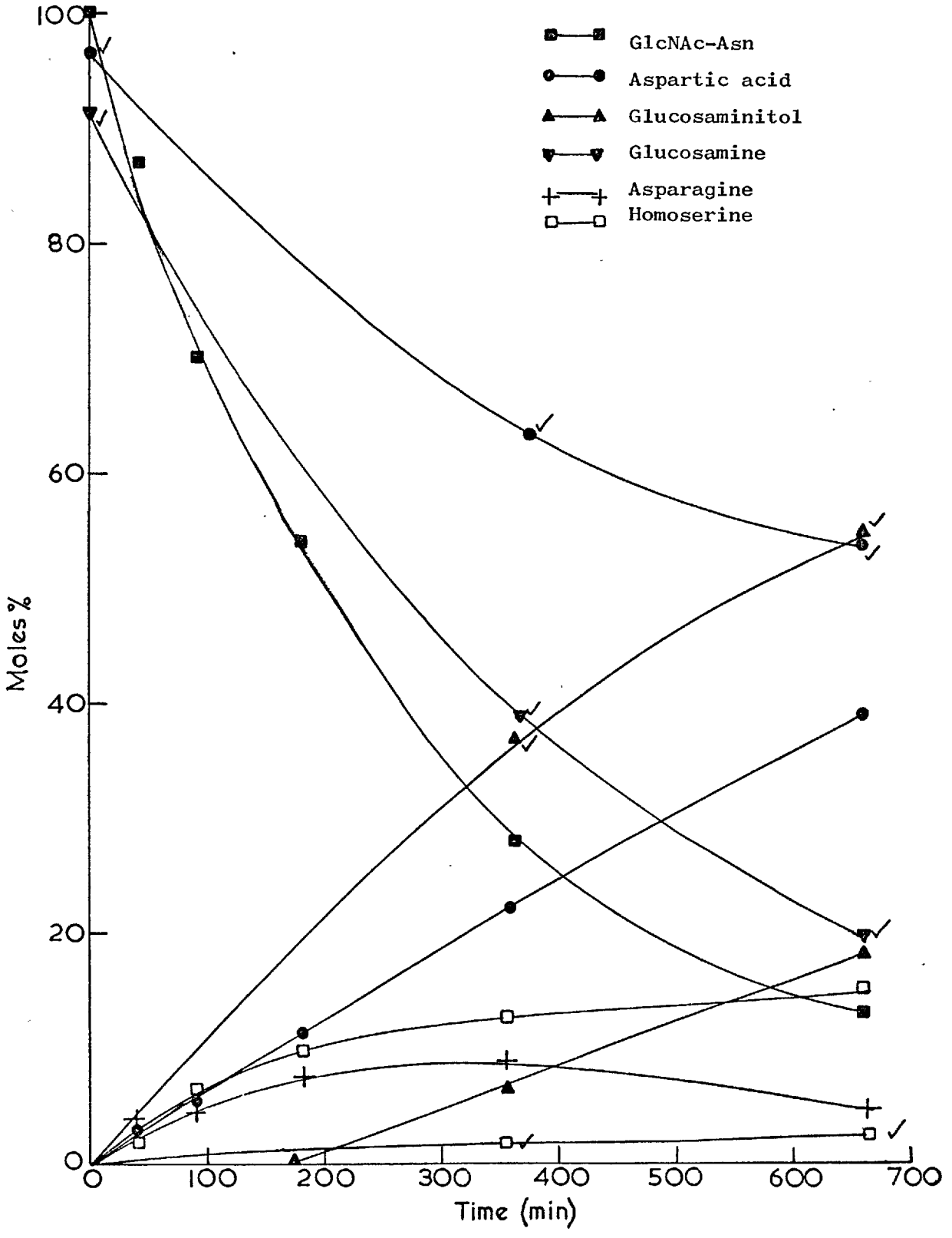
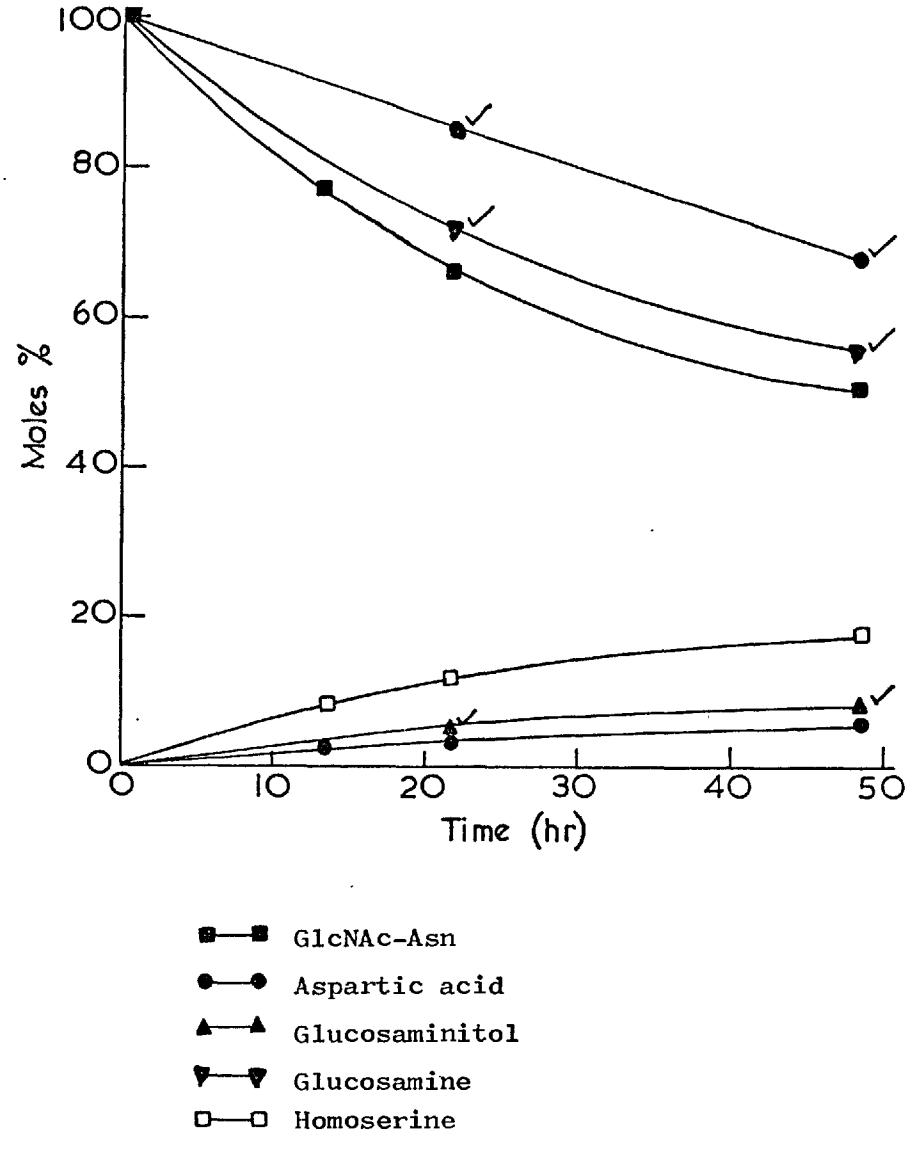


Fig. 3.25. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn (6mM) with 0.2M-NaOH and 1M-$NaBH_4$ at 82°C



Products marked ✓ represent those formed when acid hydrolysis (4M-HCl; 3hr; 100°C) followed alkaline reduction of GlcNAc-Asn.

Fig 3.26. Destruction of GlcNAc-Asn and formation of products resulting from treatment of GlcNAc-Asn with 0.05M-NaOH and 1M-$NaBH_4$ at 47°C



Products marked with ✓ are those formed when acid hydrolysis ($\frac{4}{\pi}$M-HCl; 3hr; 100°C) followed alkaline reduction of GlcNAc-Asn.

The production of reduced sugars.

D-Glucosaminitol was identified in the samples taken at the later stages of the alkaline reduction, performed in more concentrated alkali (Figs. 3.21-3.23). Increased amounts of D-glucosaminitol were found if acid hydrolysis followed the alkaline reduction. The increase observed on acid hydrolysis almost certainly represents the amount of N-acetyl-D-glucosaminitol which was originally present. The measured amounts before and after acid hydrolysis, expressed as a mole fraction of the amount of GlcNAc-Asn destroyed, are recorded in Table 3.3. It was necessary to add more sodium borohydride as the reaction proceeded (Figs. 3.20 and 3.21) to replace that destroyed, in order to produce high yields of D-glucosaminitol and of N-acetyl-D-glucosaminitol. The conditions of 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for 6 hr produced the maximum yield of alditol composed of both D-glucosaminitol and N-acetyl-D-glucosaminitol. The total yield was 69%. On the other hand, milder conditions of cleavage led to smaller yields. In 0.05M-NaOH containing 1M-$NaBH_4$ the total yield of N-acetyl-D-glucosaminitol was only 16% after 48 hr, with no D-glucosaminitol (Table 3.3).

D-Mannosaminitol, which might be expected to be formed through epimerisation of N-acetyl-D-glucosamine before reduction, was identified after reactions performed in 1M and 2M-NaOH solutions which contained 1M-$NaBH_4$

Table 3.4. Reduction in alkaline borohydride of N-acetyl-D-glucosamine and GlcNAc-NH_2

Products were measured as mole per mole of substrate after alkaline reduction, followed by acid hydrolysis in 4M-HCl at 100°C for 3 hr.

N-Acetyl-D-glucosamine

Alkaline conditions: 0.2M-NaOH, 1M-$NaBH_4$, 100°C

	10 min	3 hr
D-Glucosamine	0	0
D-Glucosaminitol	0.73	0.78
D-Mannosaminitol	0.06	0.08

Alkaline conditions: 2M-NaOH, 1M-$NaBH_4$, 100°C

D-Glucosamine	0	0
D-Glucosaminitol	0.57	0.61
D-Mannosaminitol	0.24	0.24

GlcNAc-NH_2

Alkaline conditions: 0.2M-NaOH, 1M-$NaBH_4$, 100°C

	10 min	2 hr
D-Glucosamine	0.20	0
D-Glucosaminitol	0.36	0.50
D-Mannosaminitol	0.08	0.09

Alkaline conditions: 2M-NaOH, 1M-$NaBH_4$, 100°C

	10 min	3 hr
D-Glucosamine	0.27	0
D-Glucosaminitol	0.23	0.29
D-Mannosaminitol	0.11	0.22

GlcNAc-NHAc

Alkaline conditions: 0.05M-NaOH, 1M-$NaBH_4$, 100°C

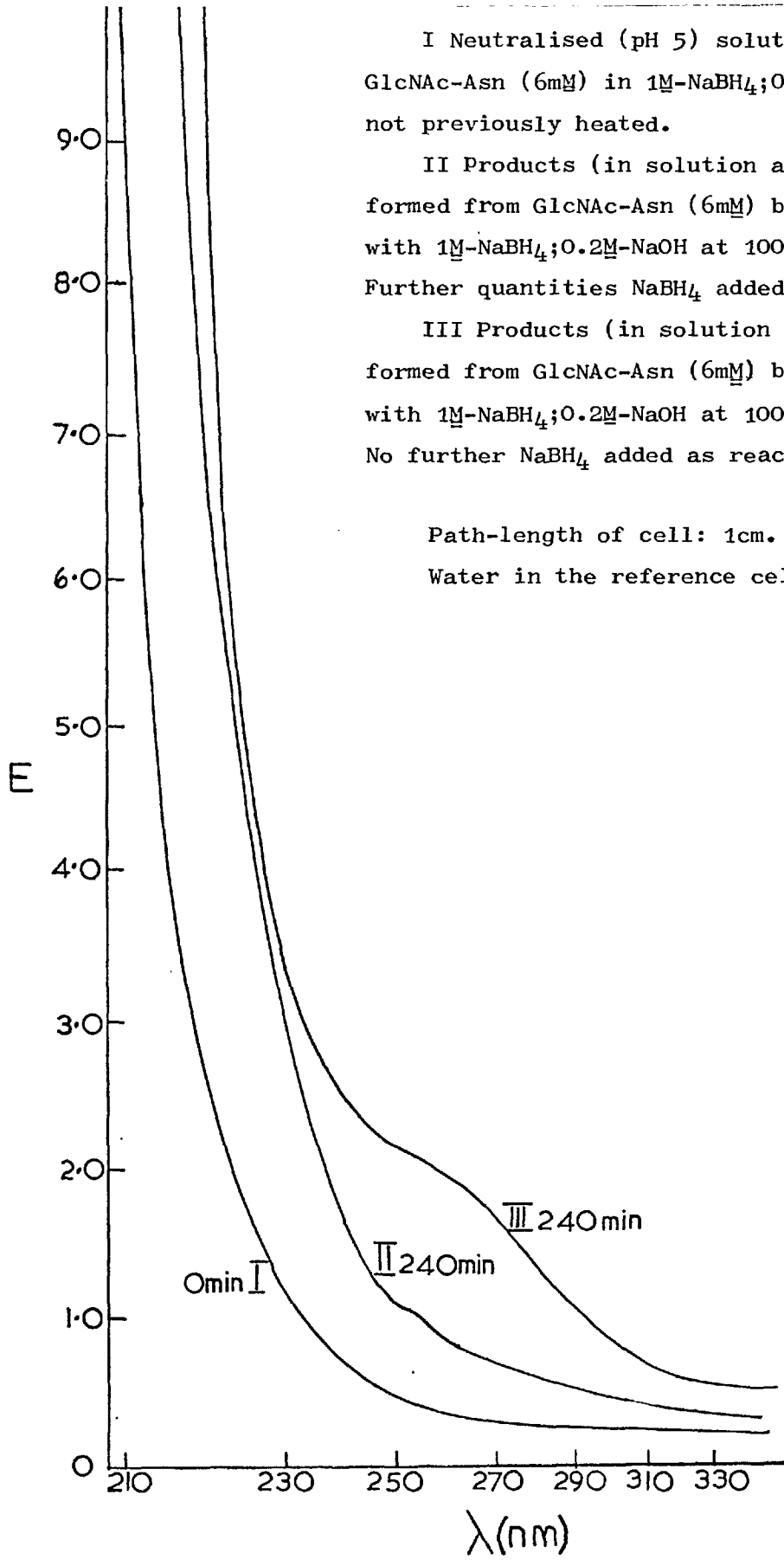
	1 hr	5 hr
D-Glucosamine	0.73	0.59
D-Glucosaminitol	0.08	0.18

(Table 3.3). This compound was more retarded on the autoanalyser than was D-glucosaminitol (Table 3.2), and was not completely resolved from it, although satisfactory measurements could be made.

Only a small amount of material absorbing in the ultra-violet between 220 and 330nm was observed, compared to that found in 0.2M-NaOH alone (Fig. 3.27). Less absorption was observed when further quantities of $NaBH_4$ were added as the reaction proceeded. Presumably the products which are formed in alkali alone and which absorb in the ultra-violet are unsaturated, so that it would not be too surprising if they underwent reduction by $NaBH_4$, or if they were not formed at all in the presence of $NaBH_4$.

N-Acetyl-D-glucosamine (6mM) was reduced to D-glucosaminitol in 78% yield in 0.2M-NaOH, 1M-$NaBH_4$ at 100°C. The yield in 2M-NaOH was less, but the yield of D-mannosaminitol was correspondingly increased (Table 3.4). The reaction appeared to be almost complete after 10 min. Relatively smaller yields of D-glucosaminitol were obtained from GlcNAc-NH_2 under the same conditions, and reduction was less rapid. Production of D-glucosaminitol from GlcNAc-NHAc in 0.05M-NaOH, 1M-$NaBH_4$ was slow. Only 0.44 mole of D-glucosaminitol per mole of D-glucosamine that had been destroyed was formed after acid hydrolysis (Table 3.4) of products formed by treatment of GlcNAc-NHAc with alkaline borohydride for 5 hr.

Fig. 3.27. Ultra-violet spectra of products formed from GlcNAc-Asn by treatment with 1M-$NaBH_4$ and 0.2M-NaOH at 100°C.



The formation of amino acids as a result of alkaline reduction of GlcNAc-Asn.

Yields of aspartic acid were less than stoicheiometric when GlcNAc-Asn had decomposed. Yields were somewhat higher at 100°C in 0.05M-NaOH in the presence of 1M-borohydride than with alkali alone, whereas the yield in 1M-NaOH, 1M-$NaBH_4$ was less than that produced by 1M-NaOH alone (cf. Tables 3.1 and 3.3). The lowest yield recorded for aspartic acid was from the reaction performed at 47°C (Table 3.3).

Two other peaks, running after aspartic acid, were found on the autoanalyser, and these had elution times of 70 and 76 min respectively, when neutralised samples from the alkaline borohydride reaction of GlcNAc-Asn were analysed directly (Fig. 3.28a). Standard samples of L-asparagine (70 min) and DL-homoserine (76 min) ran concurrently with these peaks. The ratio of the peak areas measured at 440nm to that measured at 570nm of the peak that eluted after 70 min, upon autoanalysis of the products formed by alkaline reduction of GlcNAc-Asn, was 0.237, while the ratio measured for standard L-asparagine was 0.231. The ratio of the peak occurring at 76 min that resulted from autoanalysis of the products of alkaline reduction (0.183) was also the same as that measured for standard DL-homoserine (0.187). The compound giving rise to the peak eluting at 70 min behaves as an intermediate, maximum amounts being present in the early stages of the reaction, and being destroyed as the reaction proceeds (Figs. 3.20, 3.21, 3.25 and 3.28a) This compound is

Fig.3.28a Products formed from GlcNAc-Asn by treatment with 1M-$NaBH_4$;0.2M-NaOH at 100°C for different periods of time. Products are measured on the autoanalyser.

The parts of the traces recorded up to the time of elution of valine only are shown.

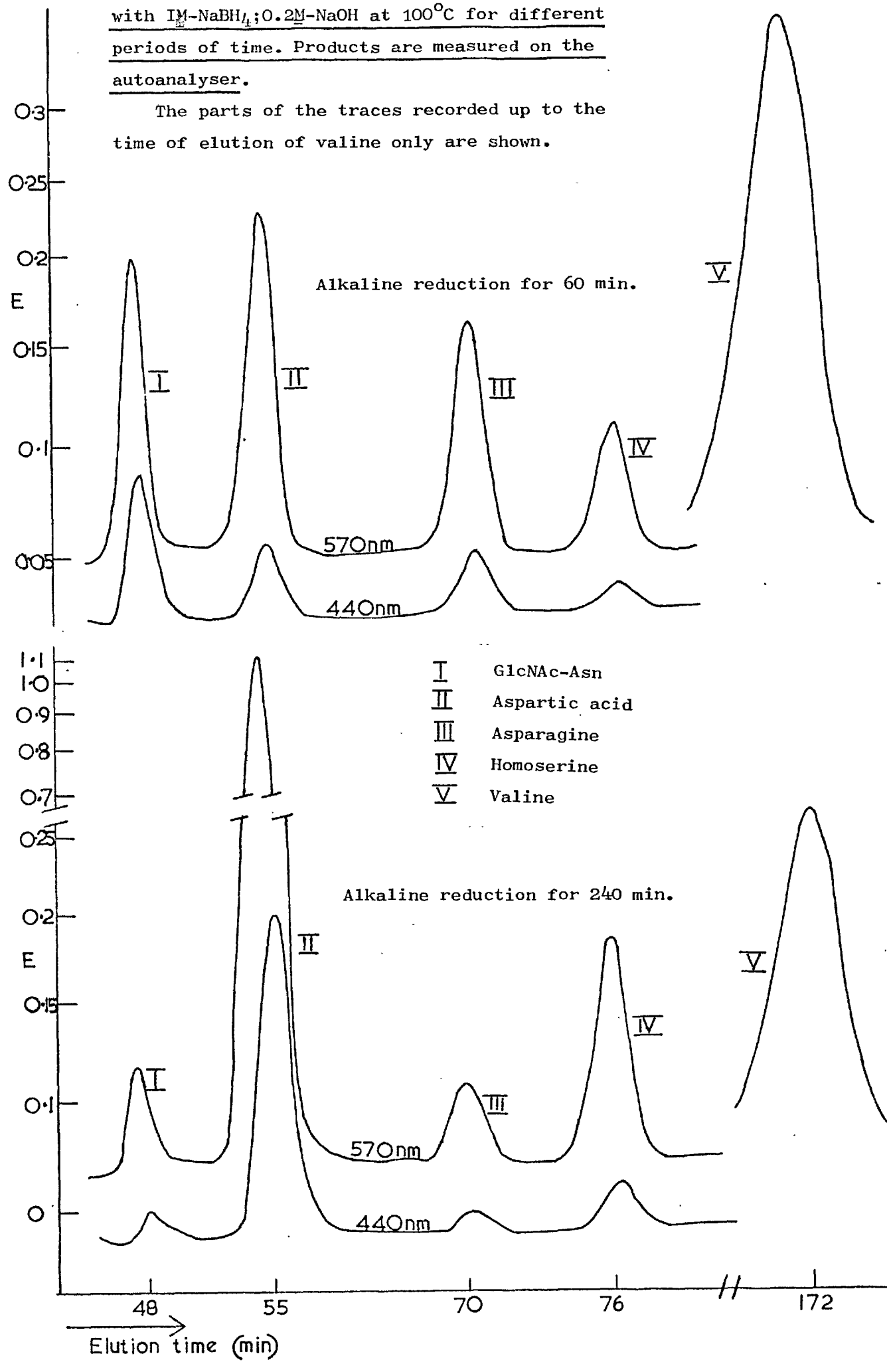
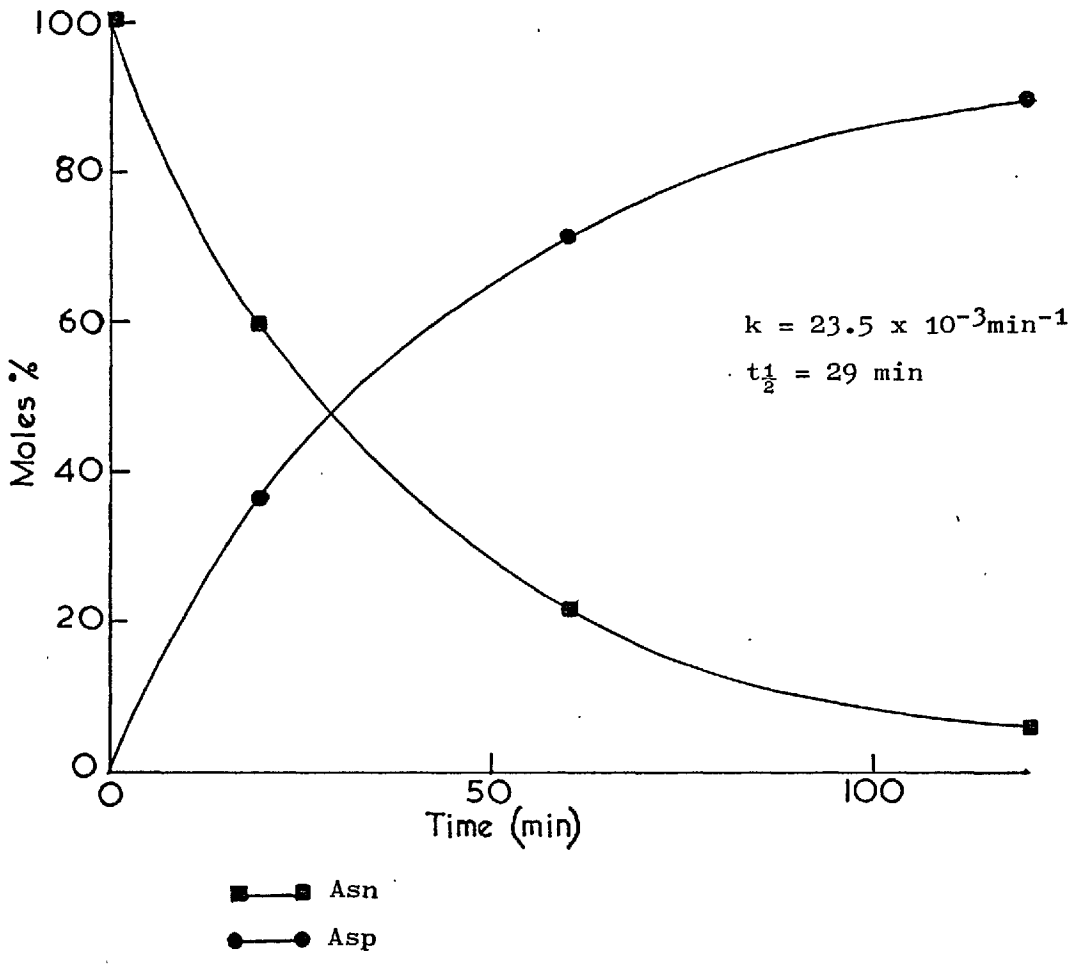


Fig. 3.28b Destruction of L-asparagine by treatment with 0.2M-NaOH and 1M-$NaBH_4$ at 100°C, and formation of aspartic acid.



asparagine, and it was shown in control experiments that the amino acid is converted, in 0.2M-NaOH, 1M-$NaBH_4$ at 100°C, almost quantitatively into aspartic acid with a half-life of 29 min (Fig. 3.28b; $k = 23.5 \times 10^{-3} min^{-1}$). There is a very small peak at the position occupied by homoserine on the chromatogram obtained by autoanalysis of the products of alkaline reduction of L-asparagine, but it is not possible to state with assurance that the substance is homoserine in view of the tiny quantities involved. In any case, the yield is quite small ($< 3\%$). The compound which has been identified as homoserine (see below) is stable for at least 3 hr under the conditions used. Standard DL-homoserine was used in the control experiments.

Under some conditions, the kinetics for the production of aspartic acid displayed an induction period (see particularly Figs. 3.20, 3.21 and 3.24). These findings are consistent with the view that a relatively large fraction of the aspartic acid arises from an initial formation of asparagine.

The yield of homoserine increased as the temperature decreased, becoming a major product at 47°C, although its rate of formation was slow (Fig. 3.26).

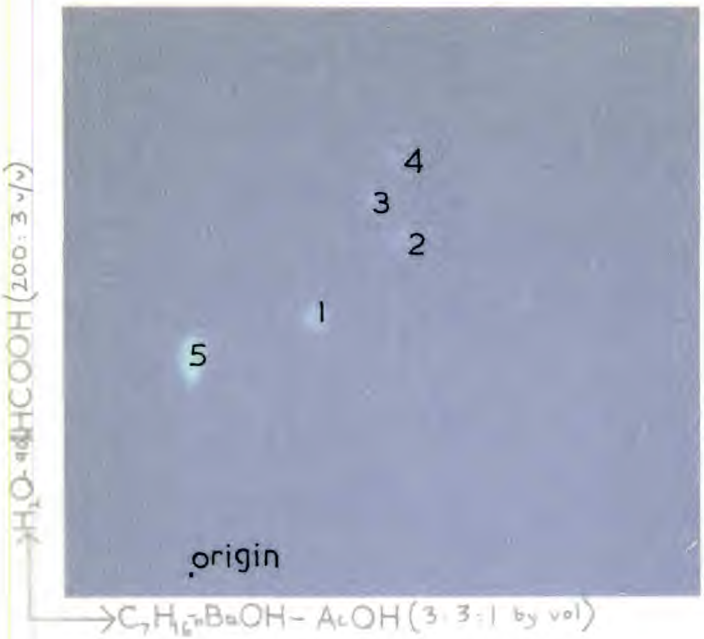
Acid hydrolysis of samples removed during alkaline reduction of GlcNAc-Asn completely destroyed asparagine as expected, whereas homoserine was partially destroyed, giving rise to the lactone. The amounts of aspartic acid measured were approximately equal to those present already

in the alkaline reaction media, plus those formed from unreacted GlcNAc-Asn, and in some cases also from GlcN-Asn, in addition to those expected from destruction of asparagine (Fig. 3.20-3.25).

It was desirable to measure the total quantities of homoserine and its lactone in acid hydrolysates of samples previously treated with alkaline borohydride, especially under the conditions where large amounts of this 4-hydroxyamino acid were formed. Thus acid hydrolysates of samples reduced for 6 and 11 hr in 0.2M-NaOH at 82°C, and 0.05M-NaOH at 100°C for 180 min, were autoanalysed with a buffer programme in which pH 6.65 buffer was replaced with pH 5.28. These modified conditions result in the resolution of ammonia and homoserine lactone. A peak eluting 1 hr after ammonia, with a total elution time of 6 hr 54 min, was found in each case. This peak had an area 1.7 times that resulting from homoserine in the same hydrolysate. Standard DL-homoserine was destroyed to an extent of 70% under the same conditions of acid hydrolysis, and also formed a peak eluting after 6 hr 54 min. This peak is attributable to the lactone of homoserine (Armstrong, 1949), and it was found to have an area ratio of 1.5 relative to the homoserine peak.

The identities of asparagine and homoserine, as products of alkaline reduction in 0.05M-NaOH, 1M-$NaBH_4$ at 100°C of GlcNAc-Asn, were confirmed by thin-layer chromatography of their dansyl derivations. Five dansyl-derivatives besides Dns-OH and Dns-NH_2 appeared on the plates. Four of these appeared at all stages of the

Plate 3.2. Chromatography of Dns-Asparagine and Dns-Homoserine.



Standards

1 Dns-Asp

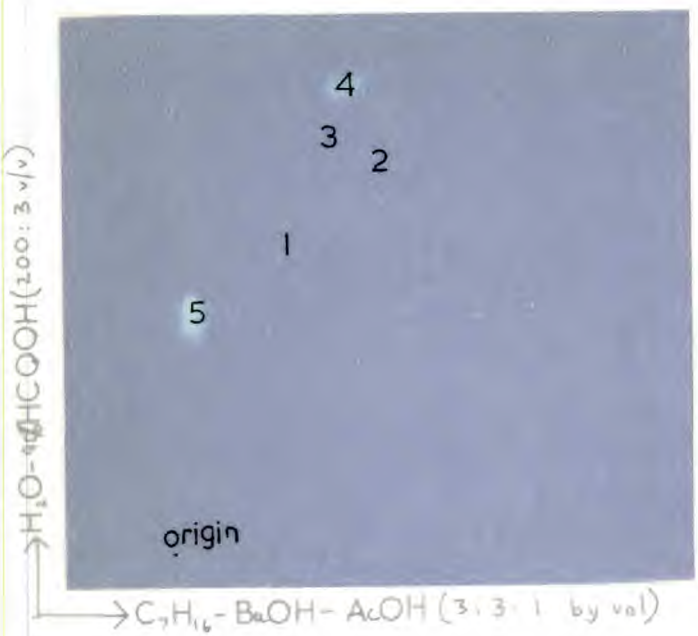
2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

5 Dns-OH

Methods of preparing dansyl derivatives, and chromatographic conditions are described in the text.



Products formed by treating GlcNAc-Asn with 0.05M-NaOH; 1M-$NaBH_4$ at 100°C for 40 min, and then Dns-Cl

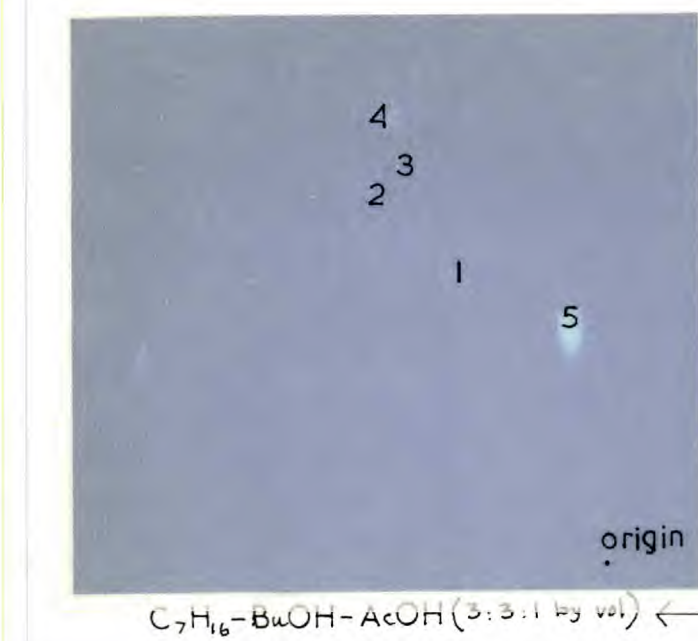
1 Dns-Asp

2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

5 Dns-OH



Products formed by treating GlcNAc-Asn with 0.05M-NaOH; 1M-$NaBH_4$ at 100°C for 2 hr, and then Dns-Cl.

1 Dns-Asp

2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

5 Dns-OH

Plate 3.2. Chromatography of Dns-Asparagine and Dns-Homoserine.

H_2O-90%HCOOH (200:3 v/v)

C_7H_{16}-BuOH-AcOH (3:3:1 by vol)

Standards

1 Dns-Asp

2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

5 Dns-OH

Methods of preparing dansyl derivatives, and chromatographic conditions are described in the text.

H_2O-90%HCOOH (200:3 v/v)

C_7H_{16}-BuOH-AcOH (3:3:1 by vol)

Products formed by treating GlcNAc-Asn with 0.05M-NaOH; 1M-$NaBH_4$ at 100°C for 40 min, and then Dns-Cl

1 Dns-Asp

2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

5 Dns-OH

C_7H_{16}-BuOH-AcOH (3:3:1 by vol)

H_2O-90%HCOOH (200:3 v/v)

Products formed by treating GlcNAc-Asn with 0.05M-NaOH; 1M-$NaBH_4$ at 100°C for 2 hr, and then Dns-Cl.

1 Dns-Asp

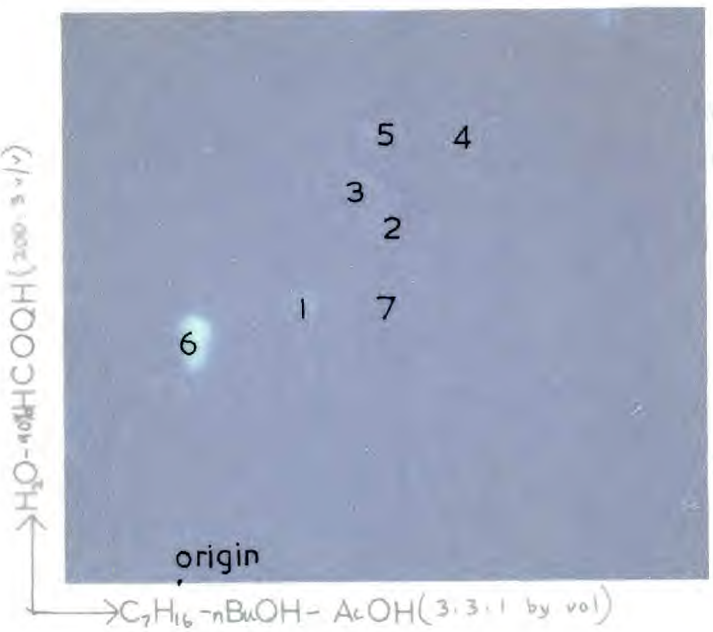
2 Dns-Homoserine

3 Dns-Asn

4 Dns-GlcNAc-Asn

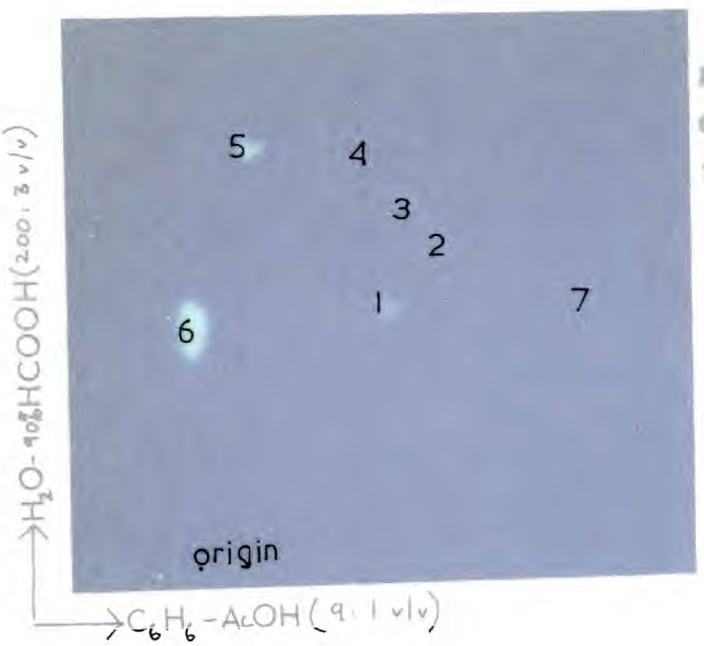
5 Dns-OH

Plate 3.2. (continued)



Products formed by treating GlcNAc-Asn with 0.05M-NaOH ; 1M-$NaBH_4$ at 100°C for 4 hr

1 Dns-Asp
2 Dns-Homoserine
3 Dns-Asn
4 Dns-Glucosaminitol
5 Dns-GlcNAc-Asn
6 Dns-OH
7 Dns-NH_2

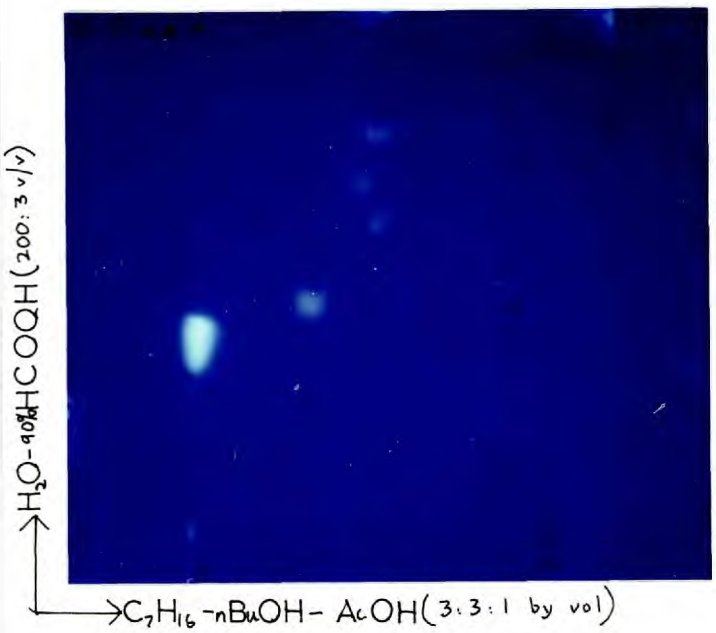


Products formed by treating GlcNAc-Asn with 0.05M-NaOH ; 1M-$NaBH_4$ at 100°C for 2 hr

1 Dns-Asp
2 Dns-Homoserine
3 Dns-Asn
4 Dns-Glucosaminitol
5 Dns-GlcNAc-Asn
6 Dns-OH
7 Dns-NH_2

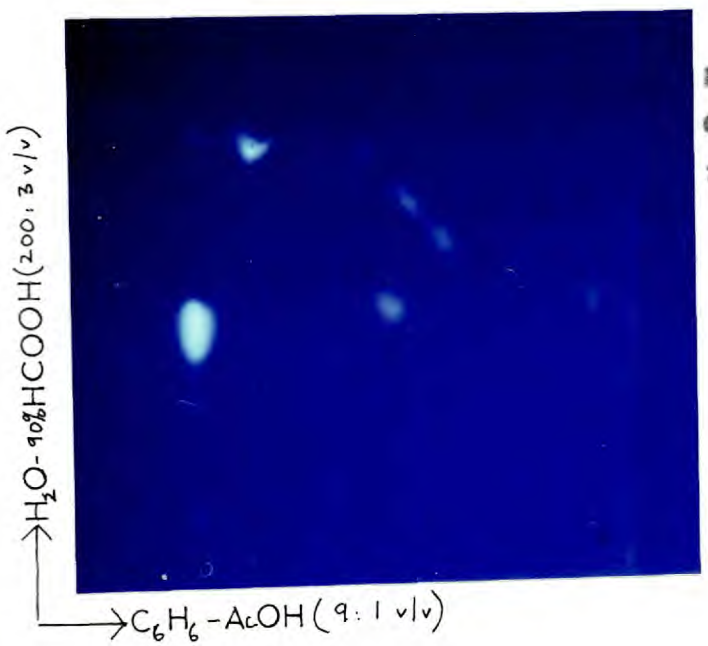
The true colours have been lost during photographic reproduction. Dns-OH and Dns-NH_2 fluoresce blue under uv light. The other derivatives fluoresce green.

Plate 3.2. (continued)



Products formed by treating GlcNAc-Asn with 0.05M-NaOH ; 1M-$NaBH_4$ at 100°C for 4 hr

1 Dns-Asp
2 Dns-Homoserine
3 Dns-Asn ←
4 Dns-Glucosaminitol
5 Dns-GlcNAc-Asn
6 Dns-OH
7 Dns-NH_2



Products formed by treating GlcNAc-Asn with 0.05M-NaOH ; 1M-$NaBH_4$ at 100°C for 2 hr

1 Dns-Asp
2 Dns-Homoserine
3 Dns-Asn ←
4 Dns-Glucosaminitol
5 Dns-GlcNAc-Asn
6 Dns-OH
7 Dns-NH_2

The true colours have been lost during photographic reproduction. Dns-OH and Dns-NH_2 fluoresce blue under uv light. The other derivatives fluoresce green.

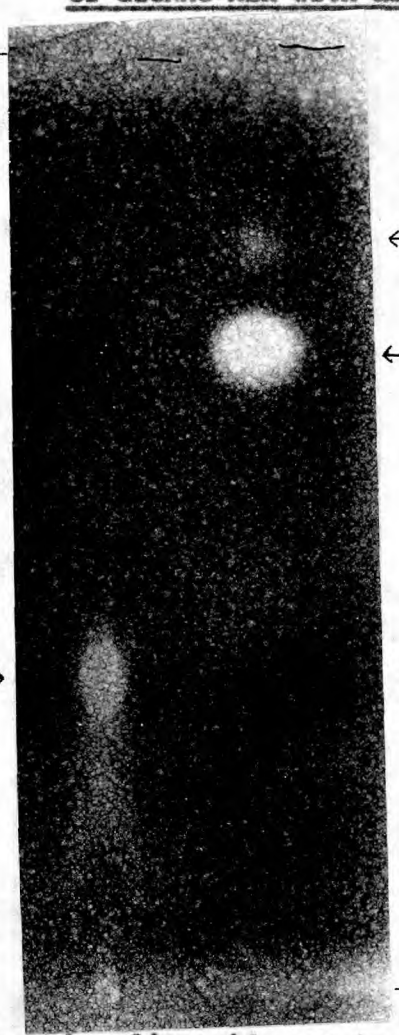
reaction (20 min, 2 hr and 4 hr) and were identified as Dns-GlcNAc-Asn, Dns-Asp, Dns-Asn and Dns-homoserine by their mobilities in three solvents (Plate 3.2). The intensities of the spots given under ultra-violet light varied with time in the same manner as the corresponding peaks measured on the autoanalyser. Dns-D-glucosaminitol was found only at the later stages of the reaction.

Standard L-2,4-diaminobuytric acid gave a peak on the autoanalyser during elution by the pH 6.65 buffer after 305 min, at a position which was just before the ammonia peak (Table 3.2). Small peaks running in this position were found upon analysis of the products of reduction of GlcNAc-Asn in 0.05M-NaOH, and 0.2M-NaOH, both before and after acid hydrolysis, but the sizes of these peaks suggested that GlcNAc-Asn is degraded to an extent which is less than 3% to this compound.

Thin-layer chromatography of acidic products formed from the alkaline reduction of GlcNAc-Asn.

Thin-layer chromatography of the fractions obtained by elution from Dowex 1 with 0.08M-HCl, and with 0.8M-HCl, (see page 257) of the products resulting from treatment of GlcNAc-Asn with alkaline borohydride 0.05M-NaOH; M-$NaBH_4$; 3 hr; 100°C) did not show fast-running spots corresponding to standard carboxylic acids considered as possible products (Plate 3.3). After prolonged re-evaporation of the material obtained from the 0.08M-HCl eluate with methanol in order to remove boric acid, one spot was obtained (R_f 0.46 in ether - formic acid - water, 7 : 2 : 1, by vol). The elemental analysis found by

Plate 3.3. Thin layer chromatography of an acid formed by treatment of GlcNAc-Asn with alkaline borohydride.



Solvent: Ether-formic acid-water (7 : 2 : 1 by vol)

A = Products formed by treatment of GlcNAc-Asn (0,2mg) with 0.05M-NaOH, 1M-$NaBH_4$ at 100°C for 3 hr, and isolated as described in the text.

B = Fumaric acid

C = Maleic acid + Succinic acid.

Other carboxylic acids run to the positions shown in Plate 3.1.

Table 3.5. Changes observed upon treatment of glycopeptide I from hen ovalbumin with alkaline borohydride.

(all values expressed as moles per mole Asp at zero time)

	Analysis of acid hydrolysate before reaction with alkaline borohydride	Changes in components after treatment under the following conditions, followed by acid hydrolysis[a]			
		0.05M-NaOH[b] (13 1/3 hr)	0.2M-NaOH[b] (20 hr)	1.0M-NaOH[b] (10.5 hr)	2.0M-NaOH[b] (3 hr)
Asp	1.00	- 0.57	- 0.54	- 0.37	- 0.19
Leu	0.96	- 0.14	- 0.11	- 0.05	- 0.04
Thr	0.82	- 0.37	- 0.41	- 0.42	- 0.39
Ser	0.96	- 0.12	- 0.23	- 0.29	- 0.21
Glu	0.14	0	+ 0.03	0	0
Gly	0.14	+ 0.19	+ 0.16	+ 0.19	+ 0.18
Ala	0.16	+ 0.06	+ 0.03	0	0
Val	0.16	0	0	0	0
GlcN	4.11	- 1.02	- 1.01	- 1.16	- 1.50
Man	5.24	- 0.42	- 0.23	- 0.76	- 0.33
2-Amino-n-butyric acid	0	+ 0.09	+ 0.12	+ 0.07	+ 0.04
Homoserine (+ lactone)	0	+ 0.05[c]	+ 0.14	+ 0.11	0
Glucosaminitol	0	+ 0.31	+ 0.39	+ 0.26	+ 0.12
Mannosaminitol	0	0	+ 0.11	+ 0.12	+ 0.10
Total nitrogen	9.94				

(a) Acid conditions for hydrolysis were 4M-HCl at 100°C for 18 hr.

(b) All solutions contained in addition 1M-$NaBH_4$ at the beginning of the reaction. Further amounts of borohydride were added at intervals according to the schedule shown in Table 3.16.

(c) The amount of homoserine lactone was not measured in this experiment because it co-chromatographed with ammonia under the conditions used.

Mrs. Doris Butterworth at the National Physical Library, Teddington, Middlesex, was C 34.51%; H 4.71%; N < 0.24%. The sample analysed for nitrogen was quite small and the value reported was stated to be a maximum figure. The nature of this carboxylic acid is unknown, but the yield is relatively high (~ 1mg from 8.4mg GlcNAc-Asn). It is not known whether it is a degradation product of the sugar or amino acid part of the original molecule.

Changes observed on treating glycopeptides with alkaline sodium borohydride.

When ovalbumin glycopeptides I and II (see page 257), differing in their D-mannose and D-glucosamine contents, were both examined at various concentrations of alkali in 1M-$NaBH_4$ at 100°C. The changes observed, expressed as moles per mole of L-aspartyl residue present at the start of the reaction, are shown as functions of time (Figs. 3.29-3.33), and the overall changes are also recorded (Tables 3.5 and 3.6). Analyses of amino acids, hexosamines and hexosaminitols were of course performed after acid hydrolysis.

Approximately 1 mole of D-glucosamine was lost, under milder conditions of alkaline borohydride, from glycopeptide I, rising to 1.5 moles in 2M-NaOH, 1M-$NaBH_4$, after 3 hr. A similar loss (1.4 moles) was recorded for glycopeptide II in 2M-NaOH, 1M-$NaBH_4$ after 10 hr. Under these strong conditions of alkali, an initial loss of D-glucosamine was followed by a slower loss as the reaction proceeded.

The maximum yield of D-glucosaminitol (0.39 mole per

Fig. 3.29. Changes observed on treating hen ovalbumin glycopeptide I with 1M-$NaBH_4$, 0.05M-NaOH at 100°C.

Components measured after acid hydrolysis (4M-HCl, 100°C, 18 hr) of material treated with alkaline borohydride.

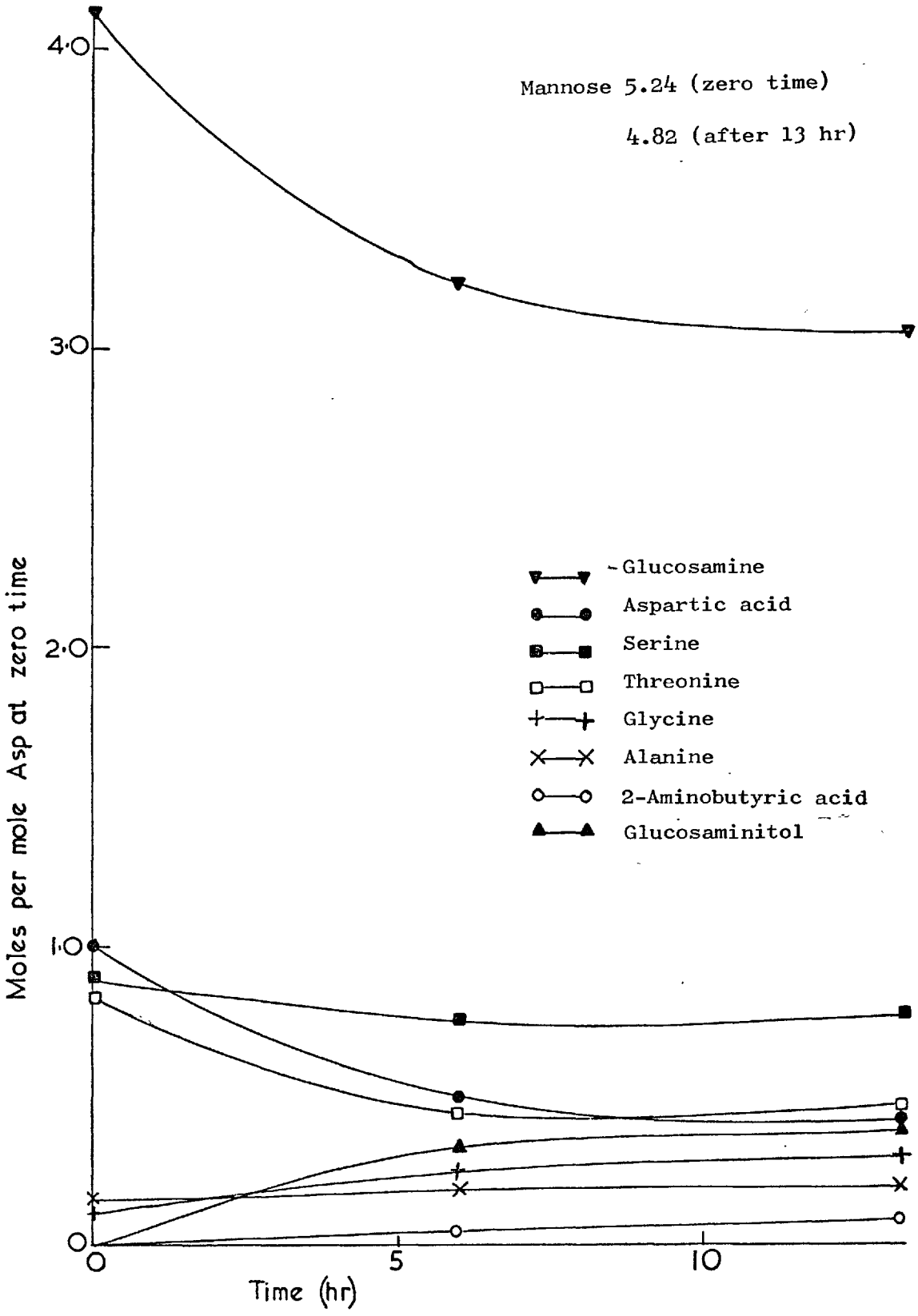


Fig. 3.30. Changes observed on treating hen ovalbumin glycopeptide I with 1M-$NaBH_4$ and 0.2M-NaOH at 100°C.

Components measured after acid hydrolyses (4M-HCl, 100°C, 18 hr) of material treated with alkaline borohydride.

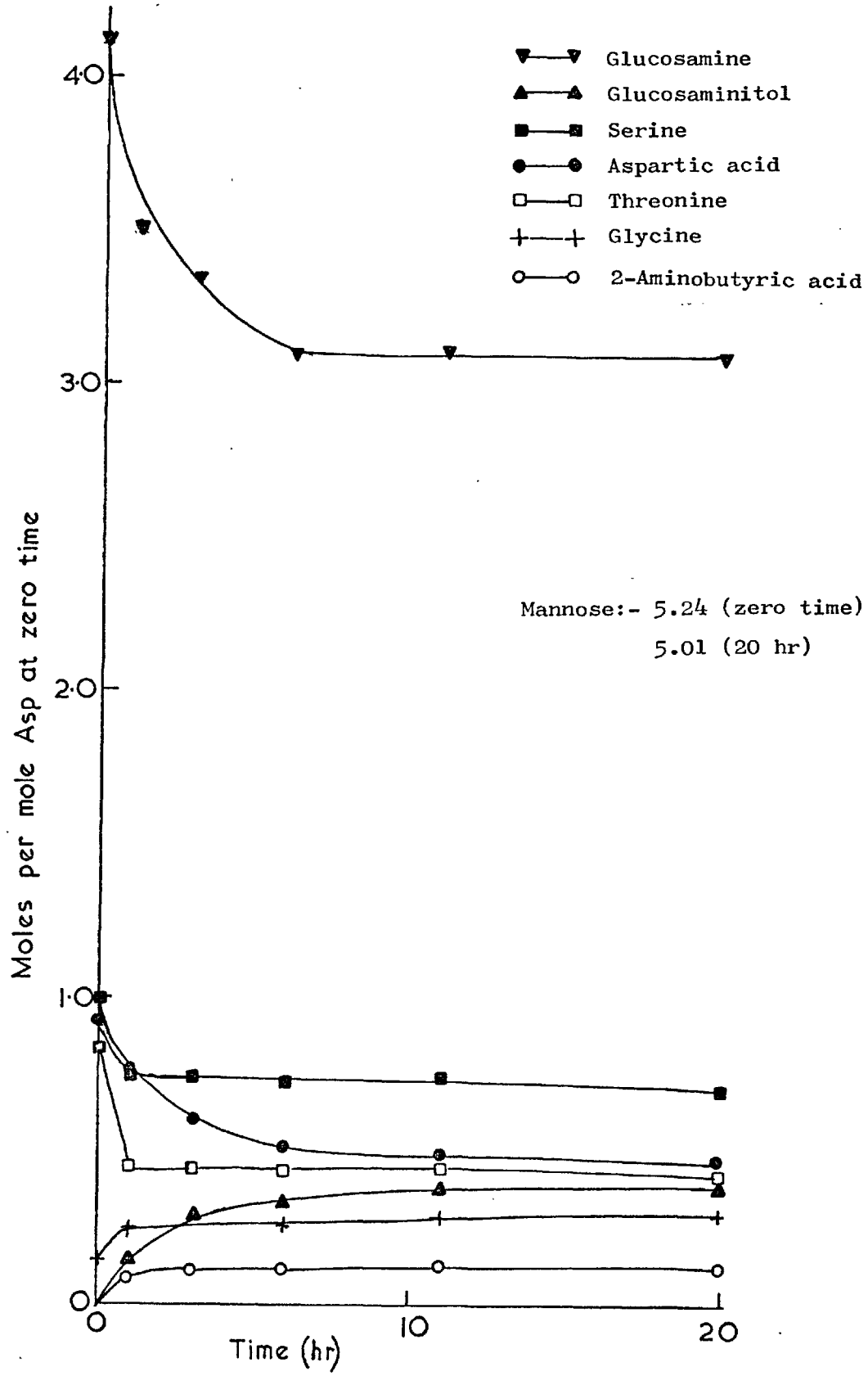


Fig 3.31. Changes observed on treating hen ovalbumin glycopeptide I with 1M-NaOH and 1M-$NaBH_4$ at 100°C for 11hr.

Components were measured after acid hydrolysis (4M-HCl; 18hr; 100°C) of material previously treated with alkaline borohydride.

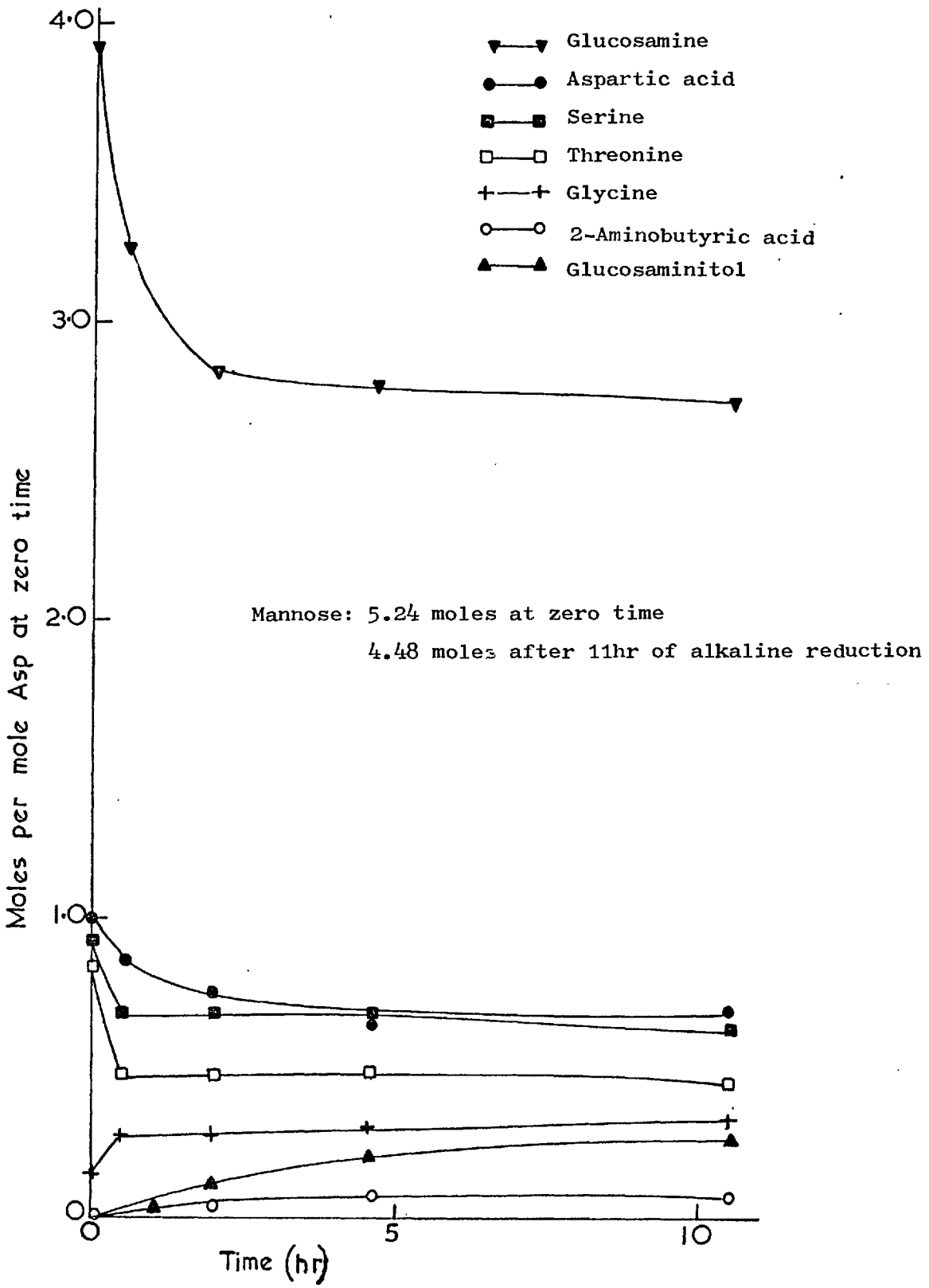


Fig. 3.32. Changes observed on treating hen ovalbumin glycopeptide I with 2M-NaOH and 1M-$NaBH_4$ at 100°C.

Components were measured after acid hydrolysis (4M-HCl; 18hr; 100°C) of materials previously treated with alkaline borohydride.

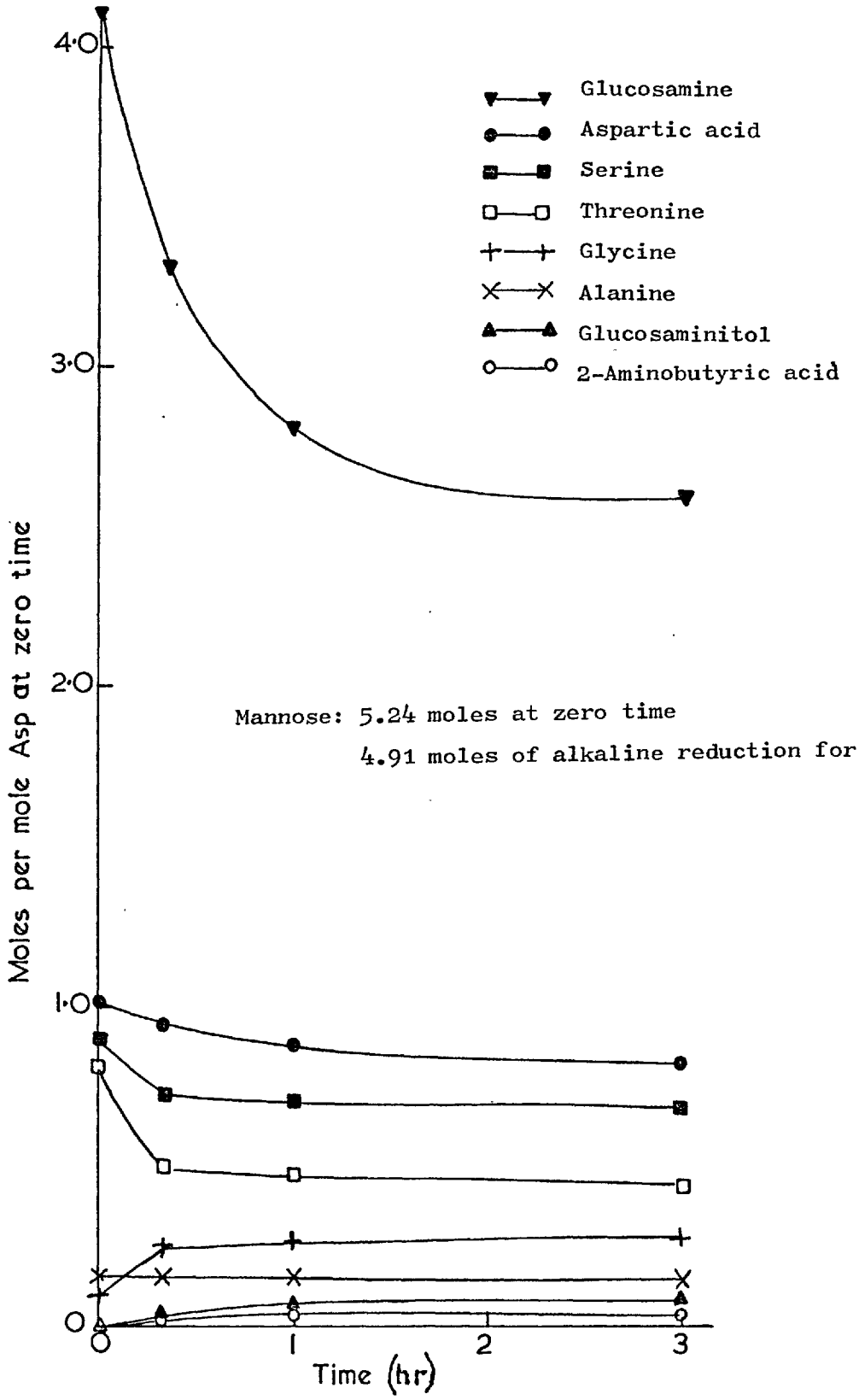


Fig. 3.33 Changes observed on treating hen ovalbumin glycopeptide II with 2M-NaOH and 1M-$NaBH_4$ at 100°C

Components were measured after acid hydrolysis (4M-HCl; 18hr; 100°C) of material that had previously been treated with alkaline borohydride.

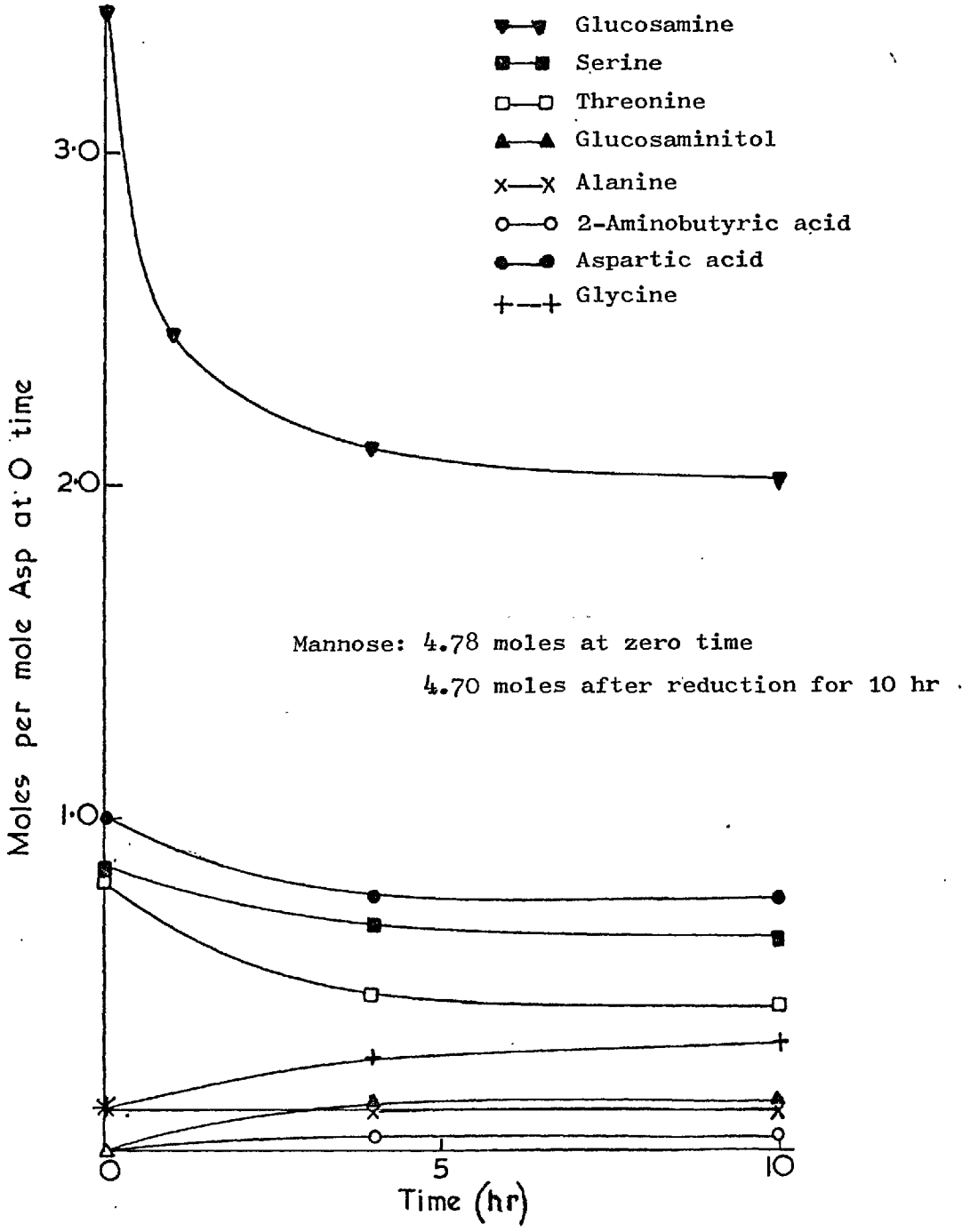


Table 3.6. Changes observed on alkaline reduction in 1M-$NaBH_4$, 2M-NaOH of glycopeptide II from hen ovalbumin.

	Analysis of acid hydrolysate before reaction with alkaline borohydride.	Change in the amounts of components after treatment for 10 hr followed by acid hydrolysis.
Asp	1.00	- 0.25
Leu	0.94	- 0.02
Thr	0.82	- 0.38
Ser	0.85	- 0.21
Glu	0.11	0
Gly	0.12	+ 0.19
Ala	0.12	0
Val	0.15	0
GlcN	3.42	- 1.38
Man	4.72	- 0.08
Total nitrogen	8.75	
2-amino-n-butyric acid	0	+ 0.04
Glucosaminitol		+ 0.16
Mannosaminitol		+ 0.08

mole of L-aspartyl residue originally present) was obtained in 0.2M-NaOH, 1M-$NaBH_4$ after about 11 hr of reaction (Fig. 3.30), but the yield compared unfavourably with that obtained from GlcNAc-Asn itself (0.69 mole per mole GlcNAc-Asn; Table 3.3) in 0.2M-NaOH, 1M-$NaBH_4$. D-Mannosaminitol was again produced with more concentrated alkaline conditions.

Fractional molar values of hexose were lost, as measured by the phenol-sulphuric acid reaction, from glycopeptide I during the reactions, but no definite relationship with the strength of alkali used could be ascertained. Practically no hexose was lost after reaction of glycopeptide II with 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr.

As in the case of treatment of GlcNAc-Asn with alkaline borohydride, losses of aspartic acid were observed, the extents of which were dependent on the concentration of alkali used (Table 3.5). The half-lives of the reactions resulting in destruction of aspartic acid in 0.2M-NaOH, 1M-$NaBH_4$, at 100°C for GlcNAc-Asn and for glycopeptide I may be compared. The values are 15 min (Fig. 3.20) and 60 min (Fig. 3.30) respectively. These values, combined with the observation that there is a greater absolute loss of aspartic acid when glycopeptide I is treated with alkali compared to that of GlcNAc-Asn, imply that the base catalysed cleavage of the linkage between the carbohydrate and the peptide chain is also slower for the glycopeptide than for GlcNAc-Asn.

Losses of serine and threonine were observed in the

Table 3.7. Composition of fractions obtained by gel filtration (see Fig. 3.34) of the products of the reaction of Glycopeptide I with alkaline borohydride.

Treatment	Pooled fractions	Molar Ratios[a]			Moles/mole glycopeptide I[a,d]		
		GlcN / Man	GlcNol / Man	Amino[b] / Man	GlcN	GlcNol	Amino[b]
0.2M-NaOH; 10.5 hr[f]	1 (25% Man)	0.758	0.053	0.098	3.80	0.27	0.49
	2 (51% Man)	0.581	0.064	0.180	2.91	0.32	0.90
	3 (24% Man	0.592	0.071	0.21	2.97	0.36	1.09
	Unfractionated[c]	0.617	0.081	n.d.	3.09	0.39	n.d.
2.0M-NaOH; 4 hr[f]	A (29% Man)	0.467	0.023	0.423	2.29	0.11	2.10
	B (71% Man)	0.606	0.023	0.570	2.98	0.11	2.80
	Unfractionated[e]	0.532	0.024	n.d.	2.61	0.12	n.d.

(a) Since deacetylation of glucosamine residues may have occurred in alkali, the question arises as to whether acid hydrolyses (4M-HCl; 100°C; 18 hr) will result in complete liberation of the sugars. This problem is considered in the discussion (page 237).

(b) The amount of free amino group was assessed from the colorimetric reaction with ninhydrin, with D-glucosamine as a standard.

(c) These data are for glycopeptide that was treated for 20 hr under the same conditions (column 4, Table 3.5).

(d) This is based on a value of 5.01 moles of mannose for the experiment carried out in 0.2M-NaOH, to take account of the loss of 0.23 moles during treatment of glycopeptide I with alkaline borohydride. It is based on 4.91 moles of mannose in the experiment carried out in 2M-NaOH for similar reasons.

(e) These data are for glycopeptide that was treated for 3 hr under the same conditions (column 6, Table 3.5).

(f) The alkaline solutions contained 1M-$NaBH_4$ and were heated at 100°C.

initial stages of the reactions involving treatment with alkaline borohydride. These losses probably occur only from those residues which have both their carboxyl and amino groups involved in peptide linkage (see page 45). Simultaneous increases in the values for glycine, alanine and ∝-amino-butyric acid were also observed, together with small amounts of homoserine and its lactone.

Gel chromatography of glycopeptides that had been treated with alkaline borohydride.

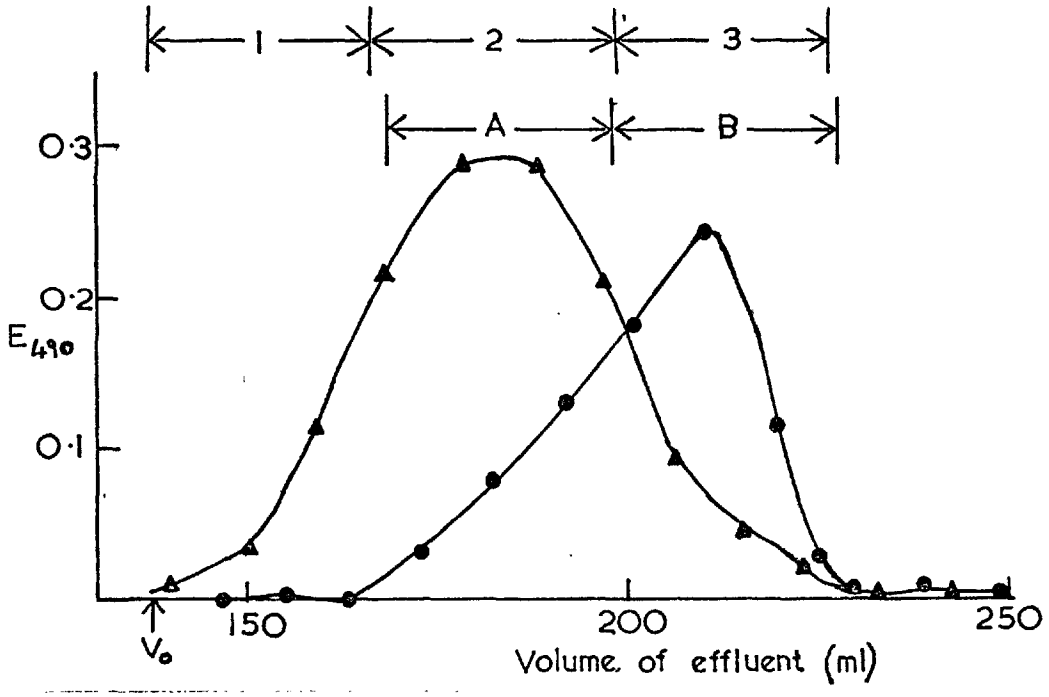
One broad peak containing hexose was obtained from gel chromatography of glycopeptide I, after reduction with 1M-$NaBH_4$ at 100°C in the presence of either 0.2M-NaOH or 2M-NaOH, the most concentrated fractions eluting at 210ml and 185ml respectively (Fig. 3.34), giving yields of hexose of 82% and 84% respectively from the amounts of starting materials used.

The fractions of effluent collected from the initial, middle and final portions of the peak containing hexose (Fig. 3.34), obtained by gel chromatography of glycopeptide I after reduction in 0.2M-NaOH, contained different ratios of D-glucosamine and D-glucosaminitol to hexose (Table 3.7), showing that the material containing hexose was heterogeneous and that fractionation on G-25 had occurred by factors involving differences in composition.

The ninhydrin reaction gave increased colour per mole of hexose for the more retarded fractions, suggesting that free amino groups were present, and that these might be factors causing fractionation. There was an overall molar ratio of 0.28 residue of free amino group per mole of D-glucosamine in the glycopeptide after it had been

Fig. 3.34. Gel chromatography of hen ovalbumin glycopeptide I that had been treated with alkaline borohydride at 100°C.

Chromatography on Sephadex G-25 (two columns each 120 x 1.2 cm) in 0.1M-acetic acid at 4°C. Columns were run in series.



Assay: Phenol-H_2SO_4 reaction on 0.2ml of each fraction

Fraction size: 9.2ml

Flow rate: 18.6ml per hr

Eluting agent: 0.1M-acetic acid.

▲—▲ Elution profile of glycopeptide I (45 µmoles mannose) previously treated with 0.2M-NaOH; 1M-$NaBH_4$ at 100°C for 11hr. Fractions were pooled(1,2 & 3)

●—● Elution profile of glycopeptide I (34 µmoles mannose) previously treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 4 hr. Fractions were pooled (A & B)

reduced with 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for 10.5 hr. A larger extent of deacetylation (0.92 moles per mole of D-glucosamine) was found in glycopeptide which had been reduced in 2M-NaOH, 1M-$NaBH_4$ at 100°C. These values do not of course include D-glucosamine residues originally present in glycopeptide I, but which had been converted to other products including D-glucosaminitol and N-acetyl-D-glucosaminitol.

The overall ratios of D-glucosamine to hexose are about the same before and after gel filtration, for given conditions of alkaline borohydride treatment of glycopeptide I. The values obtained in the reduction carried out in 0.2M-NaOH are 0.628 and 0.601, before and after filtration, respectively. In 2.0M-NaOH the values are 0.565 and 0.532 respectively. These results show that little, if any, sugar has been separated from the main fraction by gel filtration.

Only trace amounts of amino acids (less than 0.1 mole of any amino acid per 5 moles of hexose) were detected in Fractions A & B (Fig 3.34) showing that cleavage of peptide from oligosaccharide was complete and that separation of the free peptides and amino acids from the material containing hexose had been achieved by gel chromatography. The colour produced in the ninhydrin reaction is therefore probably due to N-deacetylated D-glucosamine residues, or just possibly products of them.

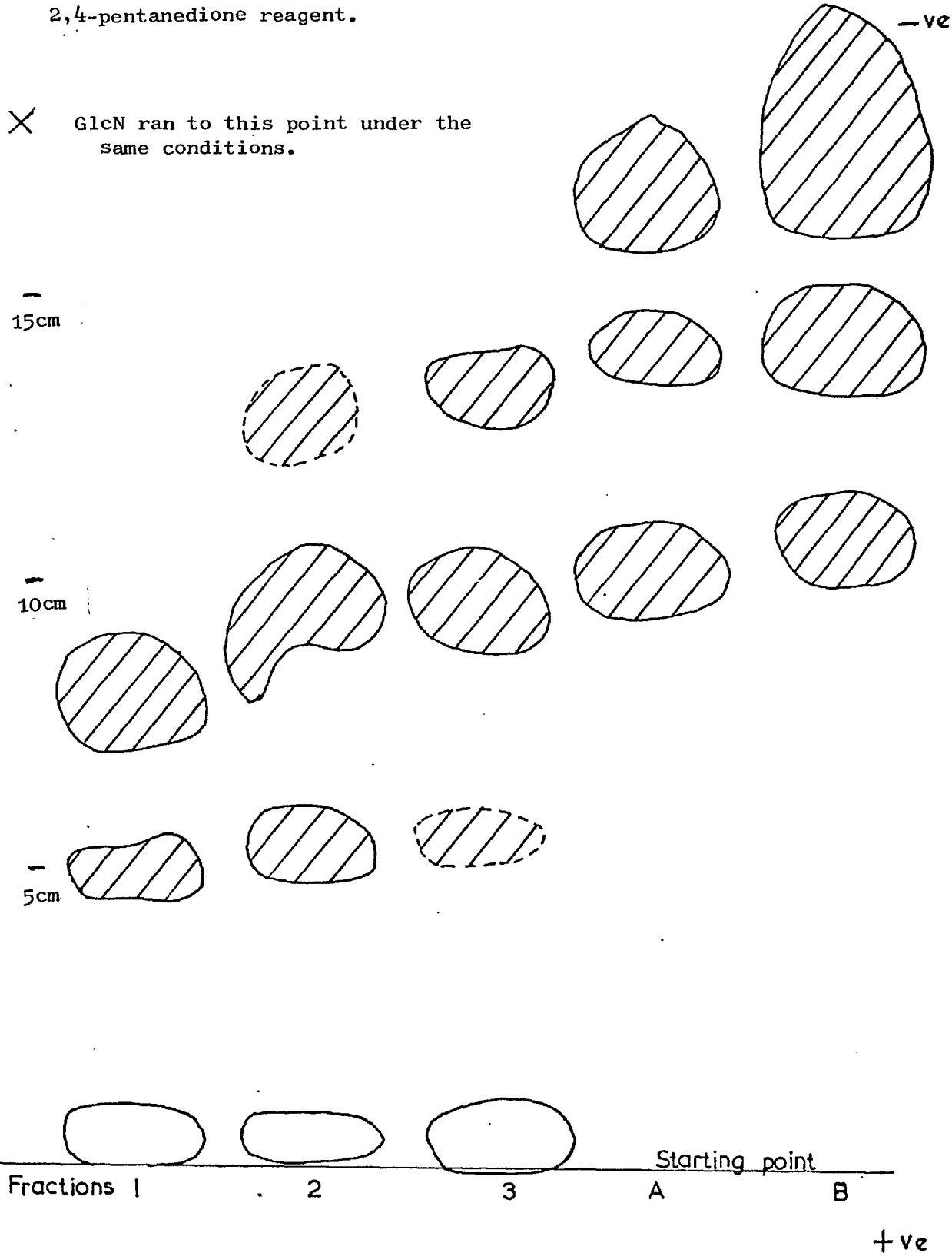
Heterogeneity of the products was further demonstrated by paper electrophoresis (Fig. 3.35). D-Glucosamine migrated 18cm under the same conditions. The periodate-

Fig. 3.35. Paper electrophoresis of reduced oligosaccharides.

Fractions 1, 2, and 3 were obtained by pooling fractions obtained by gel chromatography (Fig. 3.34) of glycopeptide I which had been treated with 1M-$NaBH_4$;0.2M-NaOH at 100°C for 11 hr. Fractions A and B were obtained similarly (Fig. 3.34) from glycopeptide I which had been treated with 1M-$NaBH_4$;2M-NaOH at 100°C for 4 hr.

Electrophoretic conditions: pH 2.0; 32v/cm; 30 min.

Shaded spots /// stained with both the periodate / 2,4-pentanedione reagent and the ninhydrin reagent used on a duplicate chromatogram. Spots not shaded developed only with the periodate / 2,4-pentanedione reagent.



2,4-pentane dione reagent produced spots which fluoresced strongly under ultra-violet light. This reagent forms fluorescent products with formaldehyde produced as a result of periodate oxidation.

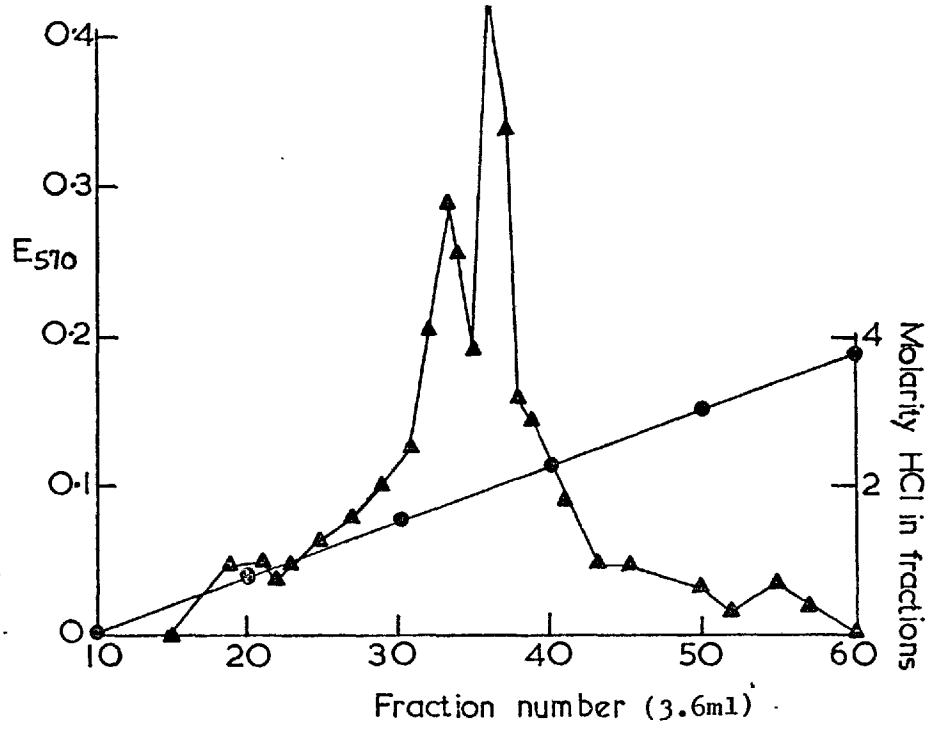
The spots that moved away from the starting line stained also with the ninhydrin reagent. The mobilities of these components under the conditions used (pH 2.0) are likely to depend in part on the total number of free amino groups present in the molecule. The products from reduction in 2.0M-NaOH included substances which migrated further than those produced from reduction in 0.2M-NaOH, in addition to ones which migrated to a similar extent. Furthermore, the more retarded fractions, which contained hexose and which were obtained by gel chromatography, contained components with higher mobility on paper electrophoresis. These results agree with those discussed earlier, in which it was demonstrated that the more retarded fractions had the larger amounts of free amino groups (Table 3.7) as determined from quantitative ninhydrin estimations. The components staining only with the periodate-2,4-pentanedione reagent and which remain close to the starting line are probably fully N-acetylated products. These substances occur among the products only when the alkali concentrations used is low.

Heterogeneity of the pooled fraction B (Fig. 3.34) could also be demonstrated on Dowex 50 by elution with increasing concentrations of HCl. Two major ninhydrin-positive peaks that also contained hexose, were obtained by elution of the material, at an acid molarity of about 2 (Fig. 3.36).

Fig. 3.36. Elution profile of reduced oligosaccharide on Dowex 50 X 4 (H^+)

Oligosaccharide was isolated by gel-chromatography of hen ovalbumin glycopeptide I that had been treated with 2M-NaOH and 1M-$NaBH_4$ for 4hr

Column: 64 x 1 cm. Resin suspended in water at 4°C.
Eluted by water (100ml) and 4M-HCl (100ml) arranged to give a linear gradient in HCl.

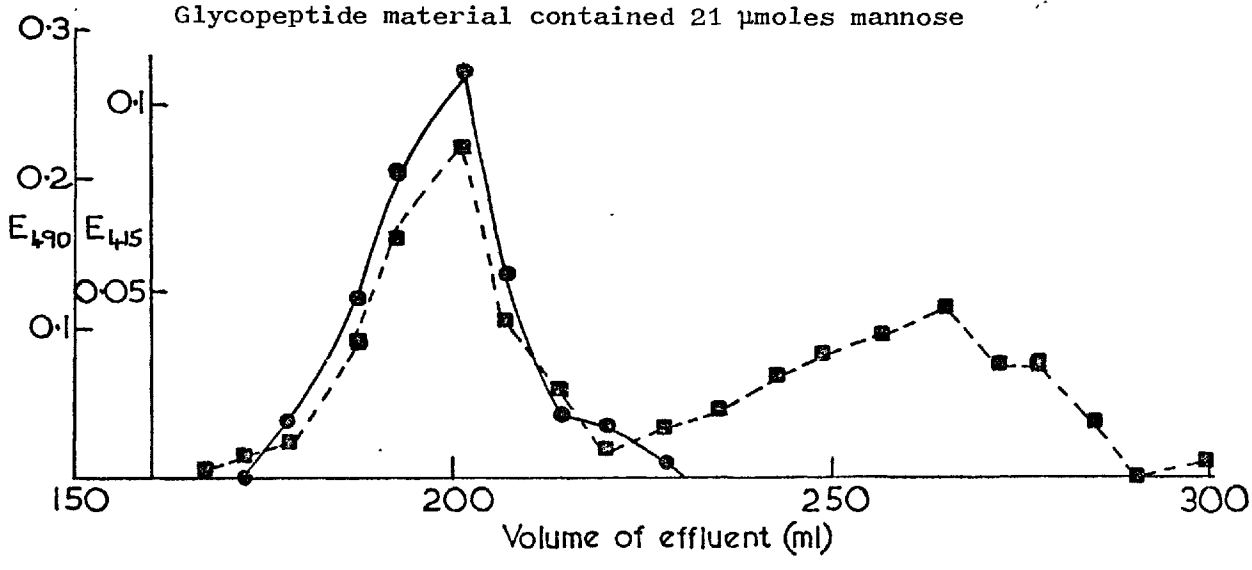


▲—▲ Ninhydrin reaction on 0.5ml of each fraction

●—● Molarity of HCl in effluent

Fig. 3.37. Gel chromatography of hen ovalbumin glycopeptide II that had been treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 10hr.

Chromatographic conditions are those described in Fig. 3.34.
Glycopeptide material contained 21 µmoles mannose



■—■ Periodate / 2,4-pentanedione reaction on 0.5ml.

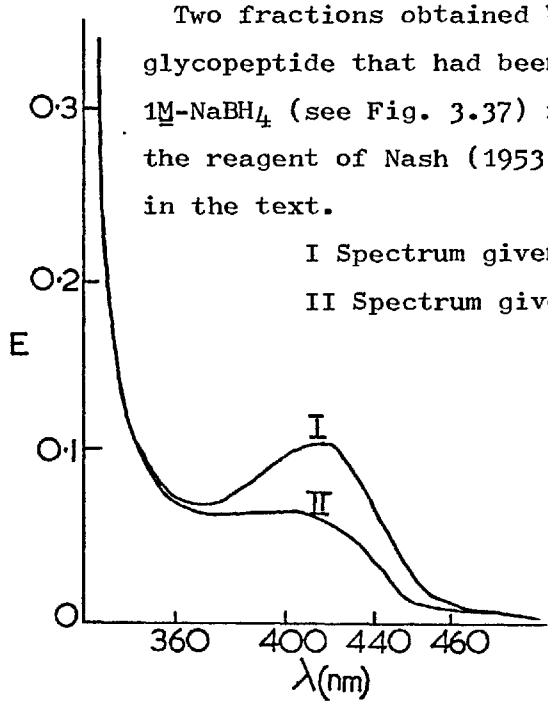
●—● Phenol-H_2SO_4 reaction on 0.3ml

The elution profile obtained by gel chromatography of the material produced by treatment of glycopeptide II with 1M-$NaBH_4$ and 2M-NaOH at 100°C for 10 hr is shown in Fig. 3.37. Fractions were assayed by the phenol-H_2SO_4 reagent and by periodate oxidation followed by treatment with 2,4-pentanedione. The 2,4-pentanedione reagent produced colour in the more retarded fractions that did not contain hexose (V_e 230-290ml; Fig. 3.37). The absorption spectrum (λ_{max} 405nm) of the coloured product formed in these fractions was sufficiently different from that (λ_{max} 413nm) formed in the fractions (V_e 170-220ml) that also contained hexose (Fig. 3.38) to suggest that acetaldehyde, as well as formaldehyde, was formed by periodate-oxidation of the material in more retarded fractions (V_e 230-290ml) (Nash, 1953).

The fractions produced by gel chromatography of material that was obtained by alkaline reduction of glycopeptide II, followed by re-N-acetylation (Fig. 3.39), were also assayed by the phenol-H_2SO_4 and periodate/2,4-pentanedione reagents. The colour produced in the more retarded fractions (V_e 220-270ml) by 2,4-pentanedione was much reduced compared to that shown in Fig. 3.37 and the substance(s) giving rise to this colour could be removed entirely by treatment with Dowex 50 (H^+). The presence of D-mannitol in these fractions could thus be excluded, and it seems reasonable to suggest that periodate-oxidation of free serine and threonine gave rise to chromophores. Autoanalysis of the fractions(V_e 230-290ml; Fig. 3.37) showed that these free amino acids were present.

To assess the extent to which N-deacetylation

Fig. 3.38. Ultra-violet spectra of products of reaction between periodate / 2,4-pentanedione reagents and products formed by alkaline reduction of hen ovalbumin glycopeptide II



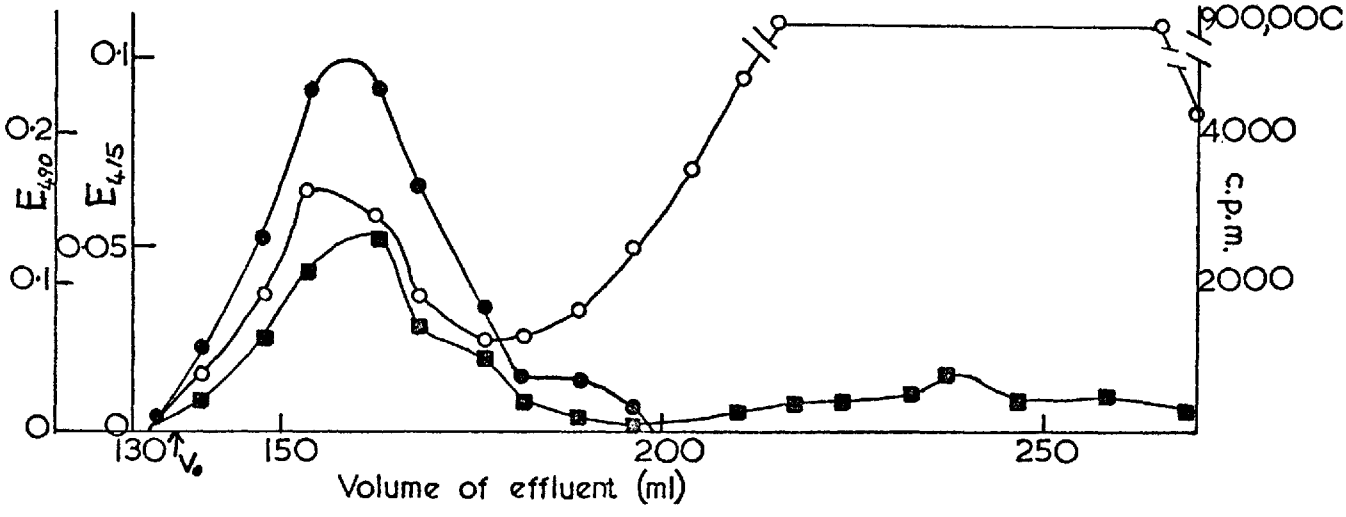
Two fractions obtained by gel chromatography of glycopeptide that had been treated with 2M-NaOH and 1M-$NaBH_4$ (see Fig. 3.37) reacted with periodate and the reagent of Nash (1953). Conditions are described in the text.

I Spectrum given by Fraction 22 (V_e=200ml)

II Spectrum given by Fraction 29 (V_e=267ml)

Fig. 3.39. Gel chromatography of hen ovalbumin glycopeptide II that had been treated with alkaline borohydride and re-N-acetylated.

Glycopeptide II (18 μmoles mannose) was treated with 2M-NaOH and 1M-$NaBH_4$ for 10hr at 100°C. The products were re-N-acetylated with [^{3}H]acetic anhydride and subjected to chromatography under the conditions described in Fig 3.34.



●—● Phenol-H_2SO_4 reaction on 0.3ml.

○—○ C.p.m. on 0.05ml + Bray's(1965) reagent.

■—■ Periodate / 2,4-pentanedione reagent on 0.5ml.

Table 3.8. Analysis of products formed as a result of gel chromatography of products of alkaline reduction of glycopeptides

Analysis of samples of glycopeptides I and II after treatment with 1M-$NaBH_4$ in either 0.2M-NaOH or 2M-NaOH at 100°C. In some cases the products were divided in to equal parts, one of which was N-acetylated. Each of the materials were separately filtered on Sephadex G-25 in 0.1M- acetic acid and the fractions containing hexose were pooled and analysed. Free NH_2 was determined by ninhydrin reaction, mannose by phenol reaction, and other components after acid hydrolysis (4M-HCl; 100°C; 18 hr.)

Substrate and conditions[a]	Molar ratios			Moles of product per mole of reduced oligosaccharide[b]			
	GlcN / Man	Glucosaminitol / Man	Free NH_2 / Man	GlcN	Glucosaminitol	Free NH_2	Mannosaminitol
Glycopeptide I 2M-NaOH; 10 hr.	0.493	0.025	0.507	2.42	0.14	2.49	0.09
Glycopeptide II 2M-NaOH; 10 hr.	0.418	0.03	0.379	1.96	0.14	1.78	0.08
Glycopeptide II[c] 2M-NaOH; 10 hr.	0.476	0.069	0.025	2.24	0.32	0.11	0.20
Glycopeptide II 0.2M-NaOH; 11hr.	0.465	0.060	0.138	2.18	0.28	0.65	0
Glycopeptide II[c] 0.2M-NaOH; 11 hr.	0.488	0.093	0	2.29	0.44	0	0
Glycopeptide II[d] 0.2M-NaOH; 11 hr.	0.326	0.02	0.495	1.53	0.09	2.32	0

Continued opposite page 201.

might make it difficult to release sugars by acid hydrolysis, measurements of the various products were made on acid hydrolysates of the substances both before and after N-acetylation. The results are summarised in Table 3.8, but discussion of these results will be deferred until later (see page 237).

Paper electrophoresis (pH 2, 30 min, 34 V/cm) was carried out on the substances obtained when glycopeptides I and II were treated with 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr and subsequently filtered on Sephadex G-25 (see items 1 and 2 of Table 3.8). On staining with the periodate-2,4-pentane dione reagent, the ultra-violet fluorescing bands (illustrated in Plate 3.4) became apparent. The proportions of products in the two cases are different, and this indicates that the material separated from the products of reaction of glycopeptide I contains larger numbers of deacetylated D-glucosamine residues. The original glycopeptides I and II are therefore different in the contents or structures, or both, of their carbohydrate moieties.

Gel-chromatography of N-acetylated glycopeptide previously subjected to alkaline reduction, and determinations of the apparent molecular weights of the products.

The elution profile of glycopeptide II reduced in 2M-NaOH, 1M-$NaBH_4$ for 10 hr at 100°C, and then re-N-acetylated is shown in Fig. 3.39. A shift after N-acetylation was observed. The elution volume had decreased by 45ml, which is equivalent to 33% of the void volume of the column. This value is much greater than that expected for the increase in molecular weight result-

Table 3.8. continued

(a) All reactions were carried out at 100°C in the presence of 1M-$NaBH_4$.

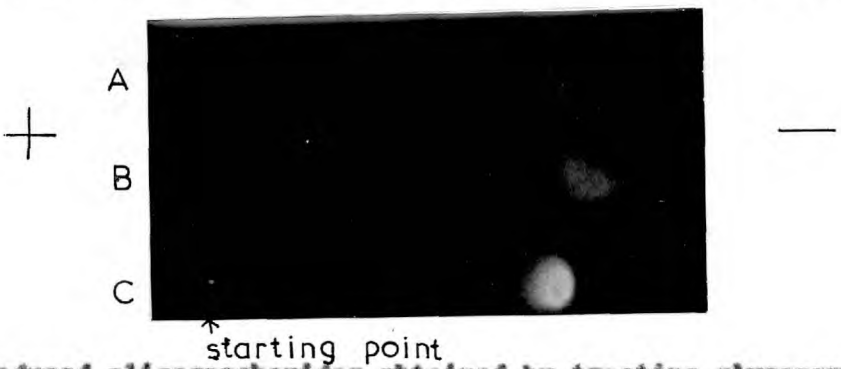
(b) Calculated on the basis of 4.91 moles of mannose for the reduced oligosaccharide from glycopeptide I, and 4.70 moles of mannose for that from glycopeptide II.

(c) This material was re-N-acetylated before gel chromatography.

(d) Glycopeptide II in this experiment was treated with 1M-$NaBH_4$ in 0.2M-NaOH for 11 hr , followed by 2M-NaOH with no further addition of borohydride, for 11 hr at 100°C.

Plate 3.4. Paper electrophoresis of reduced oligosaccharides.

Electrophoresis was performed at pH 2 and 32 V/cm for 30 min.



A = Reduced oligosaccharides obtained by treating glycopeptide I with 2M-NaOH ; 1M-$NaBH_4$ at 100°C for 10 hr.

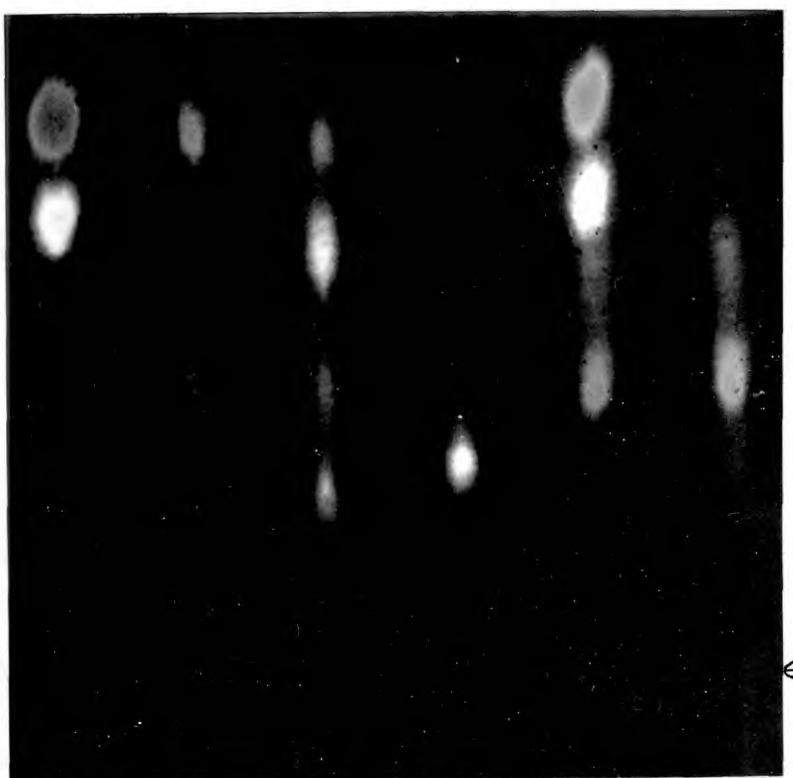
B = Reduced oligosaccharides obtained by treating glycopeptide II with 2M-NaOH ; 1M-$NaBH_4$ at 100°C for 10 hr.

C = Glucosamine

Spots were located with the periodate/2,4-pentanedione reagent

Plate 3.5. Paper chromatography of sugars obtained from re-N-acetylated oligosaccharides by hydrolysis in 2M-HCl at 100°C for 3 hr

Development by ethyl acetate-pyridine-water (120 : 50 : 40 by vol) for 14 hr.



A = Mannose

B = Mannitol

C= Acid hydrolysate of reduced oligosaccharide (see text for conditions)

D = Glucosaminitol

E = Glucosamine

Amounts of 50µg of standard sugars were also placed on the chromatogram.

Components were located with the periodate/2,4-pentanedione reagent.

ing from N-acetylation.

The elution profiles from the G-25 column of sugars of known molecular weight are shown in Fig. 3.40. The calibration curve for these compounds as well as for other substances suggested (Fig. 3.41) values for the molecular weights of reduced saccharides derived from glycopeptide II, as a result of reduction (1M-$NaBH_4$) in 2.0M-NaOH, or 0.2M-NaOH, followed by N-acetylation, of 1460 and 1480 respectively. A compound containing 3.42 moles of N-acetyl-D-glucosamine and 4.72 moles of D-mannose residues (see Table 3.6 for the sugar contents of glycopeptide II) would be expected to have a molecular weight of 1469. It was noted that the original glycopeptide II containing several amino acids (marked II in Fig. 3.41) did not fall on the calibration curve constructed from the results obtained with the sugars. Its large molecular weight (1890) might be expected to result in its exclusion from the G-25 column if it did behave as a pure sugar derivative. In fact its elution volume was 21ml greater than that of blue dextran.

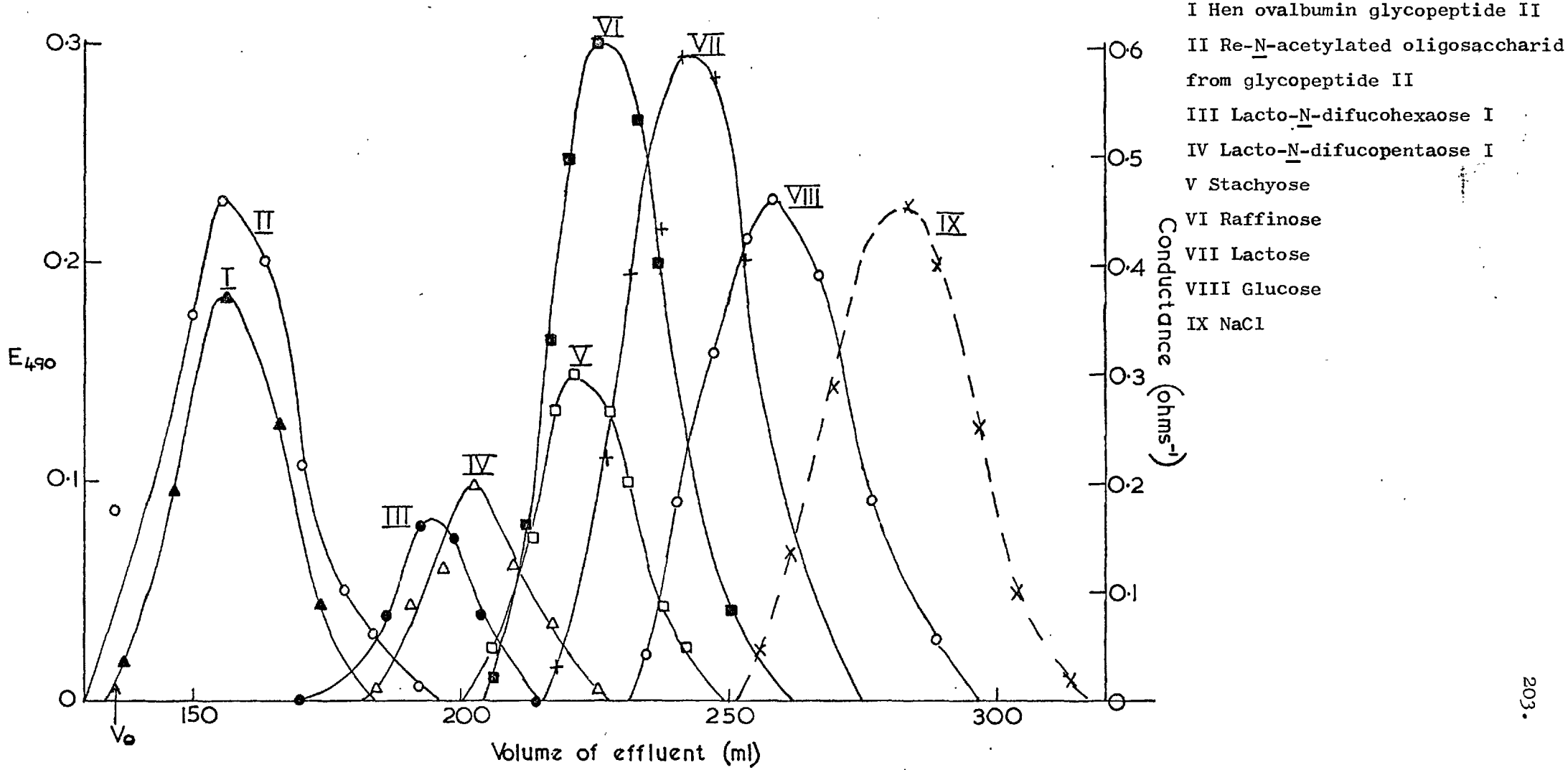
Peptides also behaved anomalously with respect to the calibration curve established with oligosaccharides. Glutathione was retarded to some extent, and oxytocin very considerably (Fig. 3.41).

The use of either 0.15M-NaCl or 0.1M acetic acid as eluting agent made no difference to the elution volumes for D-glucose, lactose, lacto-N-difucohexaose or glycopeptide II. A change of solvent altered the elution

Fig. 3.40. Separation of materials of known molecular weight and reduced oligosaccharides from hen ovalbumin glycopeptide on Sephadex G-25.

Chromatographic conditions are described in Fig. 3.34.

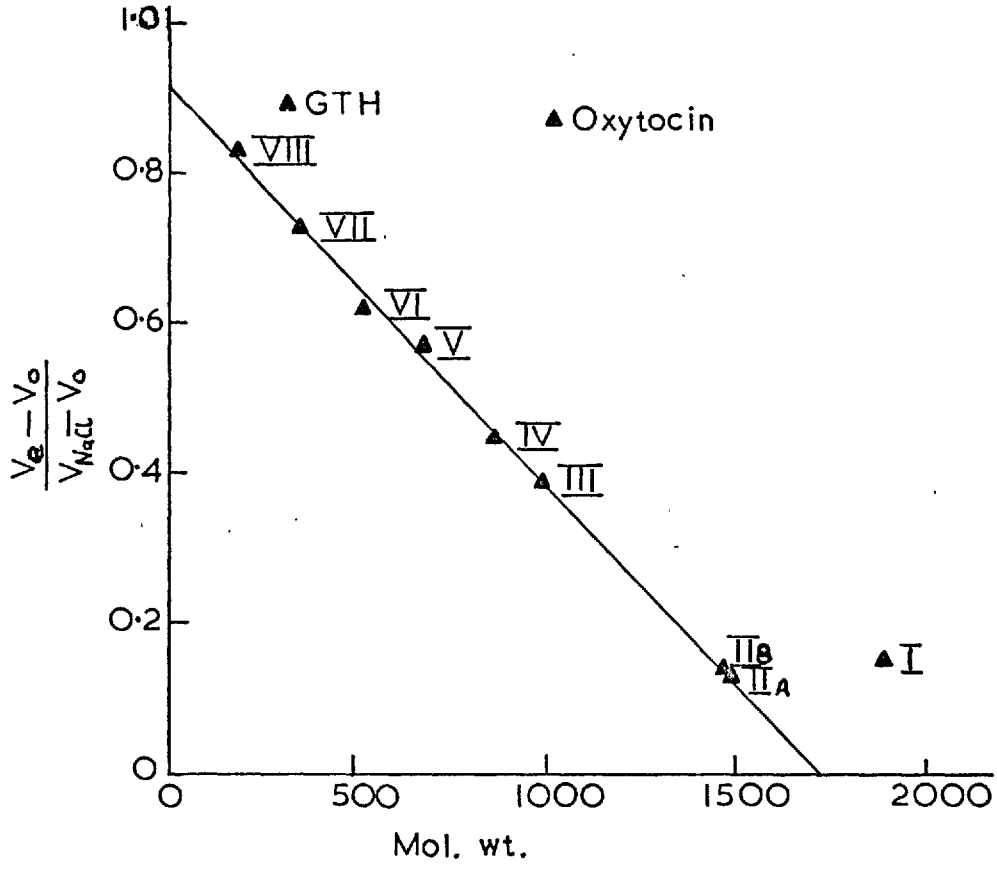
Materials (<1mg) were dissolved in 0.1M-acetic acid (0.5ml) and subjected to chromatography.



Fractions containing carbohydrate were assayed with the phenol-H_2SO_4 reagent. Salt was assayed conductometrically.

Fig. 3.41. Calibration of Sephadex G-25 with materials of known molecular weight.

Elution profiles are shown in Fig. 3.40, and chromatographic conditions are described in Fig. 3.34.



I Hen ovalbumin glycopeptide II

IIA Re-N-acetylated reduced oligosaccharide prepared from glycopeptide II by treatment with 2M-NaOH and 1M-$NaBH_4$ at 100°C for 10hr.

IIB Re-N-acetylated reduced oligosaccharide prepared from glycopeptide II by treatment with 0.2M-NaOH and 1M-$NaBH_4$ at 100°C for 11hr.

III Lacto-N-difucohexaose I.

IV Lacto-N-difucopentaose I.

V Stachyose

VI Raffinose

VII Lactose

VIII Glucose

Table 3.9. Assay of free amino groups contained in reduced oligosaccharides.

The products were separated by gel chromatography of glycopeptides that had previously been reduced with alkaline borohydride.

	Conditions of alkaline borohydride	Ninhydrin value (moles) per mole of reduced oligosaccharide	Number of N-[^{3}H]acetyl groups introduced per mole of reduced oligosaccharide	c.p.m. (per mole of GlcN[(a)]) resulting from N-[^{3}H]acetylation
Glycopeptide II	2M-NaOH; 1M-$NaBH_4$; 100°C; 10 hr	1.78	2.53	224×10^{6}[(b)]
Glycopeptide II	0.2M-NaOH; 1M-$NaBH_4$; 100°C; 11 hr	0.65	0.71	65.3×10^{6}[(b)]

(a) Measured after acid hydrolysis (4M-HCl; 100°C; 18 hr) of the reduced oligosaccharide.

(b) The mean value of the c.p.m. measured on seven samples.

volume exhibited by the major product of subjecting glycopeptide II to borohydride reduction (1M-$NaBH_4$) in 2M-NaOH at 100°C for 10 hr. In 0.15M-NaCl it was 180ml and in 0.1M acetic acid it increased to 203ml.

Extent of N-deacetylation.

Free amino groups in material separated by gel chromatography were measured by the ninhydrin reaction, with D-glucosamine as standard, and also by the uptake of radioactive N-acetyl groups (Table 3.9).

The ninhydrin assay suggested that N-deacetylation was almost complete after 4 hr in 2M-NaOH, 1M-$NaBH_4$, as there was little increase in the value measured after 10 hr. From measurements of radioactive uptake of [^{3}H]acetyl groups, somewhat higher values for the number of free amino groups per molecule, than those found by the ninhydrin assay, were obtained. The values were almost equal to the number of D-glucosamine and D-glucosaminitol residues (cf. Table 3.9 with item 3 of Table 3.8). The values obtained by the radioisotope method are likely to be more accurate than those found colorimetrically.

Infrared spectra.

The spectrum of the intact glycopeptide material is shown (Fig. 3.42). The bands at wavelengths of 1650cm^{-1} and 1550cm^{-1} may be assigned to amide I and II bands (Otting, 1958). These bands are not present at all in the material isolated after alkaline reduction in 2M-NaOH (Fig. 3.43), and are much decreased in intensity relative to the band at 1050cm^{-1}, assigned to the C-O stretching

Fig. 3.42. Infrared spectra of glycopeptide II.
c 1mg in 100mg KBr.

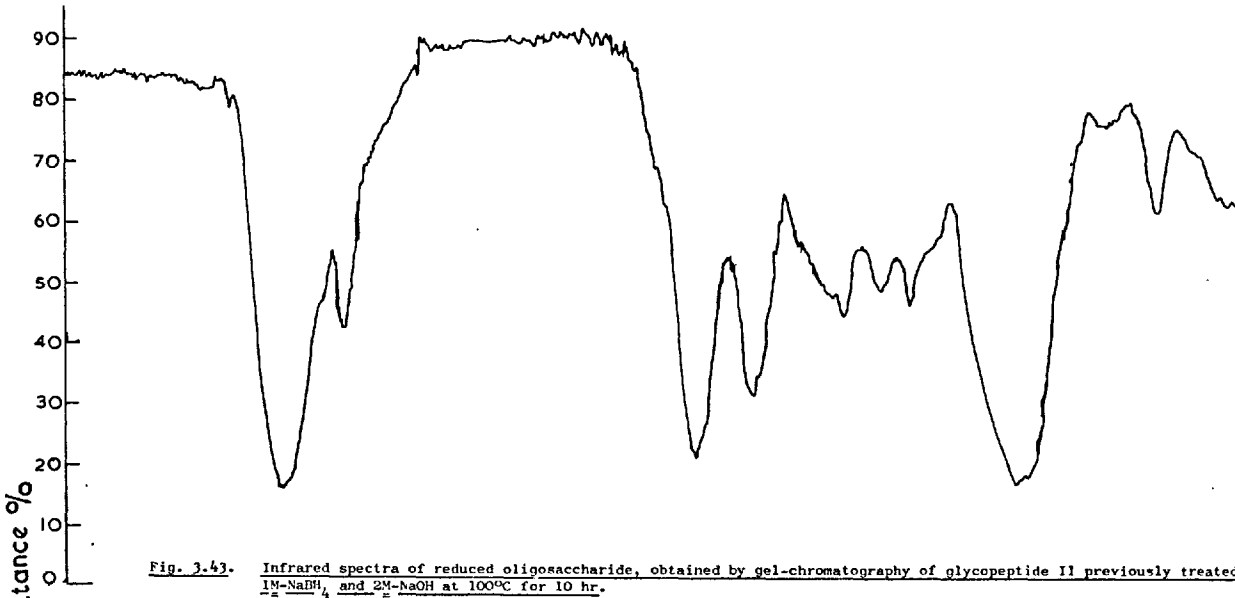
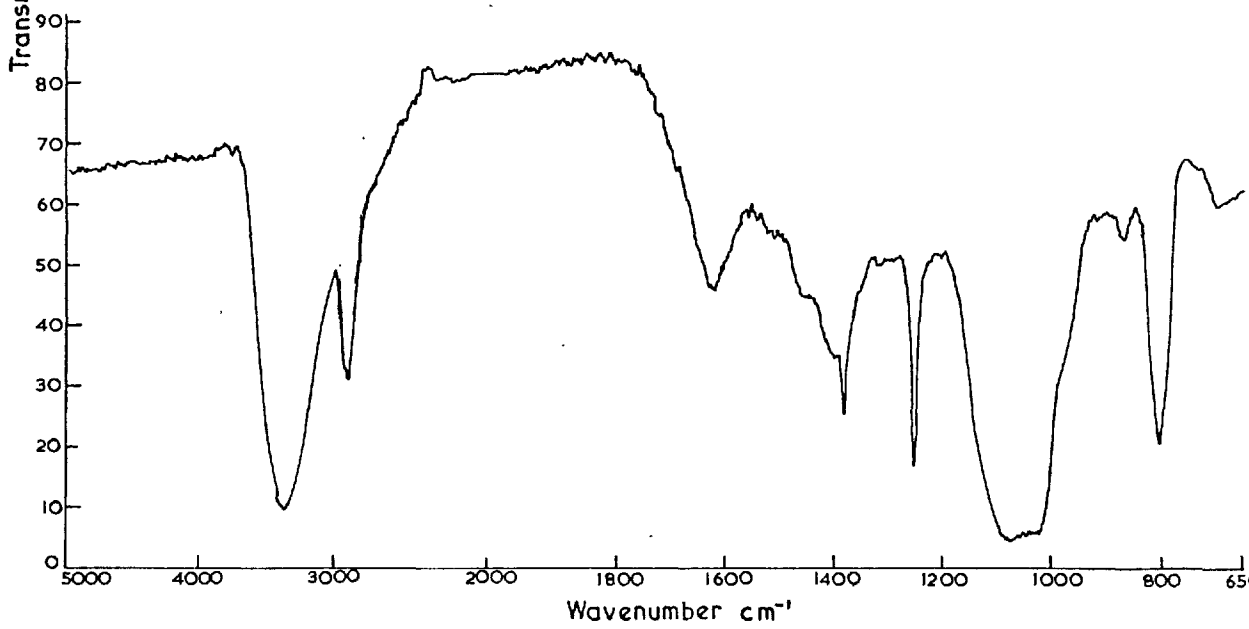


Fig. 3.43. Infrared spectra of reduced oligosaccharide, obtained by gel-chromatography of glycopeptide II previously treated with 1M-$NaBH_4$ and 2M-NaOH at 100°C for 10 hr.
c material containing 720µg mannose in 100mg KBr.



frequencies (Otting, 1958), in the material isolated from glycopeptides reduced in 0.2M-NaOH (Fig. 3.44). A weak band at 1625cm^{-1}, given by material obtained by reduction in 2M-NaOH, may be assigned to N-H deformations of primary amino groups (Fig. 3.43). Methyl-β-D-glucosaminide, not unexpectedly, shows a band in the same region (1607cm^{-1}) and this may be attributed to the amino group (Fig. 3.45). Bands at 810cm^{-1} and 1265cm^{-1} were intensified after reduction with borohydride in 2M-NaOH, and are at present unassigned.

No evidence of unsaturation was obtained from the infrared spectrum. Moreover, in support of this, no decolourisation of alkaline potassium permanganate or bromine water was detected.

Identification of components by paper chromatography.

Paper chromatograms of acid hydrolysates of material obtained by N-acetylation of glycopeptide, previously treated with 2M-NaOH, 1M-$NaBH_4$ for 10 hr, are shown in Plate 3.5. Photographs were taken under ultraviolet light after visualisation with the periodate-2,4-pentane diona reagent. The only components identified were D-mannose, D-glucosamine and D-glucosaminitol. No D-mannitol was detected.

Identification of a further ninhydrin-positive component in acid hydrolysates of glycopeptide after alkaline reduction.

An unidentified peak was found during autoanalysis of acid hydrolysates (4M-HCl; 100°C; 18 hr) of glycopeptides after alkaline reduction. It eluted after 5 hr 20 min,

Fig. 3.44. Infrared spectra of reduced oligosaccharide, obtained by gel-chromatography of glycopeptide II previously treated with 1M-$NaBH_4$ and 0.2M-NaOH at 100°C for 11 hr.
c material containing 720µg mannose in 100mg KBr.

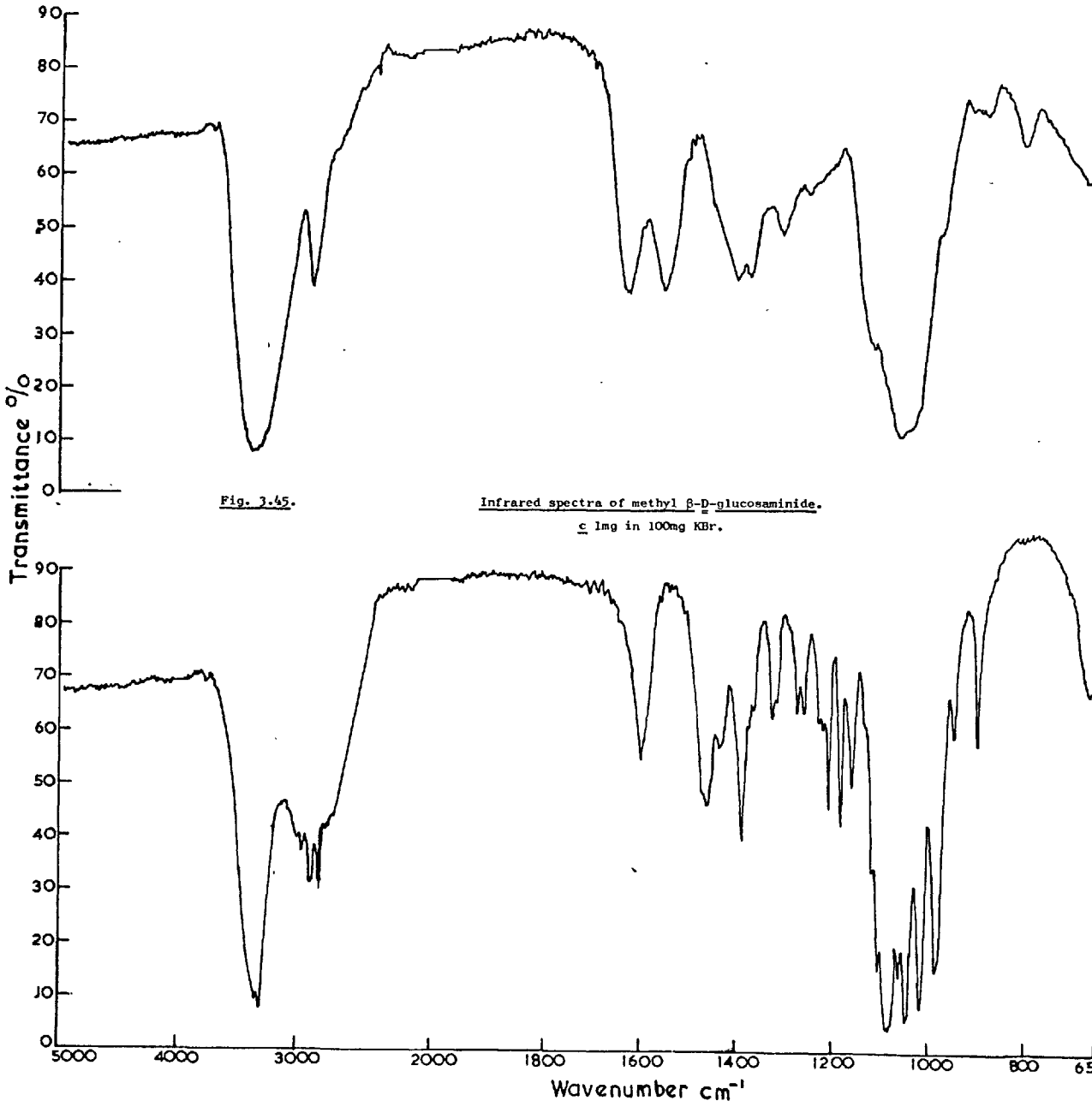


Fig. 3.45. Infrared spectra of methyl β-D-glucosaminide.
c 1mg in 100mg KBr.

Table 3.10. Products formed by acid hydrolysis (4M-HCl; 100°C; 18 hr) of products formed from N,N^1-diacetylchitobiose by reduction in 2M-NaOH; 1M-$NaBH_4$ at 100°C

Time of treatment with alkaline borohydride	20 min	1 hr
Products		
GlcN	0.40	0.24
Glucosaminitol	0.18	0.13
Mannosaminitol	0.13	0.08
"Chitobiol"(a)	0.13	0.21

The concentrations of products are expressed per mole of substrate

(a) "Chitobiol" represents a mixture of
β-D-glucosaminyl-(1→4)-D-glucosaminitol and
β-D-glucosaminyl-(1→4)-D-mannosaminitol in a ratio of 0.77

with the pH 6.65 citrate buffer. The size of this peak was greater in those experiments in which glycopeptide was reduced in the presence of higher concentrations of alkali. The highest yield of this component was obtained after treatment with 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr, followed by acid hydrolysis in 2M- or 4M-HCl for 3 hr at 100°C. The peak was present only in trace amounts in acid hydrolysates of material which had been re-N-acetylated after treatment with alkaline borohydride.

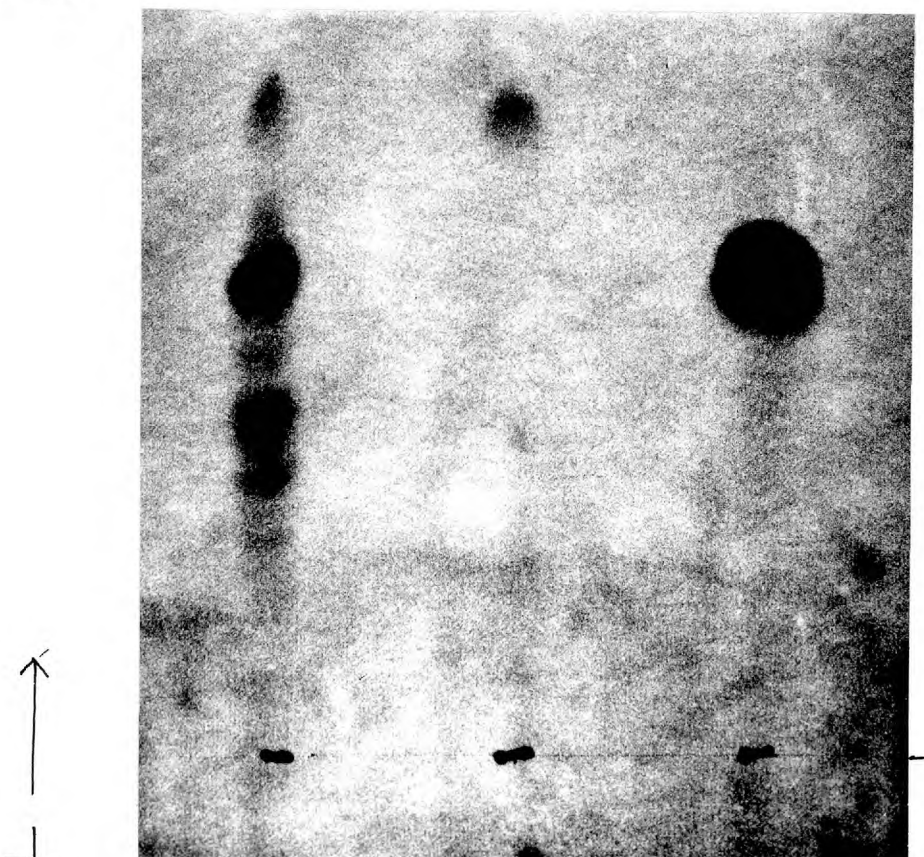
Because of the possibility that the product was reduced chitobiose, experiments to test this hypothesis were carried out.

Reduction of N,N'-diacetyl-chitobiose in 2M-NaOH, 1M-$NaBH_4$, for periods up to 10 hr, gave a peak eluting in an identical position to that of the unknown, both before and after acid hydrolysis (4M-HCl; 100°C; 16 hr). Acid hydrolysis yielded, in addition, D-glucosamine, D-glucosaminitol and D-mannosaminitol (Table 3.10).

The product formed after 10 hr alkaline reduction was deionised on a column of Sephadex G-10 equilibrated with 0.1M-ammonium acetate. Paper electrophoresis of this material produced one spot with a mobility of 13.5cm, moving further than that of D-glucosamine (10.3cm); the spot developed with the ninhydrin reagent and also with the periodate-2,4-pentane dione reagent (Plate 3.6). A spot with the same mobility was found in the 3 hr acid hydrolysates of glycopeptide II after an identical alkaline reduction. Other bands were present in the acid hydrolysate of the reduced glycopeptide, and these ran

Plate 3.6. Paper electrophoresis of 'chitobiol'.

Electrophoresis was performed at pH 2.0 and 32 V/cm for 30 min. Components were located with ninhydrin.



A = Products of acid hydrolysis (4 M-HCl ; 100°C ; 3 hr) of reduced oligosaccharides (containing about 1.2 μmole hexose) obtained from glycopeptide II by treatment with 2 M-NaOH ; 1 M-$NaBH_4$ at 100°C for 10 hr.

B = a mixture of chitobiol and β-D-glucosaminyl-(1→4)-D-mannosaminitol obtained by treatment of N,N'-diacetylchitobiose with 2 M-NaOH ; 1 M-$NaBH_4$ at 100°C for 10 hr. Products obtained from about 1 μmole were subjected to electrophoresis.

Products were isolated as described in the text.

C = Glucosamine

slower than D-glucosamine and also stained with ninhydrin.

The band with mobility of 13.5cm found in the acid hydrolysates of the reduced glycopeptide was eluted into water. The material was N-acetylated and rehydrolysed with acid (4N-HCl; 100°C; 18 hr). Autoanalysis showed that D-glucosamine, D-glucosaminitol and D-mannosaminitol had been formed in a ratio of 1 : 0.6 : 0.4.

Two components could be separated from the reduction products of chitobiose by paper chromatography. Streaking was observed after development with n-butanol - pyridine - water (6 : 4 : 3, by vol) for 20 hr, although two bands could be seen. Two bands in the same position were also found in the partial acid hydrolysates of the glycopeptide previously treated with 1N-$NaBH_4$ and 2N-NaOH for 10 hr.

Better separation was obtained by development with n-butanol - pyridine-0·1N-HCl (5 : 3 : 2, by vol) for 15 hr. The bands obtained from the reduction product of N,N'-diacetyl-chitobiose were eluted from the paper. Band A, which ran 2.5cm, gave the peak which eluted after 5 hr 20 min on the autoanalyser, and which was present in samples of reduced oligosaccharides and was formed by acid hydrolysis. This substance was N-acetylated and subjected to acid hydrolysis. D-Glucosamine and D-glucosaminitol, plus a small amount of the material eluting after 5 hr 20 min, were obtained. Band B, which ran 4.5cm, also gave a peak eluting at 5 hr 20 min before acid hydrolysis, and D-glucosamine and D-mannosaminitol plus, again, a small amount of the 5 hr 20 min peak, after N-acetylation followed by acid hydrolysis. Autoanalysis of the sample

Table 3.11. Analysis of products formed by alkaline reduction of N,N^1-diacetylchitobiose. The products were separated by paper chromatography.

Chitobiose was treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 10 hr. The products resulting from reduction of 5 μmoles of chitobiose were subjected to paper chromatography (see text). Bands were eluted from the chromatogram and the products were subjected to N-acetylation and acid hydrolysis (4M-HCl; 100°C; 18 hr) followed by autoanalysis.

	μmoles of product			
	GlcN	Glucosaminitol	Mannosaminitol	"Chitobiol"(a)
Band A	1.84	1.62		0.37
Band B	1.48	-	1.24	0.10

Molar colour yields of products with respect to DL-norleucine:-

Glucosaminitol	0.50
Mannosaminitol	0.60
β-D-Glucosaminyl-(1→4)-D-glucosaminitol	1.70
β-D-Glucosaminyl-(1→4)-D-mannosaminitol	1.81

(a) "Chitobiol" represents a mixture of the two reduced disaccharides in a ratio of 0.77

after N-acetylation, but prior to acid hydrolysis, showed that N-acetylation was complete because no ninhydrin positive peak was present. The material eluting after 5 hr 20 min in the experiments with glycopeptide and the material in the band running 13.5cm on paper electrophoresis was thus shown to be a mixture of β-D-glucosaminyl-(→ 4)-D-glucosaminitol and β-D-glucosaminyl-(1 → 4)-D-mannosaminitol.

The relative molar colour yields of β-D-glucosaminyl-(1 → 4)-D-glucosaminitol, β-D-glucosaminyl-(1 → 4)-D-mannosaminitol and norleucine were assessed. Each of the disaccharides obtained from paper chromatography was separately mixed with a known amount of DL-norleucine and part of it subjected to autoanalysis directly. The amount of disaccharide in the remainder of the sample was measured by N-acetylating the sample, hydrolysing it with acid and again analysing on the autoanalyser. The amounts of D-glucosamine and D-hexosaminitol could thus be assessed. Hence the amount of disaccharide could be determined and its colour yield relative to that of DL-norleucine calculated. The values are given in Table 3.11.

A weighted molar colour yield, that included contributions from both disaccharides, was then calculated, and used to estimate the amounts of "chitobiol" (that is the sum of the two reduced disaccharides) produced from glycopeptides I and II under various conditions during alkaline reduction. The results are shown in Table 3.12.

The amounts of reduced di-hexosaminitols measured in this way agreed with the increase in D-glucosaminitol

Table 3.12. Analyses of hexosaminitols, chitobiol and β-D-glucosaminyl-(1→4)-D-mannosaminitol contained in reduced oligosaccharides

Glycopeptides were treated with alkaline borohydride and subjected to gel chromatography. Measurements were made after acid hydrolysis (4M-HCl; 100°C; 18 hr). The concentration of products are expressed as moles per mole of reduced oligosaccharides based on the mannose content of the reduced material.

	Conditions of alkaline reduction	GlcN	GlcNol	ManNol	"Chitobiol"[a]
Glycopeptide I	2M-NaOH; 1M-$NaBH_4$; 100°C; 10 hr	2.45	0.16	0.10	0.23
Glycopeptide II	2M-NaOH; 1M-$NaBH_4$; 100°C; 10 hr	1.88	0.15	0.09	0.35
Glycopeptide II	0.2M-NaOH; 1M-$NaBH_4$; 100°C; 11 hr	2.18	0.25	-	0.16
Glycopeptide II	0.2M-NaOH; 1M-$NaBH_4$; 100°C; 11 hr[b]	1.53	0.13	-	0.30

(a) "Chitobiol" represents a mixture of β-D-glucosaminyl-(1→4)-D-mannosaminitol and chitobiol.

(b) In this experiment, treatment of glycopeptide II with alkaline borohydride was followed by treatment with 2M-NaOH for 11 hr at 100°C without further addition of borohydride.

and D-mannosaminitol measured after N-acetylation (Table 3.12). The highest yield of reduced hexosaminitols (51% of the glucosamine destroyed) was thus seen to be produced from glycopeptide II by reduction with 2M-NaOH, 1M-$NaBH_4$ for 10 hr.

Quantitative assessment of the formaldehyde released by periodate oxidation.

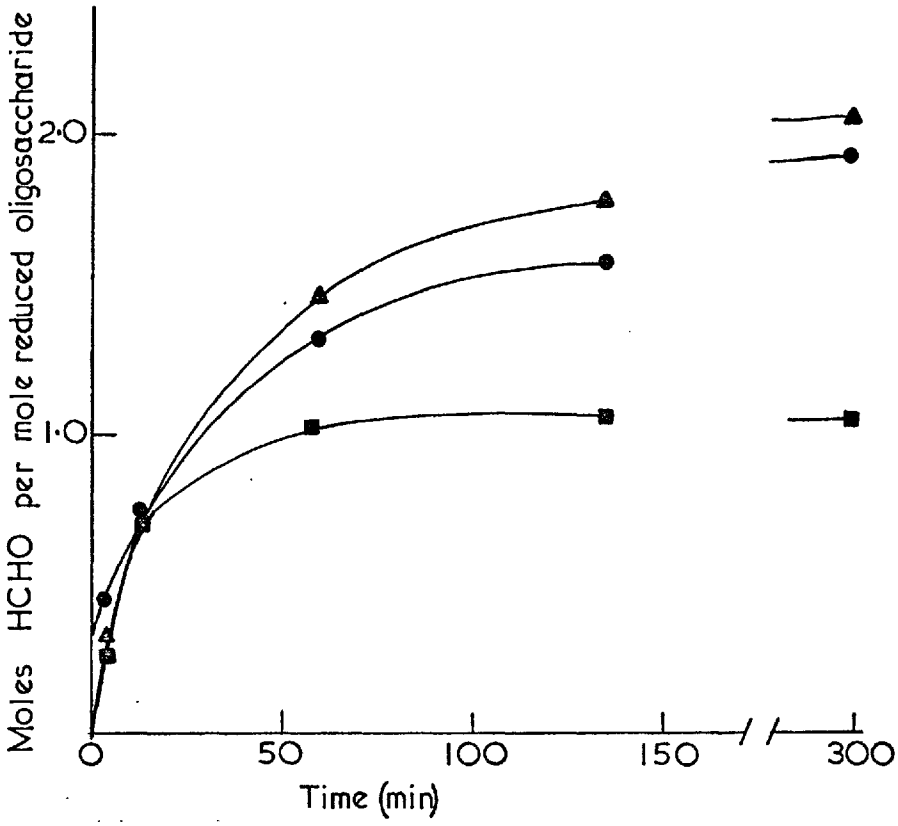
The rate of release of formaldehyde, produced by periodate oxidation of material obtained from glycopeptides I and II after reduction in 2M-NaOH, 1M-$NaBH_4$ for 10 hr, is shown in Fig. 3.46. The reaction rate decreased markedly after a few hours. The amount released approximated to two moles of formaldehyde per mole of reduced oligosaccharide, the quantity that might be expected if one residue of hexosaminitol with an unsubstituted amino group, and also an unsubstituted C_6 position, were present in the molecule. In fact, only 0.49 residues of hexosaminitol appeared to be present in the reduced oligosaccharide, formed when glycopeptide I was reduced under these conditions (Table 3.12). The amount in the reduced oligosaccharide from glycopeptide II was 0.59 residues (Table 3.12).

After N-acetylation, the release of formaldehyde was rapid initially, but decreased after about 1 hr, with a final production of about one mole.

The yields of formaldehyde arising from the reduced oligosaccharide, prepared from glycopeptide II by treatment with 0.2M-NaOH, 1M-$NaBH_4$ for 10 hr, were smaller. This finding is not too surprising in view of the observations

Fig. 3.46. Release of formaldehyde by periodate oxidation of reduced oligosaccharides

Periodate oxidation of about 0.1 μmole of reduced oligosaccharide was performed with 1.07mg sodium metaperiodate in 1ml of 0.4M-sodium acetate buffer (pH 4.2) at 0°C. Formaldehyde released was measured by the reagent of Nash (1953).



HCHO released from material isolated by gel chromatography of:

- ●—● Glycopeptide I that had been treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 10hr
- ▲—▲ Glycopeptide II that had been treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 10hr
- ■—■ Glycopeptide I that had been treated with 2M-NaOH; 1M-$NaBH_4$ at 100°C for 10hr and re-N-acetylated.

Results were calculated on the basis of 4.91 moles of mannose per mole of reduced oligosaccharide from glycopeptide I and 4.70 moles of mannose per mole of that from glycopeptide II.

Table 3.13. **Formaldehyde formed by periodate oxidation of reduced oligosaccharides separated by gel chromatography**
(for details of isolation procedure, see text)

Source of reduced oligosaccharide	Conditions of alkaline reduction[a]	Moles formaldehyde per mole reduced oligosaccharide[b]
Glycopeptide I	2M-NaOH; 10 hr	1.90
Glycopeptide II	2M-NaOH; 10 hr	2.04
Glycopeptide I	2M-NaOH; 10 hr[c]	1.03[c]
Glycopeptide II	0.2M-NaOH; 11 hr	1.10
Glycopeptide II	0.2M-NaOH; 11 hr[c]	0.68[c]
Glycopeptide II	0.2M-NaOH; 11 hr[d]	1.53[d]

(a) All reactions were carried out at 100°C in the presence of 1M-$NaBH_4$

(b) This is calculated on the basis of 4.91 moles of mannose for the reduced oligosaccharide from glycopeptide I, and 4.70 moles mannose for that from glycopeptide II.

(c) In these experiments, free amino groups which had been formed during the alkaline reduction had been fully re-N-acetylated.

(d) In this experiment, treatment of glycopeptide II with 1M-$NaBH_4$ in 0.2M-NaOH was followed by treatment with 2M-NaOH for 11 hr at 100°C without further addition of borohydride.

noted above that N-deacetylation of the amino sugar residues occurred to a lesser extent under these conditions.

Nitrogen determination.

The results of nitrogen analyses of the reduced oligosaccharide are shown in Table 3.14. In each case, the total amount of nitrogen found by direct measurement was greater than could be accounted for by the numbers of residues of D-glucosamine and hexosaminitols present in the material. It was observed however, that the number of atoms of nitrogen contained in each reduced oligosaccharide was closely similar to the number of residues of D-glucosamine present in the glycopeptide from which the material was derived.

The treatment of glycopeptide II with 0.2N-NaOH, 1N-$NaBH_4$ at 100°C for 10 hr. followed by 2N-NaOH at 100°C for a further 11 hr.

The number of D-glucosamine residues present in the reduced oligosaccharide obtained from glycopeptide II after treatment with 0.2N-NaOH, 1N-$NaBH_4$ at 100°C for 10 hr is greater than in the material obtained if this treatment is followed by treatment with 2N-NaOH at 100°C for 11 hr. It may be argued that the lower amounts of D-glucosamine found in acid hydrolysis of material are produced by the latter conditions because of an increasing extent of N-deacetylation of D-glucosamine residues brought about by the alkali, resulting in the formation of acid resistant glycosidic linkages. But this is unlikely to be the explanation of the total loss, (although it may be

Table 3.14. Nitrogen contents [a] of reduced oligosaccharides obtained from glycopeptides

Source of reduced oligosaccharide	Conditions of alkaline reduction [b]	Calc. atoms of N [c] from content of: GlcN	GlcNol	ManNol	"Chitobiol" [d]	Total atoms of N: Calc.	Found	Atoms of N: Unaccounted for	Original moles of GlcN [e]
Glycopeptide I	2M-NaOH; 10 hr	2.45	0.16	0.10	0.46	3.17	4.24	1.07	4.11
Glycopeptide II	2M-NaOH; 10 hr	1.88	0.15	0.09	0.70	2.82	3.53	0.71	3.42
Glycopeptide II	2M-NaOH; 10 hr [f]	2.24	0.32	0.20	0	2.76	3.29	0.53	3.42
Glycopeptide II	0.2M-NaOH; 11 hr	2.17	0.25	0	0.32	2.74	3.10	0.36	3.42
Glycopeptide II	0.2M-NaOH; 11 hr [g]	1.53	0.09	0	0.68	2.30	2.95	0.65	3.42

(a) The nitrogen contents are given as atoms of nitrogen per mole of reduced oligosaccharide, the values of which are based on a content of 4.91 moles of mannose for that prepared from glycopeptide I, and 4.70 for that prepared from glycopeptide II.

(b) All reactions were carried out at 100°C in the presence of 1M-$NaBH_4$

(c) The amounts of the components mentioned are determined after acid hydrolysis (4M-HCl; 100°C; 18 hr) of the reduced oligosaccharides.

(d) "Chitobiol" is a mixture of β-D-glucosaminyl-(1→4)-D-glucosaminitol and β-D-glucosaminyl-(1→4)-D-mannosaminitol (see footnote (a) of Table 3.10)

(e) These are the numbers of residues of N-acetyl-D-glucosamine in the original glycopeptide before treatment with alkaline borohydride

(f) This reduced oligosaccharide was fully N-acetylated before isolation by gel chromatography

(g) Treatment of this reduced oligosaccharide included treatment with 2M-NaOH at 100°C for 11 hr without addition of further borohydride after the initial reduction in 0.2M-NaOH

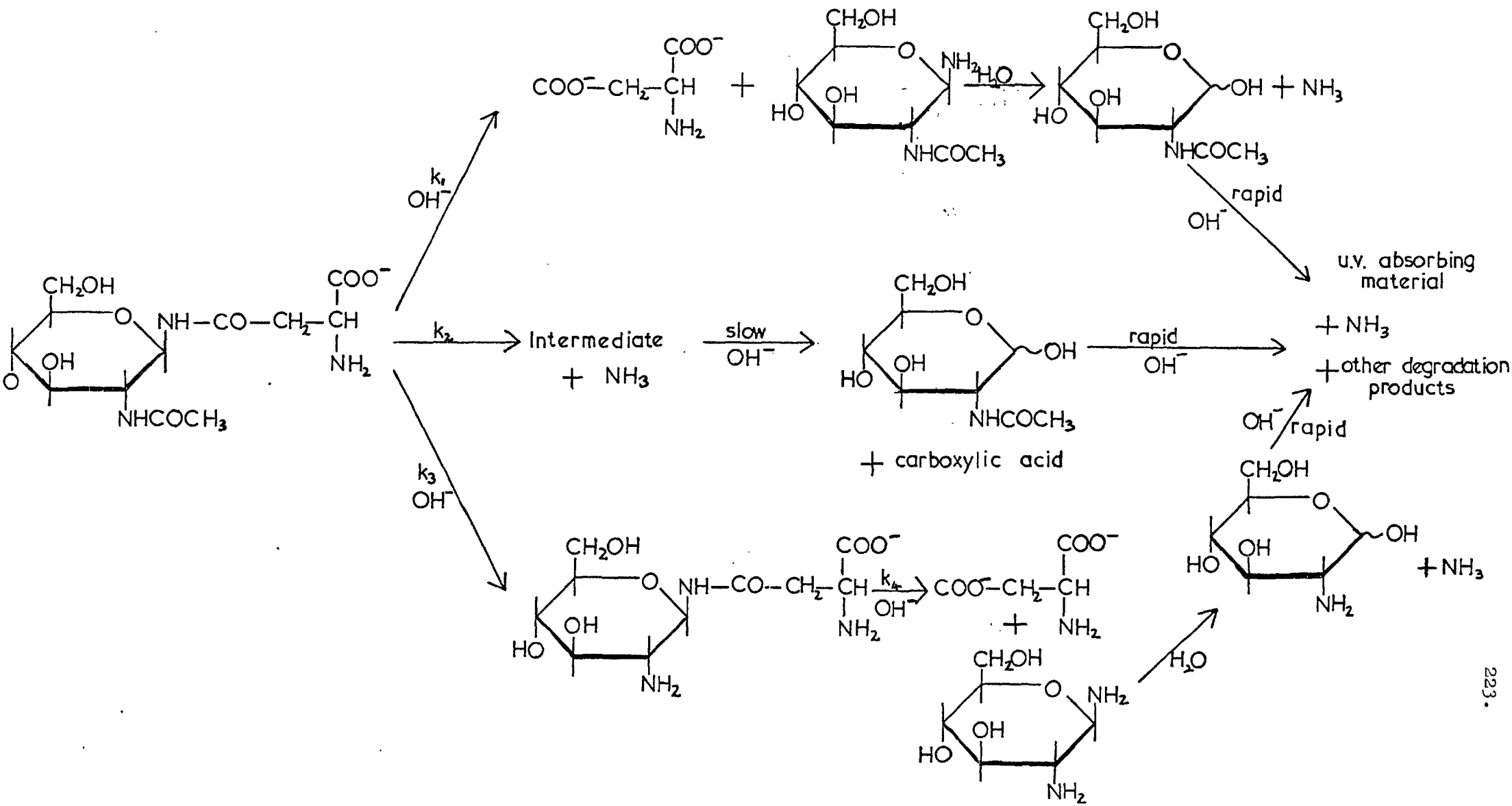
a small contributing factor), because acid hydrolysis of reduced oligosaccharide, isolated after treatment of glycopeptide II with 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr, caused the release of more D-glucosamine (1.88 residues) than arose from the material under consideration (Table 3.14). It is reasonable to deduce, therefore, that the value for the D-glucosamine to hexose ratio in the separated product was lower than that for glycopeptide material separated after treatment with 2M-NaOH, 1M-$NaBH_4$ for 11 hr at 100°C (Table 3.8), and the chitobiol formed did not account for the extra D-glucosamine loss. Thus, reduction in 0.2M-NaOH at 100°C does not result in the production of substances which are all resistant to degradation by further treatment with alkali leading to further losses of D-glucosamine.

DISCUSSION.

Treatment of GlcNAc-Asn and related compounds with alkali.

A simplified reaction scheme that is consistent with the observations made on the kinetics of, and reaction products from, GlcNAc-Asn and related compounds, when treated with alkali, is presented in Fig. 3.47. There are likely to be additional pathways, including for example N-deacetylation of GlcNAc-NH_2. Part of the compound is split by a reaction, the rate constant for which contains a first-order term in base concentration, to yield aspartic acid (Table 3.1). This finding is in agreement with the kinetics usually found in the alkaline hydrolysis of amides, which occurs by the $B_{Ac}2$ mechanism (Ingold, 1953).

Fig. 3.47. A simplified representation to indicate the pathways by which GlcNAc-Asn may decompose in alkali at 100°C.



In the present experiments it was demonstrated that splitting of GlcNAc-Asn occurs by general base catalysis (see Table 3.1). The more concentrated the alkali, the higher the amount of an intermediate X, which is probably GlcN-Asn, is produced (see page 140). Thus the production of aspartic acid from GlcNAc-Asn at 100°C in M-NaOH follows somewhat complicated kinetics (see Fig. 3.7d). In dilute alkali the kinetics are simpler and it would seem that, although the overall yield of aspartic acid is lower, very little of it is formed by way of GlcN-Asn as an intermediate. The release of aspartic acid from GlcNAc-Asn is similar to that from Glc-Asn in 0.2M-NaOH at 100°C.

The initial rates of release of aspartic acid are, to a large extent, a measure of the rates of release from GlcNAc-Asn before a significant amount of N-deacetylation has occurred. The value calculated from Fig. 3.5 for this reaction in 1M-NaOH at 100°C ($6 \times 10^{-3} min^{-1}$), when compared to the value obtained in 2M-HCl ($20 \times 10^{-3} min^{-1}$; Marshall, 1969) suggests that the release of aspartic acid from GlcNAc-Asn is faster in acid than from alkali at the same concentration. For most amides the reverse is the case (Taylor & Baker, 1937), so that the observations on GlcNAc-Asn suggest that the reaction is anomalous.

In support of this is the finding that the yield of aspartic acid is far from quantitative, the loss being higher in the lower concentrations of alkali (Table 3.1). The reaction that results in decomposition of GlcNAc-Asn

or Glc-Asn, but does not release aspartic acid, is largely independent of the base concentration (see also page 142), and has a first-order rate constant of about $2.4 \times 10^{-3} min^{-1}$. The products formed in this reaction, or these reactions, are at present unknown, although they do not include malic, maleic, fumaric or succinic acids. Pyruvic acid may have been produced as a result of the operation of this pathway, but it may also have arisen from the sugar moiety. It is perhaps of interest that pyruvic acid was isolated after treatment with 0.09M-LiOH at 100°C of blood group substances (Adams, 1965a). The total production of ammonia, when GlcNAc-Asn is treated with 0.2M-NaOH at 100°C, is about 1.5 moles, but it is unknown if any of this arises from the α-amino group of the aspartic acid residues. Further studies along these lines must await the preparation of GlcNAc-Asn in which the α-amino group of the amino acid residue is labelled with ^{15}N.

It is of particular interest that losses of aspartic acid occur when certain glycoproteins are treated with alkali. Weber & Winzler (1969) observed this when they treated various submaxillary mucins and red cell glycopeptides with alkali (see also page 47). Almost 40% of the aspartic acid content of sheep submaxillary gland glycoprotein was found to be destroyed upon treatment at pH 12.8 (I = 1.6) at 70°C for 45 min (Harbon et al., 1964). In all the glycoproteins studied by these two groups of workers, the major carbohydrate-peptide linkages are of the type in which L-serine and L-threonine are linked to N-acetyl-D-galactosamine residues, but it is possible

that linkages of GlcNAc-Asn type occur also in them. There are, of course, mechanisms for the destruction by alkali of L-aspartic acid residues in protein which are devoid of sugars. Thus, treatment of ribonuclease A in 50% (w/v) NaOH at 110°C, conditions which are admittedly severe, causes complete disappearance of cystine, appreciable losses of aspartic acid and extensive destruction of serine and threonine (Neumann, Moore & Stein, 1962). Whatever the mechanisms by which L-aspartic acid residues in proteins are destroyed, it seems likely that there is an additional pathway possible in glycoproteins which contain linkages of the form GlcNAc-Asn.

It is relevant to mention that 4-ethyl-N-benzoyl isoasparagine may be converted in sodium carbonate solution to a mixture of N-benzoylisoasparagine and N-benzoylasparagine. N-Benzoyl-2-amino-L-succinimide is suggested to be an intermediate (Battersby & Robinson, 1954). It was also shown that α-benzyl N-benzyloxycarbonyl asparagine is rapidly converted in alkali to N-benzyloxycarbonyl-2-amino-L-succinimide (Bernhard, 1959). It is not known whether any cyclic derivatives of this type are produced during the alkaline conditions presently employed for the treatment of GlcNAc-Asn. It was suggested that an intermediate of this form might be produced when 1-benzyl-2-N-benzyloxycarbonyl-4-N-(3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine was treated with alkali under mild conditions (Marks, Marshall & Neuberger, 1963).

The rate of release of ammonia from GlcNAc-Asn in 0.2M-NaOH at 100°C displays an inductive period (Fig. 3.17),

a feature not observed in the release of ammonia by alkali from hen ovalbumin glycopeptides (Neuberger, 1938; Marks, Marshall & Neuberger, 1963). The source of the ammonia is not known, but it is of interest that after treatment of glycopeptide, whose amino acid sequence was ASN.Leu.Thr.Ser (where ASN represents a glycosylated L-asparaginyl residue) with 0.2M-NaOH at 100°C for 2 hr, about 0.7 moles of ammonia were released (Marks, Marshall & Neuberger, 1963), whereas glycopeptide, which probably contained few, if any, amino acids other than L-asparagine, released about 1 mole of ammonia in 1M-NaOH in the same time (Neuberger, 1938). Moreover, about 0.8 moles of ammonia were released from GlcNAc-Asn after 2 hr in 0.2M-NaOH at 100°C (Fig. 3.17). Hence the rates of release of ammonia are of the same order in all cases. These results contrast with those reported by Miller, Neidle & Waelsch (1955) who found a very much faster rate of release of ammonia from the peptide Asn.Gly than from Gly.Asn or from asparagine itself when the compounds were treated at pH 12 at 70°C.

The rate of release of aspartic acid from GlcNAc-Asn was much slower than that from L-asparagine ($1.69 \times 10^{-2} min^{-1}$) or the rate of breakdown of GlcNAc-NH_2 ($2.31 \times 10^{-2} min^{-1}$). The rate of liberation of ammonia from L-asparagine under the same conditions has been measured previously (Marks & Neuberger, 1961)($1.45 \times 10^{-2} min^{-1}$), and is very similar to the value presented here, and the rate of release of ammonia from D-glucosylamine ($4.22 \times 10^{-2} min^{-1}$) has also been measured previously and

is of the same order as the rate of breakdown of GlcNAc-NH_2 measured in these studies. The value of the first-order rate constant for the production of aspartic acid from GlcNAc-Asn ($3.28 \times 10^{-3} min^{-1}$) is about twice that of the destruction of GlcNAc-NHAc ($1.3 \times 10^{-3} min^{-1}$) in 0.2M-NaOH at 100°C, a reaction which probably involves cleavage of a similar bond.

The behaviour of glycosylamines in alkali is not well understood. The mutarotations of various glycosylamines under different conditions of pH have been studied by Isbell & Frush (1951). In weakly alkaline solutions, the sugars show a decrease in rotation, suggested to be due to anomerisation, followed by a slow increase, suggested to be due to hydrolysis. Both these changes were sensitive to general acid catalysis and very slow at high pH; the formation of an intermediate immonium ion was proposed. However, the reactions are more complex than those proposed, and it is likely that the initial changes in rotation observed under mild conditions are due, at least in part, to reactions in which diglycosylimines are produced.

The change in rotation of GlcNAc-NH_2 in 0.2M-NaOH at 25°C (Fig. 3.16) is not simply due to an initial hydrolysis to N-acetyl-D-glucosamine, which may be followed by further reactions, as shown by the differences in the values for the final rotations reached by these two compounds. The differences observed in the mutarotation of GlcNAc-NH_2 in 0.2M-NaOH and in water may indicate that completely different reactions of this compound are

occurring under the two sets of conditions. It is unlikely to be a result of a change of the extent of ionisation of the free amino group and its effect on the total rotatory strength, because the pK_a value is less than 5 (Marshall, 1967). At 100°C, GlcNAc-NH_2 had a half-life of about 30 min, forming coloured and ultra-violet absorbing products of a similar nature to those formed by N-acetyl-D-glucosamine, and it is probable that preliminary hydrolysis does, in fact, occur at these temperatures.

Maximum ultra-violet absorption at about 230nm was observed by Aminoff et al., (1952) on heating N-acetyl-D-glucosamine in 0.025M-Na_2CO_3 at 100°C for 4 min. After the addition of Ehrlich's reagent to this reacted solution, a purple colour was produced and it was suggested that the ultra-violet absorbing (λ_{max} 230nm) material was the Kuhn chromogen (see page 60). They found that the ultra-violet absorption, and ability to produce colour with Ehrlich's reagent, decreased after extended periods of heating.

In the present studies it has been shown that the Kuhn chromogen, that was initially formed from N-acetyl-D-glucosamine when it was treated with 0.2M-NaOH at 100°C, decomposed after 20 min, with the formation of ultra-violet absorbing material whose peak, at pH 5 is, at 264nm, similar to, but not identical with that produced from GlcNAc-Asn, GlcNAc-NHAc and GlcNAc-NH_2. This ultra-violet absorbing material may be related to those products formed from most monosaccharides in

alkaline solution. Berl & Feazel (1951), for example, studied a substance that forms in alkaline solutions of glucose after several days at room temperature. The maximum absorption of this material was at 295nm in alkaline, and at 265nm in acid solution.

Treatment of GlcNAc-Asn with alkaline borohydride.

There is a marked increase in the rate of destruction of GlcNAc-Asn when $NaBH_4$ is included in the alkaline reaction media. Indeed, reaction occurs rapidly in the presence of 1M-$NaBH_4$ alone. It may be noted that the presence of borohydride increases the rate of cleavage in alkali of carbohydrate-peptide linkages involving L-serine and L-threonine and, indeed, Carlson (1968) found that borohydride alone without addition of alkali caused splitting of these types of linkages in pig submaxillary mucin. It is, however, not relevant to discuss further, at this point, the cleavage of the L-serine and L-threonine types of carbohydrate-peptide linkage, because the observations made on the rate of splitting of GlcNAc-Asn, and the products formed from it, are specific for this latter type.

This greater rate of splitting of GlcNAc-Asn, when borohydride is present in the alkaline solution, may be attributable to a general base catalysis, to which the reaction of this substituted amide is susceptible (see above). But there are other factors which may be more important, in quantitative terms. Thus, there are additional pathways for the breakdown of GlcNAc-Asn in alkaline borohydride compared with those observed in

alkali alone. The presence of asparagine in the products, formed when GlcNAc-Asn is reduced with borohydride in alkaline solution, suggests that cleavage of the C_1 to amide N bond occurs under these conditions. About 14% of the degradation products of GlcNAc-Asn may be estimated as asparagine when reaction occurs in 0.2M-NaOH, 1M-$NaBH_4$ or in 1M-$NaBH_4$ alone at 100°C (Table 3.3). Asparagine is destroyed under the former set of conditions mentioned here, with a half-life of the order of 30 min (Fig. 3.28b). These data imply that somewhere in the region of 20 to 25% of the GlcNAc-Asn may be initially converted to asparagine. It should be emphasised that no asparagine was found among the products when GlcNAc-Asn was treated with alkali without borohydride, although under these conditions the half-life for the destruction of asparagine is of the order of 35 min.

Another interesting product formed from GlcNAc-Asn when treated with alkaline borohydride is homoserine, with especially large amounts (34%) being produced in 0.05M-NaOH, 1M-$NaBH_4$ at 47°C (Table 3.3). This may have been formed by way of cyclic imide intermediates (Fig. 3.48), by a reaction which may be similar to that whereby dioxo-dihydropyrimidines are reduced by sodium borohydride (Cerutti & Miller, 1967).

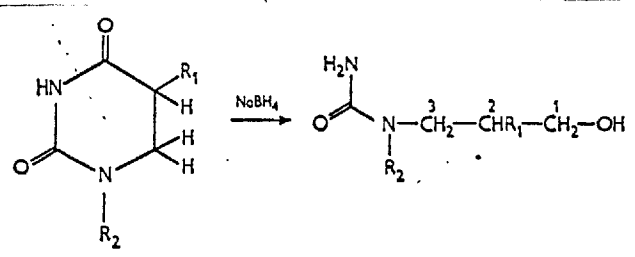
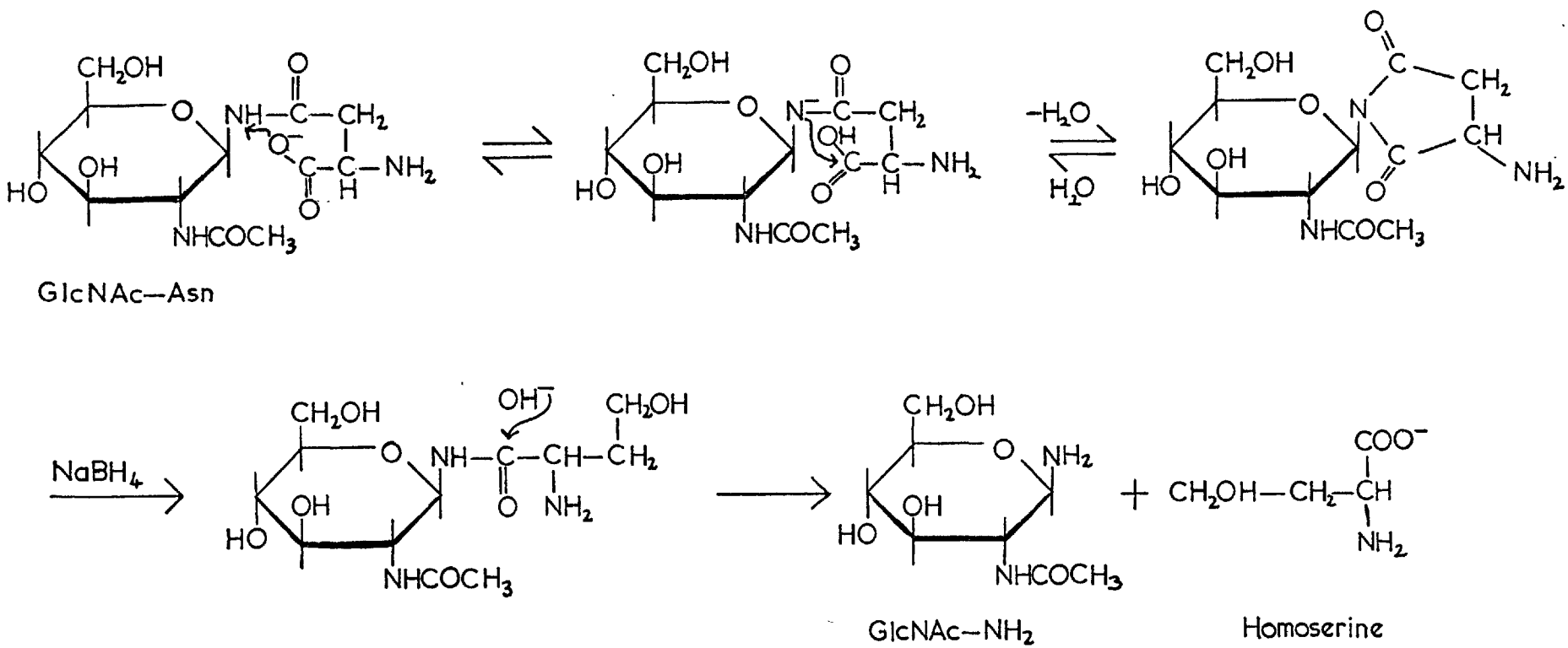


Fig. 3.48. A possible mechanism whereby homoserine might be formed by the action of alkaline borohydride on GlcNAc-Asn.



A further example of the reduction by aqueous sodium borohydride of a cyclic imide is given by the formation of 2-hydroxymethyl-benzoic acid from ε-N-phthaloyl lysine esters (Gallop, Blumenfeld, Henson & Schneider, 1968).

The possibility must not be ruled out, however, that direct scission of the amide bond may occur under the influence of sodium borohydride (Crestfield, Moore & Stein, 1963), but whether aspartic acid or homoserine would arise from this cleavage is uncertain.

It might be mentioned that the source of homoserine, arising from the reduction with lithium borohydride of sheep and cattle submaxillary gland glycoproteins, has not been explained satisfactorily, although it was suggested (Gottschalk & König, 1968) that direct reduction of aspartic acid residues under the action of diborane, which may be formed when lithium borohydride is destroyed by anhydrous methanolic HCl, had occurred (see also Chapter 1).

A high energy of activation (26.7Kcal) was found for the destruction of GlcNAc-Asn in 0.2M-NaOH, 1M-$NaBH_4$. Moreover the production of reduced sugars was greater at higher temperatures. It seemed therefore most reasonable to carry out the reduction of glycopeptides at the higher temperatures employed.

Higher yields of reduced hexosaminitols are obtained from N-acetyl-D-glucosamine and N,N'-diacetyl-chitobiose than from either GlcNAc-NH_2 or GlcNAc-Asn under similar conditions. The lower yields obtained from GlcNAc-Asn, as well as from glycopeptide material as discussed later, may therefore be a result of the intermediary production

of the glycosylamine from these compounds.

Treatment of hen egg albumin glycopeptide with alkaline borohydride.

The production, as a result of treatment of alkaline borohydride, of reduced sugars from glycopeptide showed some differences from that found with GlcNAc-Asn. The total molar amount of reduced sugar obtained from glycopeptide (44% of that theoretically possible) is not as great as that formed from GlcNAc-Asn (69%) in 0.2M-NaOH, 1M-$NaBH_4$ (see Tables 3.3 and 3.8). Moreover, the time required to produce this maximum yield from glycopeptide, (11 hr) is longer than that (4 hr) required for the model compound. Both the amino acid and sugar residues involved in the carbohydrate-peptide are further substituted in the glycopeptide.

The use of more concentrated alkali (2M-NaOH) during reduction of the glycopeptide produced only slightly reduced yields of total reduced hexosamines when these were measured after acid hydrolysis of re-N-acetylated material.

Various side reactions occurred. About half of the threonine content, and about a quarter of the serine content of the glycopeptide material prepared from hen ovalbumin were destroyed during the initial stages of the alkaline reduction reaction, and appeared to be the most labile components present (Tables 3.5 and 3.6; Figs. 3.29-3.33). The sequence of amino acids around the carbohydrate moiety in the glycopeptides employed is ASN-Leu-Thr-Ser-Val, and it is likely that destruction of these amino acid

residues occur when both their α-amino and carboxyl groups are involved in peptide linkage. This is not surprising because treatment of ovalbumin with 0.5M-NaOH at 30° or 37° for 20 hr (Arima, Muramatsu, Saigo & Egami, 1969) or of other proteins, which are free of carbohydrate, in 0.2M-NaOH at 23°C for 24 hr, leads to losses of between 5 and 15% of the threonine and serine (see also Marshall & Neuberger, 1970).

Glycine was formed in the reaction of glycopeptide studied here. Previous studies (Adams, 1965b) have shown that treatment of mucin derived from human colloid breast carcinoma with 0.18M-LiOH at 100°C for 20 min resulted in a loss of threonine, and production of glycine (30% based on the destruction of threonine) and α-oxobutyric acid (70%). Adams proposed a mechanism whereby L-threonyl carbohydrate-protein linkages were cleaved in alkali to yield glycine and α-oxobutyric acid, but it would appear possible that this reaction may occur with L-threonyl residues not bound to carbohydrate.

Small amounts of α-amino butyric acid were formed during alkaline reduction of the hen ovalbumin glycopeptide (Tables 3.5 and 3.6). This compound probably arises from the reduction of the α-aminocrotonic acid formed by the β-elimination of the hydroxyl group from L-threonyl residues.

Under the conditions of reduction in alkali employed (in most of the experiments 0.2M- and 2M-NaOH at 100°C was used with 1M-$NaBH_4$), all the peptide was separated from the carbohydrate moiety (see page 192). Free amino

Table 3.15. Some rate constants for reactions which cleave glycosidic bonds

Compound	Conditions of acid	$k(min^{-1})x10^3$	Ref.
Methyl 2-amino-2-deoxy-β-D-glucopyranoside	2.5M-HCl; 100°C	1.24	1
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	1M-H_2SO_4; 100°C	180(a)	2
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose	1M-H_2SO_4; 100°C	38.4(a)	2
2-Amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucose	1M-H_2SO_4; 100°C	0.23	2
2-Amino-2-deoxy-β-D-glucopyranosyl-(1→6)-D-galactose	3M-H_2SO_4; 100°C	0.75	3
2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-D-galactose	3M-H_2SO_4; 100°C	115(b)	3

Ref.

1. Moggridge and Neuberger (1938)
2. Shively and Conrad (1970)
3. Lloyd and Evans (1968)

(a) The rate of N-deacetylation was stated to be slow

(b) The rate of N-deacetylation was not measured

groups as part of D-glucosamine and of hexosaminitol residues were formed, and this appeared to be essentially complete after treatment with 2M-NaOH, 1M-$NaBH_4$ for 10 hr. The deacetylation reaction is likely to be useful in structural determination of glycopeptides. Not only may partially N-deacetylated reduced oligosaccharides be separated by their charge characteristics (see for example Fig. 3.35), but partial acid hydrolysis will lead to the production of oligosaccharides containing acid-resistant glucosaminidic linkages (see for example Etzler et al., 1970). Some rate constants for glycosidic bonds of this nature are listed in Table 3.15. The half-life of 6-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-D-galactose in 3M-HCl, for example, is about 15 hr.

The reduced rate of splitting with acid of these types of glycosidic linkage clearly made it essential to carry out a re-N-acetylation of the material formed by treatment of glycopeptide with alkaline borohydride before the acid hydrolysis step used in the analysis for hexosamine (Table 3.8).

Partially N-deacetylated oligosaccharides may be useful also in other ways. Thus the action of nitrous acid on these materials is likely to produce valuable breakdown products.

Gel chromatography of glycopeptides treated with alkaline borohydride.

The elution volumes of various oligosaccharides on gel chromatography on Sephadex G-25 in 0.1M-acetic acid or in 0.15M-NaCl were shown to exhibit a simple relationship with respect to molecular weight (see also Cunningham

& Simkin, 1966; Bhatti & Clamp, 1968).

The molecular weight of the re-N-acetylated oligosaccharides, as assessed from chromatography on Sephadex G-25, was found to be that expected from cleavage of the peptide portion of the glycopeptide without any significant loss of sugars from the reduced oligosaccharide. No hexose, D-glucosamine or glycitols were detected in the more-retarded fractions obtained after G-25 gel chromatography, and only small (<5%) overall losses of hexose were observed after alkaline reduction of the glycopeptide material. It would appear unlikely, therefore, that the N-acetyl-D-glucosamine residue directly attached to the L-asparagine moiety in the whole glycopeptide is substituted at its 3-position by other sugars.

Prior to re-N-acetylation, the reduced oligosaccharides were more retarded on Sephadex G-25 than would be expected from the calculated molecular weight. The absorption of the materials to the gel is a result of the presence of basic amino groups (see also Isemura & Schmid, 1971). The acidic groups in Sephadex gels (Gelotte, 1960; Flodin, 1962) have been implicated in causing retention of strongly basic proteins unless the pH of the eluant is kept below pH 2 (Cruft, 1961), or the ionic strength of the eluant is 0.01 or higher (Determann, 1967). The ionic strength of the eluant used in most of the present studies (0.1M-acetic acid) was about 1.3×10^{-3}. Increasing the ionic strength to that of 0.15M-NaCl was only partly effective in eliminating the electrostatic interaction. The apparent molecular weight (1,200), calculated from

the elution volume in 0.15M-NaCl from the calibration curve depicted in Fig. 3.36, showed that ionic interactions with the gel had not been completely eliminated by increasing the ionic strength.

The original glycopeptide containing several amino acids in its peptide portion was also retarded relative to the elution behaviour expected from its calculated molecular weight. Andrews (1964) noted that glycoproteins, on gel chromatography, behave as more expanded structures than proteins of the same molecular weight. The behaviour of glycopeptides containing several amino acids on gel chromatography is clearly influenced by the characteristics of the peptide portion. The dangers of using values of the elution volumes of glycopeptides to assess the molecular weights are obvious, if the column has been calibrated with oligosaccharides.

The observed behaviour of glutathione compared to that of the oligosaccharides studied provides further confirmation of the need for care in interpreting data. The marked retardation of oxytocin is probably caused by an additional factor involving interaction of aromatic residues with the gel (Carnegie, 1965).

Properties of reduced oligosaccharides.

The most useful method for obtaining reduced oligosaccharides would appear to be treatment of glycopeptide or glycoprotein containing the N-acyl-glycosylamine type of linkage with 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for about 11 hr, with continued addition of alkaline borohydride throughout. Separation from the amino acids or peptides produced in

the reaction is readily achieved by gel chromatography. During the course of this work, a preliminary report on the production of a reduced trisaccharide (Man → $GlcNH_2$-glucosaminitol) by the action of alkaline borohydride (1M-$NaBH_4$, 1M-NaOH; 100°C; 4 hr) on a glycopeptide containing one mole of D-mannose, two moles of N-acetyl-D-glucosamine and one mole of L-asparagine has been published (Lee, 1971). The glycopeptide had been prepared by digestion with protease and α-mannosidase of both ovalbumin and α-amylase.

Estimation of formaldehyde released as a result of periodate oxidation shows that there are differences in the structures of the reduced substances, isolated by gel chromatography of glycopeptide material reduced in 0.2M-NaOH and 2M-NaOH. Part, at least, of these differences involves the extent of N-deacetylation of the terminal reduced hexosaminitol residue.

The amount of formaldehyde released suggested that the C_6 position of the D-hexosaminitol residue formed was unsubstituted. It will be shown below that the N-acetyl-D-glucosamine residue, which in the intact glycopeptide is attached to the asparagine residue, is itself substituted at its 4-position and, as discussed above, it is unlikely that this sugar carries a 3-substituent. It is extremely unlikely, therefore, that this sugar residue provides a branch point, and it is probable, therefore, that this aspect of the structure discussed by Huang, Mayer & Montgomery, 1970; (see also Chapter 1) is incorrect (see also Sukeno, Tarentino & Plummer & Maley, 1971).

Reduced oligosaccharide, which had been isolated after treatment with 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr, produced two moles of formaldehyde on periodate oxidation (Table 3.13). The substance, however, contains a total of 0.52 residues of hexosaminitol (Table 3.6). Hence there is a loss of about 1½ moles of D-glucosamine, 0.52 of which can be accounted for as the reduced sugar. The remaining 0.6 moles are unaccounted for, but it would appear that the nitrogen of this hexosamine is still contained in some form as part of the structure of reduced oligosaccharide material (Table 3.14). Whatever this modified substance is, it appears to yield formaldehyde on periodate oxidation.

The possibility of obtaining reduced disaccharides, after alkaline reduction in the presence of strong alkali, has been demonstrated by the isolation of a mixture of chitobiol and 4-(2-amino-2-deoxy-β-D-glucopyranosyl)-D-mannosaminitol from an acid hydrolysate of the reduced oligosaccharides separated by gel chromatography. These reduced disaccharides have approximate half-lives of about 18 hr in 4M-HCl at 100°C. Acid hydrolysis of re-N-acetylated forms of these reduced disaccharides produced, in addition to D-glucosamine, D-glucosaminitol and D-mannosaminitol, small amounts of chitobiol (19%) and 4-(2-amino-2-deoxy-β-D-glucopyranosyl)-D-mannosaminitol (8%). Perhaps in contrast with these findings is the report that N,N'-diacetyl-chitobiose is hydrolysed in 1M-H_2SO_4 at 100°C almost exclusively by glycosidic fission, N-deacetylation occurring only subsequently (Shively &

Conrad, 1970). These data support the contention that the structure of hen ovalbumin in the neighbourhood of the carbohydrate peptide linkage is β-D-GlcNAc-(1 → 4)-β-D-GlcNAc-L-Asn (see Chapter 1).

Additional ninhydrin-positive bands were produced upon electrophoresis of partial acid hydrolysates of glycopeptide material subjected to alkaline reduction in 2M-NaOH for 10 hr (Plate 3.6). The structures of these remain to be elucidated, but the results so far presented demonstrate that fission in alkaline borohydride solution of glycoprotein linkages of the type GlcNAc-Asn is likely to be a valuable additional method for studies of the structures of the carbohydrate moieties of these interesting macromolecules.

EXPERIMENTAL.

Materials.

GlcNAc-Asn, Glc-Asn, GlcNAc-NAc and GlcNAc-NH_2 were synthesised (Chapter 4). N-Acetyl-D-glucosamine was obtained from Koch-Light, Colnbrook, Bucks, and was purified by passage through a column of Dowex 50-X4 (400 mesh, H^+ form), followed by recrystallisation from 90% ethanol with the addition of ether to turbidity. N-Acetyl-D-mannosamine was obtained from Sigma Chemical Co., St. Louis, Missouri, and D-glucosaminitol hydrochloride was a gift from Dr. W.M. Watkins of the Lister Institute of Preventive Medicine, London. N,N'-Diacetyl-chitobiose and β-methyl-D-glucosaminide had been prepared previously in this laboratory (Neuberger & Wilson, 1967; Neuberger & Wilson, 1971).

DL-Homoserine (Biochemical Research, Los Angeles), recrystallised from 90% ethanol, and L-asparagine monohydrate (British Drug Houses) recrystallised from water, L-2,4-diamino-n-butyric acid (Koch-Light), DL-2-amino-n-butyric acid (Koch-Light), L-valine (Roche Biochemicals, Welwyn, Herts), 4-fluoro-DL-β-phenylalanine (British Drug Houses) and DL-norleucine (Hopkins & Williams, Chadwell Heath, Sussex) were used as standards on the autoanalyser. D-Mannitol (micro-analytical reagent) was obtained from Hopkins & Williams and recrystallised from 90% ethanol.

The following materials were used as standards in gel filtration experiments for molecular weight determinations. Lacto-N-difucohexaose I, lacto-N-fucopentaose, isomaltopentaose, raffinose and stachyose were gifts from Dr. W.M. Watkins. Lactose (Hopkins & Williams) and D-glucose (British Drug Houses) were also used. Synthetic oxytocin acetate was a gift from Dr. D.B. Hope, Pharmacology Dept., Oxford, and reduced glutathione was obtained from British Drug Houses.

Sodium hydroxide solutions were prepared just before use from constant volumetric solutions (British Drug Houses) diluted with freshly deionised water, and 2M- and 4M-HCl was prepared from twice-distilled constant boiling HCl. [^{3}H] -Acetic anhydride (100 mCi/mmole) was obtained from the Radiochemical Centre, Amersham. Dowex ion-exchange resins were from Sigma Chemical Co. Polyamide sheets (15 x 15cm) were obtained from Cheng Chin Trading Co. Ltd., Taipei, Taiwan, and were purchased from Micro-

Bio Laboratories, 46 Pembridge Road, London. Sephadex G-10 and G-25 (fine grade) were obtained from Pharmacia, Uppsala, Sweden.

Pyridine (Analar) was refluxed over NaOH pellets, and distilled through a fractionating column. The fraction with boiling point 115°-116°C was collected. Acetic anhydride was distilled from anhydrous sodium acetate through a fractionating column, and the fraction with boiling point 104°C was collected. Methanol was refluxed with magnesium methoxide, and distilled. Atmospheric moisture was excluded from these solvents during purification. n-Butanol was redistilled and the fraction with boiling point 116°-118°C was collected.

2-Thiobarbituric acid was bought from Eastman Kodak, and para-dimethylaminobenzaldehyde from Fisons Ltd., Loughborough, England, and was purified by the method of Adams and Coleman (1948).

METHODS.

The autoanalyser systems, methods for hexose and amino acid determinations etc., are described in Chapter 6.

Measurement of rates of reaction in alkali.

Small amounts of GlcNAc-Asn or Glc-Asn were weighed out, together with L-valine. These substances were dissolved in water or in NaOH solution whose concentrations were 0.05M, 0.2M or 1M. A solution was also prepared in 0.2M NaOH which contained 1M-KCl. The solutions were all 6mM in reactant.

Small tubes were made by sealing 10cm lengths of thick-walled borosilicate tubing (3mm internal diameter) at one

end, and 50µl portions of the solutions under investigation were pipetted into them. The tubes were evacuated with a high-vacuum oil-pump and sealed off in a hot flame. The tubes were immersed in a boiling water-bath. Sets of duplicate tubes were removed, after various lengths of time, cooled in a dry ice-acetone mixture and opened. The contents of one set of tubes were taken up in 1ml volumes of pH 2.2 citrate buffer (0.2M-Na^+) and the ninhydrin-positive components measured in either 250µl or 200µl portions, usually on the Locarte autoanalyser, but the Technicon was also sometimes used. The contents of the other set of tubes were taken up in 4M-HCl (3ml) and hydrolysed in sealed, evacuated tubes for three hours in a boiling water-bath. These tubes were then opened and, after cooling, were placed in a vacuum desiccator over NaOH pellets and conc. H_2SO_4 at room temperature. The dry residues were taken up in pH 2.2 buffer and placed on the Locarte autoanalyser.

The stability of GlcNAc-Asn was also studied in deionised water alone for times up to 48 hr at 100°C, and in pH 10.2 buffer that was 0.5M in HCO_3^- plus CO_3^{2-}, and 0.81M in Na^+, for 260 min at 100°C, by the same experimental procedure.

In control experiments, solutions (6mM) of L-aspartic acid, together with L-valine in 0.2M-NaOH both in absence and presence of N-acetyl-D-glucosamine (6mM), were heated to 100°C in the same manner for 12 hr, taken up in pH 2.2 buffer and autoanalysed.

The rate of conversion of L-asparagine to L-aspartic acid was studied in 0.2M-NaOH at 6mM concentration for

for times up to 4 hr at 100°C. The solutions were taken up in pH 2.2 buffer and autoanalysed.

The disappearance of D-glucosamine, measured after acid hydrolysis, from N-acetyl-D-glucosamine, GlcNAc-NH_2 and GlcNAc-NAc, during reaction in 6mM solutions in 0.2M-NaOH, was also studied by the same method with DL-norleucine as internal standard. In these experiments the solutions of the substances which had been treated with alkali were taken up in 2ml 4M-HCl and hydrolysed at 100°C for 3 hr before autoanalysis.

Separation and measurement of the reaction products on the Locarte autoanalyser.

The standard amino-acid programme of the Locarte autoanalyser was modified as follows (see Table 3.2). The pH of the starting buffer was lowered from the standard conditions recommended by the manufacturers by 0.1 unit to pH 3.15 in order to obtain good resolution of GlcNAc-Asn, and Glc-Asn from aspartic acid. The percentage of methanol in the pH 3.15 buffer added to the loading column was increased from 20% to 30% (v/v) in order to produce a sharp peak for GlcNAc-Asn.

The molar colour yields of standard components were determined in the presence of fixed concentrations of L-valine as internal standard in weighed samples containing various molar ratios of GlcNAc-Asn, Glc-Asn and L-aspartic acid, and L-valine. With an approximately constant area (4.7) for the L-valine peak, the areas of the peaks of GlcNAc-Asn and Glc-Asn ranged from 1.0 to 7.3, and that of L-aspartic acid from 2.5 to 19.0. Between these limits,

the relative molar colour yields of GlcNAc-Asn and Glc-Asn increased by 7% and that of aspartic acid by 10%. From these data, calibration curves were constructed and used in the kinetic experiments. The relative (to valine) molar colour yield of GlcNAc-Asn was found to vary from 0.424 to 0.456 and that of Glc-Asn from 0.543 to 0.578.

Measurement of ammonia evolved by treatment of GlcNAc-Asn and GlcNAc with alkali.

The ammonia produced during the reaction of GlcNAc-Asn with 0.2M-NaOH was measured by absorption into boric acid. The experiments were performed in Thunberg tubes. Measured volumes (300µl) of 6mM solutions of GlcNAc-Asn in 0.2M-NaOH were placed in the bottoms of the Thunberg tubes and 1ml quantities of 2% boric acid solution were pipetted into the top sections. Tubes containing 300µl of 0.2M-NaOH in the base were treated in the same way as blanks. The tubes were then evacuated on a water pump for 2 min and sealed. These tubes were then completely immersed in boiling water for varying lengths of time. Complete immersion was found necessary to avoid distillation of the solutions. For the longer times of reaction, it was found more convenient to place the tubes in a constant temperature oven. The tubes were removed and completely immersed in cold water. After standing for 30 min at room temperature, the tubes were opened and 1µl volumes of a solution of 20mg methyl red and 10mg methylene blue dissolved in 10ml ethanol were added to the boric acid. The boric acid solutions from the blank tubes were titrated to a grey-coloured end-point with 0.02M-NaOH added from a micrometer syringe. The boric acid solutions containing absorbed ammonia were

also titrated to the same colour using either 0.02M-NaOH or 0.02M-HCl, and the difference in μ moles of titrant required were equated to the number of μ moles of ammonia absorbed.

Control experiments were performed in order to measure the amounts of ammonia evolved from aqueous ammonium sulphate solutions (10 moles in 0.3ml) under these conditions. The solutions were kept apart from the alkali (0.3ml, 0.4M-NaOH) until the tubes had been closed and sealed by placing the 0.4M-NaOH in small containers made from 1cm lengths of borosilicate tubing. To start the reaction, the tubes were held at an angle of about 60°C from the vertical and shaken until the two solutions were thoroughly mixed. These tubes were heated for 10 min and treated as described above. It was found that 99.0 ± 0.7% of the ammonia contained in the ammonium sulphate solution was absorbed into the boric acid under these conditions.

The evolution of ammonia from N-acetyl-D-glucosamine (6mM) was measured in the same way, for various periods of time of heating at 100°C, after mixing with 0.2M-NaOH contained in the small containers as described above.

Measurement of ultra-violet absorption of products formed by alkaline treatment of compounds.

Solutions (6mM) of GlcNAc-Asn, GlcNAc-NAc, N-acetyl-D-glucosamine or 2-acetamido-2-deoxy-β-D-glucopyranosyl-amine were flushed with nitrogen and heated in a boiling water-bath in flasks fitted with reflux-condensers, while nitrogen bubbled through the solution. Measured volumes

(300µl) were withdrawn after various times and diluted ten-fold with water. The pH was brought to 5 by addition of 0.4M-hydrochloric acid from a micrometer syringe, and the ultra-violet absorption spectra recorded from 220 to 420nm against a water blank. The spectrum was also measured at different pH values after addition of small amounts of 0.1M-HCl or 0.1M-NaOH to the solution from a micrometer syringe.

Estimation of colour formed with Ehrlich's reagent.

Aliquots (1ml) of the reacted solutions were diluted to 10ml with water, and 0.5ml amounts were added to 3ml of Ehrlich's reagent. The mixture was heated at 36°C for 20 min, cooled, and the extinctions at 585nm read in 1cm cells, against water. A reagent blank was also carried out.

Test for colour formation with 2-thiobarbituric acid.

To 90ml water were added 0.71g crystalline 2-thiobarbituric acid and 0.7ml 1.0M-NaOH. Dissolution was facilitated by shaking and warming. The volume was adjusted to 100ml with water. Aliquots (0.5ml) of the reaction products of 6mM solutions of GlcNAc-Asn, GlcNAc and GlcNAc-NH_2 that had been heated in 0.2M-NaOH for at least 24 hr, were neutralised to about pH 7 with 0.4M-HCl, and brought to 1ml with water. The thiobarbituric acid (2ml) was added, and the solutions were shaken and heated at 100°C in a stoppered tube. The tube was then cooled and the extinctions at 532nm of the solutions were measured against a water blank.

Examination of acidic products by thin-layer chromatography.

A solution of GlcNAc-Asn (13μmoles) in 0.2M-NaOH (2ml) was heated in a sealed, evacuated tube for 24 hr at 100°C. The reaction was placed on a column of Dowex 1-X2 (200-400)(Cl^- form; 6 x 2cm), which was eluted successively with water (50ml), 0.05M-HCl (50ml) and finally with 0.5M-HCl. The effluents were collected and evaporated in a rotary evaporator and then in a vacuum desiccator. Pellets of NaOH were also present in the evaporator and the desiccator. The residue was taken up in 200μl water, and 2μl aliquots were chromatographed on cellulose thin-layer plates in ether - formic acid - water (7 : 2 : 1, by vol) or in phenol - water - formic acid (75 : 25 : 1, w/v/v), together with fumaric and maleic, malic and pyruvic acids (2.5mg/ml solutions) as standards. The substances were located with a spray indicator consisting of 40mg bromocresol green in 100ml ethanol previously neutralised with 0.1M-NaOH. The substances appeared as yellow spots on a green background.

For reasons which have been discussed (see page 153), the solution obtained from the 0.05M-HCl eluate of Dowex 1 was placed on a Dowex 50-X4 (400 mesh)(H^+ form) (1 x 4cm) and eluted with 12ml water. The effluent was freeze-dried and taken up in 100μl water, and run on thin-layer cellulose plates as before.

Mutarotation of GlcNAc-NH_2.

The optical rotations of solutions of GlcNAc-NH_2 (40mg/ml) both in water and also in 0.2M-NaOH, were measured

Table 3.16. The conditions under which various N-acylglycosylamines and other compounds were heated in the presence of 1M-$NaBH_4$.

Compound	Conditions of alkali	Temp(°C)	Longest time of heating	Conditions of acid hydrolysis(100°C)
GlcNAc-Asn	0.05M-NaOH	100	6 hr	4M-HCl; 3 hr[a]
	0.2M-NaOH	100	6 hr	4M-HCl; 3 hr[a]
	1.0M-NaOH	100	3 hr	4M-HCl; 3 hr[a]
	2.0M-NaOH	100	5 hr	4M-HCl; 3 hr[a]
	(1M-$NaBH_4$ alone)	100	4 hr	4M-HCl; 3 hr[a]
	0.2N-NaOH	82	11 hr	4M-HCl; 3 hr[a]
	0.05M-NaOH	47	50 hr	4M-HCl; 3 hr[a]
	0.5M-phosphate buffer(pH 7.0)	25	48 hr	4M-HCl; 3 hr[a]
L-Asn	0.2M-NaOH	100	2 hr	-
DL-Homoserine	0.2M-NaOH	100	3 hr	-
GlcNAc	2.0M-NaOH	100	3 hr	4M-HCl; 18 hr[b]
GlcNAc-NH_2	0.2M-NaOH	100	2 hr	4M-HCl; 18 hr[b]
NN'-Diacetyl-chitobiose	2.0N-NaOH	100	3 hr	4M-HCl; 18 hr[a]
GlcNAc-NHAc	0.05M-NaOH	100	5 hr	4M-HCl; 3 hr[b]
Hen ovalbumin glycopeptide	0.05M-NaOH	100	13 hr	4M-HCl; 18 hr[b]
	0.2M-NaOH	100	11 hr	4M-HCl; 18 hr[b]
	1.0M-NaOH	100	10 hr	4M-HCl; 18 hr[b]
	2.0M-NaOH	100	10 hr	4M-HCl; 18 hr[b]

(a) Measurements were made on products of alkaline reduction both before and after subjecting them to acid hydrolysis.

(b) Measurements were made on the products of alkaline reduction only after acid hydrolysis.

Sodium borohydride is of course destroyed in hot aqueous solutions. The reducing agent was therefore replenished at various stages during the reactions, according to the following schedules.

Conditions of alkali	Vol. alkaline $NaBH_4$ added (% of initial volume)	Intervals of addition
0.05M-NaOH	25	40 min
0.2M-NaOH	20	60 min
1.0M-NaOH	16	60 min
2.0M-NaOH	16	60 min
1.0M-$NaBH_4$ alone	25	20 min

at 365 nm at 25°C in a thermostated cell with a 1 dm. path-length over periods of time up to 24 hr. A small portion (50μl) was withdrawn from the solution in 0.2M-NaOH after 24 hr and hydrolysed with 4M-HCl (3ml) for 3 hr at 100°C together with DL-norleucine. The D-glucosamine content of the dried hydrolysate was measured.

Study of the decomposition of N-acyl glycosylamines in alkaline borohydride.

Solutions (6mM) of the substrates listed in Table 3.16 in 1M-$NaBH_4$, together with the concentrations of alkali shown, were heated in a 5ml round-bottomed flask fitted with a reflux condenser on a boiling water-bath, together with known amounts of one or other of the internal standards, L-valine or 4-fluoro-DL-β-phenylalanine.

It was noted that the borohydride was gradually destroyed as the reaction proceeded because of its reaction with water. Acidification of a solution of 1M-$NaBH_4$ in 0.2M-NaOH that had been kept at 100°C for 2 hr, no longer produced evolution of hydrogen. The borohydride appeared to be destroyed more rapidly in the presence of lower concentrations of alkali (0.05M-NaOH) than with the higher concentrations (2.0M-NaOH). In order that substrates should be continuously in the presence of reducing agent, further amounts of sodium borohydride dissolved in alkali were added as the reaction proceeded in the amounts, and at the intervals, described in Table 3.16. Duplicate (50μl) samples of the reaction mixture were removed after various periods of time and brought to about pH 5 with 4M-acetic acid. To one sample citrate buffer (pH 2.2)

was added, and the ninhydrin-positive components measured on the Locarte autoanalyser. The second set of aliquots (50µl) of the reaction mixtures were acidified with 4M-HCl (2ml) and hydrolysed in sealed, evacuated tubes for either 3 hr or for 18 hr (see Table 3.16) at 100°C. The hydrolysates were dried in a vacuum desiccator over conc. H_2SO_4 and solid NaOH, taken up in pH 2.2 citrate buffer and placed on the autoanalyser. The products formed in the reactions of L-asparagine and of DL-homoserine in 0.2M-NaOH, 1M-$NaBH_4$ at 100°C were examined without acid hydrolysis.

Autoanalysis of reaction products.

Most of the ninhydrin-positive components of the products could be separated on the Locarte autoanalyser under the conditions mentioned previously (Table 3.2).

The lactone of homoserine was not separated from ammonia by this buffer programme, but could be separated by replacing the pH 6.65 buffer with one of pH 5.28 (0.5M-Na^+; 140 min elution). Peaks obtained on the autoanalyser were identified by running standard amino acids and amino sugars, under the same conditions as those applied to the reaction products, and by comparing the times of elution. In some cases further confirmation was provided by measurements of the peak areas obtained at 570nm and 440nm. The standard compound was then run together with the products of reaction. The increased area of the peak was measured, and the peak was checked for any signs of asymmetry. Molar colour yields were determined by running together known amounts of the

standard amino acids or hexosamines and the internal standards L-valine or 4-fluoro-DL-β-phenylalanine.

DL-Homoserine (about 1 μmole) was heated together with L-valine for 3 hr in 4M-HCl (5ml) at 100°C in a sealed, evacuated tube. After the hydrolysate had been dried, the residue was taken up in citrate buffer pH 2.2, and placed on the autoanalyser. Peaks of both homoserine and homoserine lactone were found and measured. The molar colour yield of homoserine lactone was computed on the assumption that all homoserine destroyed had been converted to the lactone; this is a reasonable assumption in view of the concentrated acid employed in the hydrolysis (see for example Greenstein & Winitz, 1961).

A standard for the recognition of D-mannosaminitol was prepared in the following way. N-Acetyl-D-mannosamine (50 μmoles) together with L-valine (20 μmoles) was weighed out, dissolved in 1M-$NaBH_4$ (2ml) and left at +4°C for 48 hr. Aliquots (10μl) were withdrawn and hydrolysed in 4M-HCl for 3 hr at 100°C. The dried residue was autoanalysed. No peak at the position previously determined for D-mannosamine was found. The molar colour yield for D-mannosaminitol was computed from the areas of the peaks found, on the assumption that the acetyl amino sugar had been reduced quantitatively (Mayo & Carlson, 1970).

Dansylation of the products of GlcNAc-Asn formed by treatment with alkaline borohydride.

GlcNAc-Asn (4mg, 12 μ moles) was dissolved in 0.5ml 0.05M-NaOH, 1M-$NaBH_4$ and placed in a 5ml round-bottomed flask fitted with a reflux-condenser. The solution was heated on a boiling water-bath. Further quantities (125μl)

of 0.05M-NaOH, 1M-$NaBH_4$ were added every 40 min as the reaction proceeded. After periods of time of 40 min, of 2 hr and of 4 hr, 100µl, 250µl and 500µl volumes respectively of the reaction mixture were removed. In each case the pH was adjusted immediately to 8.5 with 4M-acetic acid. Solutions of dansyl chloride in acetone (125µl; 15mg/ml) were added to each, and to the samples withdrawn after 2 hr and 4 hr additional quantities of 150µl and 400µl acetone respectively were also added. These latter additions ensured that the compositions of the solvents in the dansylation reactions were the same in all samples. The reaction mixtures were incubated at 37°C for 1 hr, and the volumes adjusted with water to one ml.

Dansyl chloride (100µl; 7.5mg/ml solution in acetone) was added separately to 100µl amounts of 10mM solutions of L-aspartic acid, L-asparagine, DL-homoserine, GlcNAc-Asn and D-glucosaminitol, and the solutions were incubated at 37°C for 1 hr. To these solutions were added portions (100µl) of 0.05M-NaOH, 1M-$NaBH_4$ which had previously been neutralised with 4M-acetic acid, and the dansylated solutions were diluted to 2ml with water.

Small amounts (4µl) of the dansylated samples of the products obtained by reacting GlcNAc-Asn with alkaline borohydride were chromatographed in two dimensions on polyamide sheets, on the reversed sides of which a mixture of the dansylated standards had been placed. On some sheets, the standards were run together with the treated compound. Development in the first direction was performed with water - 90% formic acid (200 : 3, v/v), and

in the 90° vector, either with heptane-n-butanol-glacial acetic acid (3 : 3 : 1, by vol), or with benzene-glacial acetic acid (9 : 1, v/v) (Woods & Wang, 1967). The plates were dried at 40°C for 2 hr before development with the second solvent. The first solvent migrated about 12cm in 40 min at about 22°C; the other solvents took about 2 hr to travel the same distance. The plates were dried at about 40°C for 30 min and subsequently photographed under ultra-violet radiation from two high-pressure mercury lamps, using Kodak high-speed Ektachrome or panchromatic film, with a blue lens filter.

Examination of acidic products formed by alkaline reduction of GlcNAc-Asn.

A solution of GlcNAc-Asn (8.4mg/4ml) in 0.05M-NaOH, 1M-$NaBH_4$ was heated at 100°C for 3 hr, with the addition of further quantities (0.5ml) of 1M-$NaBH_4$/0.05M-NaOH every 30 min. The reaction mixture was cooled, neutralised with 4M-acetic acid, and placed on a column of Dowex 50-X8 (400 mesh; H^+, 10 x 1cm) which was eluted with 50ml water. The eluate was evaporated on the rotary evaporator and then repeatedly evaporated under reduced pressure with absolute methanol in order to remove boric acid. The water-soluble portion of the residue was neutralised with 0.2M-NaOH and placed on a column of Dowex 1-X2; 400 mesh; Cl^-; 5 x 1cm). The column was washed with 40ml deionised water, and eluted with 0.08M-HCl (50ml) followed by 0.8M-HCl (50ml). Each acidic fraction was evaporated to dryness on the rotary evaporator with solid NaOH in part of the condenser, followed by re-evaporation several times

with water. The oily residues were taken up separately in 100μl volumes of water, and 2μl amounts were chromatographed on thin-layers of cellulose with a mixture of ether - formic acid - water (7 : 2 : 1, by vol). The solvent used was that described by Myers & Huang (1969) for the separation of carboxylic acids. Standards were also run. These consisted of 2.5μg amounts of succinic, fumaric, maleic and malic acids. The spots were located with a spray indicator which was a solution of 40mg bromocresol green in 100ml ethanol and which had been neutralised with 0.1M-NaOH. The positions of the acids were indicated as yellow spots on a green background.

The fraction obtained from the Dowex 1 colums by elution with 0.08M-HCl was re-evaporated with methanol several times and then left with dry ether at +4°C. A white solid residue was obtained ($\sim$1mg), which was dried over P_2O_5 *in vacuo* at 60°C and sent for elemental analysis.

Analyses of the components of the glycopeptides.

Two fractions of glycopeptide material with differing contents of D-mannose and N-acetyl-D-glucosamine were used in these experiments. These were labelled glycopeptide I and II and their compositions are described in Tables 3.5 and 3.6. Amino acid and hexosamine analyses were performed on material after hydrolyses in 4M-HCl for 18 hr at 100°C, at a concentration $<$0.05% (w/v).

D-Glucosamine hydrochloride samples (0.2 moles) were treated under the same conditions of acid for periods of time of 18 hr and also of 40 hr with DL-norleucine as internal standard, in the presence and the absence of 50μl

amounts of a neutralised solution of 1M-$NaBH_4$ and 0.2M-NaOH. The recovery of D-glucosamine was 90.1% (18 hr) and 82.4% (40 hr) after these periods of acid hydrolyses both in the presence and the absence of the added amounts of neutralised solutions of alkaline sodium borohydride. The D-glucosamine analyses performed on glycopeptide material both before and after reaction with alkaline borohydride were therefore corrected for a loss upon acid hydrolysis of 10%. D-glucosaminitol was destroyed to an extent of 4% by acid hydrolysis, under the same conditions, for 13 hr, and corrections were also applied to the analyses of D-glucosaminitol performed on reduced oligosaccharide.

Samples of glycopeptide were made up to approximately 6mM with respect to the L-aspartic acid content of the glycopeptide, with solutions of 1M-$NaBH_4$, which were 0.05M-, 0.2M-, 1.0M- or 2.0M in NaOH concentration, together with either 4-fluoro-DL-β-phenylalanine or DL-norleucine as internal standards.

An assessment of the effects of neutralised solutions of sodium borohydride on the phenol-sulphuric acid procedure for neutral sugars was made in the following way. Aliquots (100μl) of a solution of 0.2M-NaOH, 1M-$NaBH_4$, previously neutralised with 4M acetic acid, were added to the D-mannose standards employed in the phenol-H_2SO_4 reaction. Addition of neutralised solutions of sodium borohydride in this way reduced the colour yield given by D-mannose by 8.5%. Standards containing neutralised solutions of sodium borohydride were also employed when hexose assays

were performed on glycopeptide that had been subjected to conditions of alkaline borohydride.

Treatment of glycopeptide with alkaline borohydride.

Glycopeptide was heated under reflux on a boiling water-bath and samples were withdrawn after measured periods of time. In all cases the glycopeptide concentrations were 6mM. Further quantities of alkaline sodium borohydride were added according to the schedule described in Table 3.16. Portions of samples (25 to 100µl) were withdrawn, cooled, acidified with 4M-HCl (2ml), and hydrolysed for 18 hr at 100°C in sealed, evacuated tubes. The hydrolysates were dried *in vacuo* over H_2SO_4 and NaOH at room temperature and analysed using the buffer programme described (Table 3.2). After an appropriate period of time (Table 3.16), the pH of the residual reaction mixture was brought to about 5 with 4M-acetic acid. The hexose content was determined. All measured values are expressed relative to the amount of aspartic acid measured at the start of the reaction.

The analyser programme (Table 3.2) that separated the lactone of homoserine from ammonia was employed with some of the acid hydrolysed samples.

Gel chromatography of glycopeptide material treated with alkaline borohydride.

Glycopeptide I (34µ moles of D-mannose, 6mM) was heated in 2M-NaOH, 1M-$NaBH_4$ at 100°C with further additions of 2M-NaOH, 1M-$NaBH_4$ [20% (v/v) of the initial volume] every hour. After 4 hr, the solution was cooled and the

pH was brought to about 5 with 4M-acetic acid. A precipitate formed which was filtered off, and the supernatant was evaporated on a rotary evaporator at about 40°C, followed by continuous evaporation in the presence of anhydrous, redistilled methanol. The residue was taken up in about 1ml water and placed on the first of two columns, which were connected in series by fine-bore (0.5mm internal diameter) tubing, of Sephadex G-25 (fine grade) equilibrated with 0.1M-acetic acid at +4°C. Each column measured 120 x 1.2cm. The columns were eluted with 0.1M-acetic acid pumped at a fixed flow rate of 18.6ml/hr. Fractions (9.3ml) were collected and 0.2ml portions of these were assayed for hexose by the phenol-H_2SO_4 reaction. A total of 28.4μ moles of hexose was recovered. Those fractions containing hexose were collected in two distinct portions (Fig. 3.34).

Glycopeptide I (45μ moles of D-mannose, 6mM) was also heated in 0.2M-NaOH, 1M-$NaBH_4$ at 100°C, with the further addition at the end of each 60 min of an amount equivalent to 20% (v/v) of the initial volume of 0.2M-NaOH, 1M-$NaBH_4$. After 11 hr, the reaction mixture was cooled, brought to about pH 5. It was treated, as described above, with methanol, and subjected to gel chromatography. Fractions of 4.3ml were collected and 0.2ml aliquots assayed for hexose with the phenol-H_2SO_4 reaction. A total of 36.7μmoles hexose was recovered. Fractions containing hexose were collected into three portions (Fig. 3.34) and freeze-dried.

Portions (0.1μ moles D-mannose) pooled as shown in Fig. 3.34, were compared by electrophoresis on paper in

the pH 2 buffer (Chapter 6), followed by staining either by the periodate/2,4-pentane dione indicator or with the use of ninhydrin.

Free amino groups in the fractions were assayed by the quantitative ninhydrin procedure (Chapter 6), using D-glucosamine as standard.

A portion of Fraction B (Fig. 3.34) containing about 8 μ moles hexose was placed on a column of Dowex 50-X4(64 x 1cm) (200-400 mesh; H^+form, suspended in water at +4°C; Horowitz, Roseman & Blumenthal, 1957). The sample was washed in with water (5ml) and elution was effected with a linear gradient of HCl (2 x 250ml containers with 100ml water in the one directly connected to the column, and 100ml 4M-HCl in the other). The eluant was pumped on to the column at a constant flow rate of 16ml/hr and fractions of 3.6ml were collected. Aliquots (0.5ml) were neutralised with 3.5M-NaOH, made up to 1ml, and assayed by the ninhydrin reaction. The amount of alkali required to neutralise the fractions was used to determine the normality of HCl in each fraction.

A further quantity of glycopeptide I (21 μ moles D-mannose, 6mM) was subjected to alkaline reduction in 2M-NaOH, 1M-$NaBH_4$ at 100°C. The reaction was continued for 10 hr, with the addition of 16% of the initial volume of 2M-NaOH, 1M-$NaBH_4$ every hour. The cooled, neutralised product was subjected to gel chromatography as described above, yielding material containing 16.7 μ moles hexose. Portions (0.5ml) of the fractions obtained were also subjected to periodate-oxidation for 30 min, and assayed

for formaldehyde under the conditions described below. The fractions containing hexose were pooled and later fractions (260-300ml of effluent, see Fig 3.37) that contained no hexose, but produced formaldehyde as a result of periodate-oxidation, were also collected.

Glycopeptide II (36 μmoles mannose, 6m$\underline{M}$) was treated with 2$\underline{M}$-NaOH, 1$\underline{M}$-$NaBH_4$ at 100°C for 10 hr as before. The reacted material was divided into two equal parts and one part subjected to gel chromatography to yield 15 μmoles hexose. The other portion was acetylated, as described in the following section, before being filtered on the G-25 columns. The analyses obtained on acid hydrolysates of the unacetylated and the re-$\underline{N}$-acetylated products are described (Table 3.8).

Portions of the pooled reduced oligosaccharides formed when glycopeptides I and II were subjected to alkaline reduction for 10 hr, and to gel chromatography, each containing about 0.4 μmoles hexose, were compared by electrophoresis on paper at pH 2.0 for 30 min. The paper was developed as described above.

Further material was obtained from glycopeptide II (72 μmoles $\underline{D}$-mannose; 6m$\underline{M}$) after treatment with 0.2$\underline{M}$-NaOH, 1$\underline{M}$-$NaBH_4$ at 100°C for 11 hr with the addition of 20% (v/v) of the initial volume of 0.2$\underline{M}$-NaOH, 1$\underline{M}$-$NaBH_4$ every 40 min. A third of the reacted material was placed on the G-25 column as before and a further one third was acetylated before gel filtration.

The remaining third of the neutralised reaction mixture (24 μmoles hexose) was made up to 2$\underline{M}$ with respect

to NaOH by addition of an appropriate amount of 5M-NaOH. The mixture was heated in a sealed, evacuated tube at 100°C for a further 10 hr. The solution was then neutralised with 4M-acetic acid and placed on the G-25 column. The fractions containing hexose in the effluent (21 μmoles hexose) were pooled. Analyses of these three fractions are described (Table 3.8).

Portions containing about 0.1 μmoles hexose of the pooled fractions containing reduced oligosaccharides obtained by gel chromatography as a result of alkaline reduction of glycopeptides I and II as described above, were hydrolysed in 4M-HCl (2ml) at 100°C for 18 hr in sealed, evacuated tubes; the hydrolysates were dried *in vacuo* over conc. H_2SO_4 and solid NaOH. The residues were taken up in pH 2.2 citrate buffer and autoanalysed on the Locarte machine as described previously. Hexose was determined by the phenol-H_2SO_4 reaction. The more retarded fractions that contained no hexose, but produced formaldehyde as a result of periodate-oxidation, were autoanalysed before and after acid hydrolysis in 4M-HCl at 100°C for 18 hr.

Gel chromatography after N-acetylation.

The other halves of the products resulting from alkaline reduction of glycopeptides I and II in 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr, and the other third of the product resulting from the treatment of glycopeptide II with 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for 11 hr were evaporated to dryness and re-evaporated several times with anhydrous methanol. The soluble portions of the residues were

dissolved in 200µl water and N-acetylated by the method described by Etzler et al., (1970), conditions which lead to a small amount of O-acetylation also.

After removal of pyridine and acetic acid by evaporation, the residues were left with 0.5ml 0.2M-NaOH at 4°C for 1 hr in order to remove any O-acetyl groups present. After neutralisation with 4M-acetic acid, the solutions were fractionated by gel chromatography as described previously. The fractions were assayed for hexose, and for formaldehyde released after periodate-oxidation. The fractions containing hexose were pooled, re-assayed for hexose and portions were hydrolysed in 4M-HCl for 18 hr at 100°C, and placed on the autoanalyser as described previously.

The procedures of N-acetylation followed by gel filtration did not lead to any extensive losses of hexose from the reduced materials. The amounts of reduced substances, obtained from glycopeptides I and II after reduction in 2M-NaOH which were subjected to these further treatments contained 21µ moles and 24µ moles hexose respectively. Recoveries of 15µ moles and 21µ moles respectively were obtained. Similarly, reduced glycopeptide (24µ moles hexose) produced by reduction in 0.2M-NaOH was recovered in a yield of 88% (21µ moles hexose).

Molecular weight determination of re-N-acetylated, reduced oligosaccharides.

The columns of Sephadex G-25 described above were calibrated with compounds of known molecular weight. Unreacted hen ovalbumin glycopeptide I, lacto-N-difucohexaose I, lacto-N-difucopentaose I, stachyose, raffinose,

lactose and glucose were each dissolved separately in 0.5ml 0.1M-acetic acid, placed on the G-25 columns in series (page 260) and eluted with 0.1M-acetic acid as described. Fractions were monitored with the phenol-H_2SO_4 reaction and the elution volume (V_e) for each substance was determined. The diffusible volume (V_{NaCl}) was determined as the elution volume of sodium chloride, for which the fractions were assayed conductometrically. The void volume (V_o) was determined as the elution volume of blue dextran. The quotient $\frac{V_e - V_o}{V_{NaCl} - V_o}$ was plotted against molecular weight for each of the standard substances (Fig. 3.41).

The elution volumes for glucose, lactose, lacto-N-difucohexaose, unreacted glycopeptide II and reduced oligosaccharide prepared from glycopeptide II by treatment with 2.0M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr, were also determined after equilibration of the same columns with 0.15M-NaCl and elution with 0.15M-NaCl (Bhatti & Clamp, 1968).

The elution volumes in 0.1M-acetic acid of the reduced oligosaccharide obtained by N-acetylation of glycopeptide II which had previously been treated with 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for 11 hr and that obtained by N-acetylation after treatment with 2.0M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr were measured.

The elution volumes of glutathionine and oxytocin in 0.1M-acetic acid were also determined. The ninhydrin reaction was used to identify the position in the effluent of

glutathione and the absorbance at 280nm was used for oxytocin.

Determination of free amino groups.

Reduced oligosaccharides obtained by gel chromatography were assayed with the ninhydrin reagent (Chapter 6) using D-glucosamine as the standard.

[^{3}H]Acetic anhydride was diluted 100-fold with acetic anhydride and those materials obtained as a result of treating glycopeptide II with 2.0M-NaOH or with 0.2M-NaOH in 1M-$NaBH_4$ for 10 hr and 11 hr respectively were N-acetylated with radioactive acetic anhydride as described above for the unlabelled nucleophilic reagent. The products were subjected to gel chromatography. Portions (50µl) of the fractions obtained were neutralised with 0.1M-NaOH, and 10ml of Bray's (1965) reagent were added. The radioactivity was counted on a Nuclear-Chicago Mark I liquid scintillation counter. The fractions containing hexose were pooled and portions were withdrawn, hydrolysed in acid (4M-HCl 100°C, 18 hr) and placed on the autoanalyser. The radioactivities in further portions were measured.

Methyl-β-D-glucosaminide (20µ moles)(m.p. 127-128°C) in 0.3ml water was N-acetylated as described above. The residue was taken up in 0.1M-NaOH (1ml), left at +4°C for 2 hr and neutralised with 4M-acetic acid. The solution was placed on a column of Dowex 50-X8 (200-400 mesh; H^+ form; 10 x 2cm) and eluted with 50ml water. The effluent was evaporated to dryness on the rotary evaporator at 40°C and dried further in a desiccator over conc. H_2SO_4

and solid NaOH at room temperature overnight. The residue was placed on a Dowex 1-X2 (200-400; OH^- form; 12 x 2cm) and eluted with 50ml water. The effluent was freeze-dried and the residue taken up in 10ml water. A portion (100μl) was withdrawn and the D-glucosamine content determined on an acid hydrolysate. Further portions (50μl) were withdrawn and the radioactivity measured. The radioactivity per μmole of D-glucosamine was calculated and this value was used to calculate the number of N-acetyl groups introduced by N-acetylation of glycopeptide material that had been subject to alkaline reduction.

Infrared spectroscopy.

Material containing hexose (720μg mannose) that had been separated by gel chromatography of glycopeptide II previously treated with either 2M-NaOH, 1M-$NaBH_4$ for 10 hr or 0.2M-NaOH, 1M-$NaBH_4$ for 11 hr, was added to about 1ml water containing 100mg KBr. The solution was freeze-dried, and left in an evacuated desiccator over conc. H_2SO_4 for 24 hr. KBr discs were made in a press and the infrared spectra recorded. Infrared spectra of KBr discs of unreacted glycopeptide II and methyl β-D-glucosaminide were also obtained at concentrations of 1mg per 100mg KBr.

Tests for unsaturation.

To about 200μl of material containing hexose ($\sim$1μmole of mannose) obtained from gel chromatography of glycopeptide II which had been treated with either 2M-NaOH, 1M-$NaBH_4$ at 100°C for 10 hr or 0.2M-NaOH, 1M-$NaBH_4$ at 100°C for

11 hr, was added solid sodium bicarbonate to saturation, followed by a drop of cold 1% aqueous potassium permanganate. A drop of water saturated with bromine was added to each of the samples which had been derived in the same way.

Paper chromatography of acid hydrolysates of hen ovalbumin glycopeptide after alkaline borohydride treatment and re-N-acetylation.

Samples of glycopeptide (5μ moles mannose) which had been reduced under alkaline conditions were hydrolysed either in 2M-HCl or in 4M-HCl (3ml) at 100°C for 3 hr in a sealed, evacuated tube.

The hydrolysates were placed on columns of Dowex 1-X2 (200-400 mesh; acetate form; 8 x 2cm), which were eluted with 100ml water, and the eluted fractions were freeze-dried. The residues were taken up in 200μl water and portions of 16μl and 8μl from both samples were examined by paper chromatography, together with standards of D-mannose (50μg) and D-glucosaminitol (50μg or 25μg). The paper (Whatman 3MM; 46 x 57cm) was serrated at the bottom edge and developed for 12 hr with ethyl acetate : pyridine : water (120 : 50 : 40, by vol; Smith, 1969), using the descending technique. The vapour in the tank was presaturated with that of the solvent. The spots were located with the periodate/2,4-pentane dione reagent and the results are shown in Plate 3.5.

Partial acid hydrolysis of hen ovalbumin after alkaline borohydride treatment.

Material used was obtained from hen ovalbumin glycopeptide II after treatment with 2M-NaOH, 1M-$NaBH_4$

at 100°C for 10 hr, followed by collection of the peak containing hexose, produced by gel chromatography, as previously described.

Small amounts (0.1 μ moles hexose) were hydrolysed separately in the presence of internal standard in 2M-HCl, and also in 4M-HCl at 100°C for 3 hr in sealed, evacuated tubes. The acid was removed over conc. H_2SO_4, and solid NaOH at room temperature in vacuo, and each residue was taken up in pH 2.2 citrate buffer, and placed on the Locarte autoanalyser. The ninhydrin-positive components were estimated after elution with the buffer programme described in Table 3.2 with a slight modification: The pH 3.15 buffer was continued for 140 min.

A further sample containing about 14 μ moles hexose was hydrolysed in 4M-HCl at 100°C (5ml) for 3 hr. The solution was cooled and de-ionised on a Dowex 1-X2 resin as before from which it was eluted with 50ml water. The effluent was freeze-dried. The residue was taken up in 100μl water and a portion (8μl) subjected to paper electrophoresis at pH 2.0 for 30 min. The paper was dipped through the ninhydrin reagent (Chapter 6).

Samples (8μl) were also chromatographed by descending technique on a sheet of Whatman 3MM (46 x 57cm) paper, which was serrated at the lower end. The chromatogram was developed with butanol : pyridine : water (6 : 4 : 3, by vol) for 20 hr; the tank was pre-saturated with the vapour of this solvent.

Preparative paper electrophoresis (pH 2, 30 min, 32V/cm) was performed (Chapter 6) on a 15μl sample on prewashed

Whatman 3MM, spread on a line of about 6cm at the starting point. A small sample spotted separately, was developed with the ninhydrin reagent and corresponding bands were cut out and eluted with water. The eluates (~2ml) were freeze-dried and the residues were taken up in 250µl volumes of water. Portions (100µl) of each were acetylated by the method of Etzler et al., (1970), together with DL-norleucine as internal standard, hydrolysed with 4M-HCl at 100°C for 18 hr, and auto-analysed with the buffer programme described above.

Alkaline reduction of N,N'-diacetyl chitobiose.

N,N'-Diacetyl-chitobiose (7.6mg), together with DL-norleucine standard, was added to 2M-NaOH, 1M-$NaBH_4$ at 100°C (3ml) and heated at 100°C with the addition of further quantities (0.6ml) of 2M-NaOH, 1M-$NaBH_4$ every hour for 3 hr. Samples were removed after 20 min and after 3 hr, acidified with 4M-HCl (2ml) and were hydrolysed at 100°C for 18 hr in sealed, evacuated tubes. The hydrolysates were dried in vacuo over H_2SO_4 and solid NaOH at room temperature and analysed on the Locarte auto-analyser with the buffer programme described above. The yields of D-glucosamine, D-glucosaminitol and D-mannos-aminitol were calculated, and the area relative to that of the DL-norleucine standard of a peak that eluted after 340. min, was recorded.

N,N'-Diacetyl-chitobiose (48mg) was heated with 2M-NaOH, 1M-$NaBH_4$ (5ml) at 100°C for 10 hr, with the addition of further quantities (1ml) of 2M-NaOH, 1M-$NaBH_4$

every hour. At the end of the reaction, the mixture was cooled, brought to about pH 5, centrifuged, and evaporated to dryness on the rotary evaporator at about 40°C, followed by re-evaporation five times with anhydrous methanol. The residue was taken up in 1ml 0.1M ammonium acetate and placed on a column of Sephadex G-10 equilibrated with 0.1M-ammonium acetate at +4°C. Fractions of 4.9ml were collected, and each was assayed by developing 10µl volumes with the periodate/2,4-pentane dione reagent on Whatmann No. 1 paper. The conductivity of each fraction was also measured. Fractions 10-14 all produced fluorescent spots after treatment with the indicator, with a peak at fraction 12, and contained no salt. These fractions were collected and freeze-dried several times after further additions of water, in order to volatilise the ammonium acetate. The yield of product was 44mg. It was taken up in 4ml distilled water.

Duplicate 20µl samples were subjected to electrophoresis (pH 2.0, 30 min, 32V/cm) on paper (see Chapter 6) for 30 min. Strips containing each sample were dipped through the ninhydrin or the periodate/2,4-pentane dione reagents.

Samples (20µl) were also examined by paper chromatography in butanol—pyridine—water (6 : 4 : 3, by vol) as previously described.

Preparative descending paper chromatography was performed on pre-washed Whatmann 3MM paper (46 x 57cm): developed with n-butanol—pyridine—0.1M-HCl (5 : 3 : 2, by vol; Nasamune & Yosizawa, 1956).

A sample (200µl) was placed on a line about 40cm long, and development was continued for about 15 hr. n-Butanol and pyridine were distilled before use, and the 0.1M-HCl was prepared from double-distilled constant-boiling HCl, diluted with de-ionised water. A strip (7cm) was cut off one side of the chromatogram and dipped through the ninhydrin reagent. Strips, corresponding to the bands that stained, were cut from the rest of the chromatogram and eluted with de-ionised water. The eluate was collected (~25ml), freeze-dried and taken up in 5ml water. Samples (200µl) were each added to an aqueous solution of DL-norleucine (50µl), and N-acetylated by the method of Etzler et al., (1970). The residues were each taken up in 500µl de-ionised water and half was diluted with pH 2.2 citrate buffer and eluted from the Locarte autoanalyser with the programme of buffers described above. The remaining 250µl volumes were hydrolysed in 4M-HCl (3ml) for 18 hr at 100°C in a sealed, evacuated tube. The hydrolysate was dried and autoanalysed as described previously.

Formaldehyde production resulting from periodate oxidation of the reduced products of glycopeptide.

The extent of formaldehyde production after various times of periodate oxidation was determined on various samples (Fig. 3.46). Portions containing about 0.5µmoles of mannose in 0.5ml water were placed in foil-covered test tubes cooled in an ice bath. Portions (0.5ml) containing sodium meta periodate (2.14mg/ml) in 0.4M-sodium acetate buffer brought to pH 4.2 with glacial acetic acid, previously cooled in the

ice bath, were added. The tubes were shaken and left in the dark at 0°C in the ice bath for various periods of time up to 5 hr. Excess periodate was destroyed by addition of 250µl potassium iodide solution (17mg/ml) in water, followed by 250µl sodium thiosulphate (55mg/ml) in water. The tubes were shaken between each addition.

The 0.02M-2,4-pentane dione reagent (1.5ml) described by Nash (1953) was added and the absorbance at 415nm determined after 12 hr at room temperature. The quantities of formaldehyde produced from standards (0.05-0.2 µmoles D-mannitol) were measured after 5 hr of periodate oxidation. A reagent blank was also determined.

Nitrogen determination.

Analyses for total nitrogen were performed on material separated by gel chromatography of glycopeptides I and II previously treated with alkaline borohydride. The method is described in Chapter 6.

CHAPTER 4.

SYNTHESES AND PROPERTIES OF N-ACYLGLYCOSYLAMINES.

The studies described in this thesis required the preparation of a number of compounds. The physical properties of a number of the glycosylamines were described by Isbell and his colleagues at the National Bureau of Standards in Washington, D.C., some 30 years ago. However, the biochemical importance of the N-acylglycosylamines was emphasized as a result of the realization that glycinamide ribonucleotide was a naturally occurring intermediate in the biosynthesis of purines (Goldthwait, Peabody & Greenburg, 1954; Buchanan & Hartman, 1959). It was shown later that a structure of a similar type was present in a large number of glycoproteins (see Chapter 1). These latter studies led to a number of attempts to prepare GlcNAc-Asn, but all of the methods used led to disappointingly low yields. One of the purposes of the present work was to try to develop methods for preparing this substance in rather better yields.

Problems in the syntheses of GlcNAc-Asn.

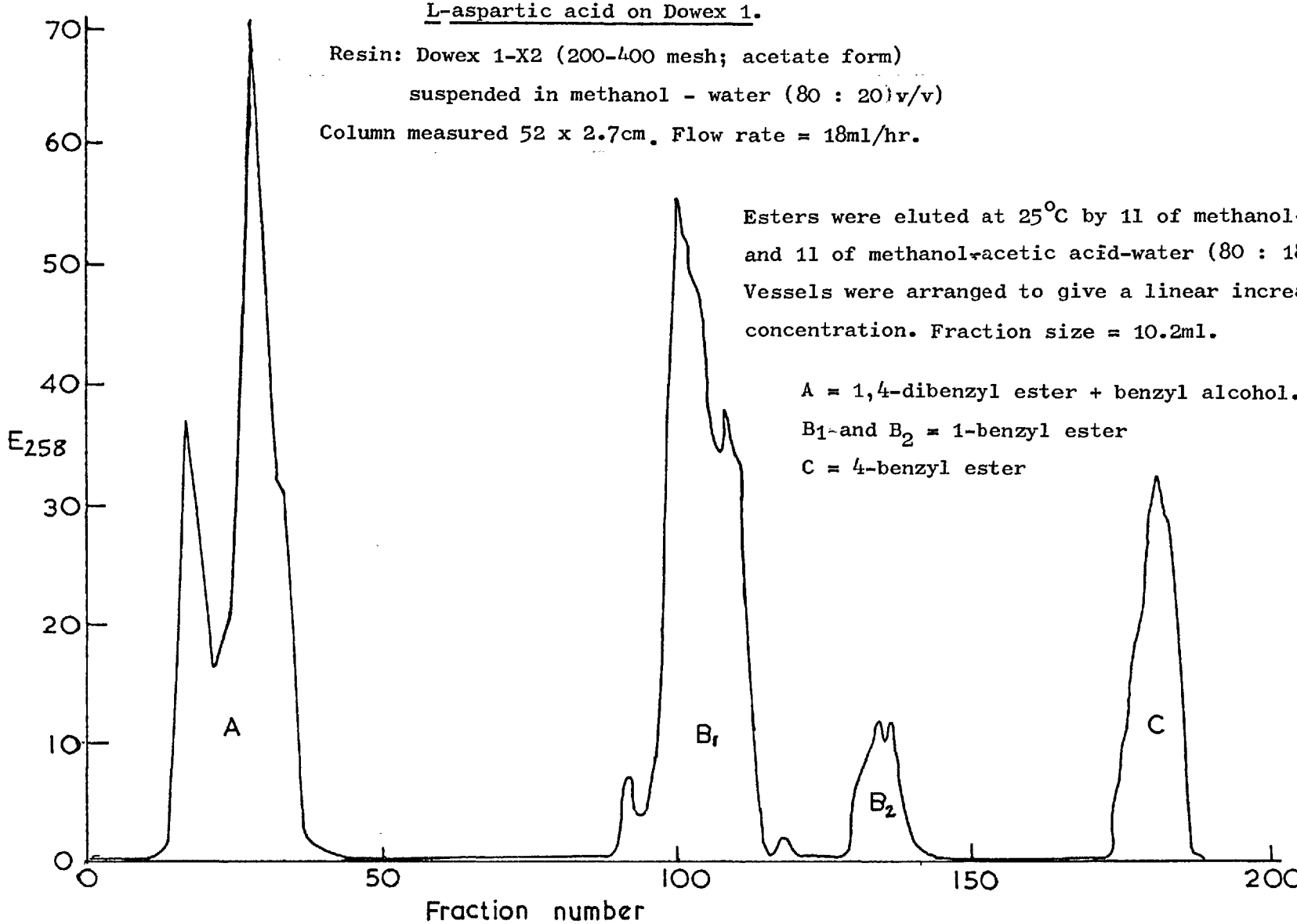
All the methods for preparing these compounds have involved the condensation of a 1-ester of an N-acyl-L-aspartic acid derivative, most commonly the 1-benzyl ester of N-benzyloxycarbonyl-L-aspartic acid, with an appropriately protected glycosylamine. The method developed in this work used this derivative of aspartic acid in the condensation reaction, but a novel method of obtaining this substance has been developed. The

procedure was developed from that of Busch, Hurlbert & Potter (1952) and some preliminary studies had been made by Dr. D.A. Lowther (see Marks & Neuberger, 1961), but it was necessary to make a careful choice of solvent.

Previous methods of separating esters of N-benzyloxycarbonyl-L-aspartic acid have relied on solvent extraction (Le Quesne & Young, 1952), countercurrent distribution (Marshall & Neuberger, 1964), chromatography on a silica gel column (Spinola & Jeanloz, 1970). The dicyclohexylammonium salt of 1-benzyl N-benzyloxycarbonyl-L-aspartate was also used as a method of obtaining this particular ester (Cowley, Hough & Peach, 1971). The present studies show that about 5g of the 1-benzyl ester may be separated from the reaction mixture on a column of Dowex 1 (52 x 2.7cm) in 80% methanol (see page 302). Free N-benzyloxycarbonyl-L-aspartic acid which may be formed by a transesterification (Marshall & Neuberger, 1964) was not eluted from the column under the conditions used. The order of elution of esters from the column was the 1,4-dibenzyl followed by 1-benzyl and then the 4-benzyl esters (Fig. 4.1) as might be predicted from the pK_a values of the carboxyl groups. The 4-benzyl ester was eluted after replacement of the gradient of acetic acid in 80% methanol by a mixture of formic acid, water and methanol. A minor peak (B_2) which was due to material absorbing at 258nm, eluted after the major peak due to the 1-benzyl ester (B_1). This quantitatively smaller product was shown by thin-layer chromatography to be the 1-benzyl ester also. This fractionation may have

Fig. 4.1. Chromatography of the benzyl esters of N-benzyloxycarbonyl-L-aspartic acid on Dowex 1.

Resin: Dowex 1-X2 (200-400 mesh; acetate form)
suspended in methanol - water (80 : 20 v/v)
Column measured 52 x 2.7cm. Flow rate = 18ml/hr.



Esters were eluted at 25°C by 1l of methanol-water (80 : 20 v/v) and 1l of methanol-acetic acid-water (80 : 18 : 2 by vol). Vessels were arranged to give a linear increase in acetic acid concentration. Fraction size = 10.2ml.

A = 1,4-dibenzyl ester + benzyl alcohol.
B_1 and B_2 = 1-benzyl ester
C = 4-benzyl ester

occurred because of its limited solubility in the eluting solvent employed, and it may have partly precipitated out on the resin at some time during the chromatographic process and separated from the major portion of the 1-benzyl ester.

The general procedure for preparing GlcNAc-Asn is described in Fig. 4.2. There has been some discussion as to the optical rotation of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylazide (compound I) and values of $[\alpha]_D^{24}$ as negative as -60° (c 2 in $CHCl_3$) have been reported (Bolton, Hough & Khan, 1966). The possibility that some of the α-anomeric form was produced (Marshall & Neuberger, 1964) was raised, but proof was not given. The substance prepared $[[\alpha]_D^{20} = -44.6°$ (c 2 in $CHCl_3)]$ was found to exhibit only one spot on thin-layer chromatography in those solvents frequently used for separating anomeric mixtures of acetylated sugars (Lewis & Smith, 1969). Fractional crystallisation of the crude material did not lead to recovery of an azide with a positive rotation. In addition, chromatography on Dowex 1 (OH^- form) of GlcNAc-N_3 produced only one peak of fractions containing material that rotated polarised light, and from it a compound was isolated that had the same specific rotation as that of the material placed on the column. Chromatography on Dowex 1 (OH^- form) has not been used previously for purifying sugar azides, but it has been used for separating anomeric glycosides (Austin, Hardy, Buchanan & Baddiley, 1963; Neuberger & Wilson, 1971). It is probable that the $[\alpha]_D^{20}$ value for compound I is

about -45° (c 2 in $CHCl_3$), but the reasons for reports of lower rotation are not clear.

In many of the earlier studies 2-acetamido 3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine (compound II; Fig. 4.2), formed by reduction of the azide, was condensed with the aspartic acid derivative. It was found that better yields of the final product were obtained if the azide (compound I) were first O-deacetylated in alkali. These findings were confirmed (Kiyozumi *et al.*, 1970) after this aspect of the work had been completed. An overall yield of 55% of GlcNAc-Asn was obtained from the protected L-aspartic acid derivative by coupling with the de-O-acetylated glycosylamine (GlcNAc-NH_2) in aqueous pyridine. Previous yields of 39% were reported when the O-acetylated glycosylamine (compound II) was used in the coupling step (Marks, Marshall & Neuberger, 1963). The two synthetic pathways are compared in Fig. 4.2.

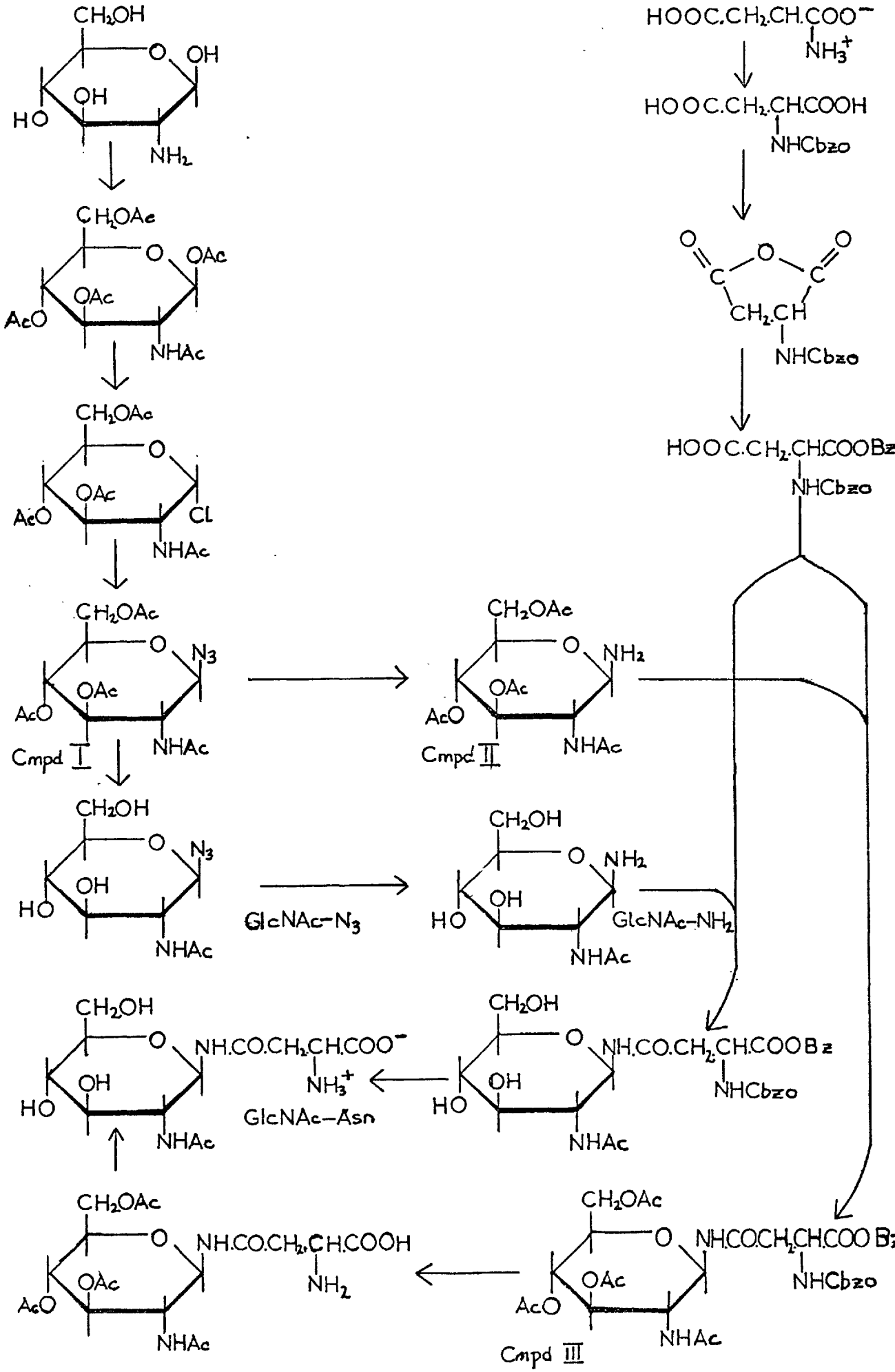
It was also found that the related derivative of β-D-glucose, Glc-Asn, could be prepared in a similar way from β-D-glucosylamine.

There were considerable difficulties encountered in the earlier attempts to reduce, with Adams' catalyst, both compound I and GlcNAc-N_3. These resulted from flushing the apparatus before use with "white-spot" nitrogen, a procedure which led to a rapid inactivation of the catalyst.

Dimerisation of the glycosylamine during the hydrogenation step or during isolation of the product has been noted previously (Bolton, Hough & Khan, 1966), and the $\beta\beta$ and $\alpha\beta$ isomers of di-(2-acetamido-3,4,6-tri-O-acetyl-

Fig. 4·2

Syntheses of GlcNAc–Asn



D-glucopyranosyl)-imine have been isolated after hydrogenation of compound I and concentration of the glycosylamine at temperatures above 40°C. The amounts and physical properties of the O-deacetylated and O-acetylated glycosylamines obtained by the procedures used here and previously (Kiyozumi *et al.*, 1970; Marshall & Neuberger, 1964), showed that good yield of the monomer could be obtained by performing the hydrogenations in methanol and ethyl acetate respectively, followed by removal of the solvent *in vacuo* at temperatures below 25°C. Bolton, Hough & Khan (1966) claimed that the dimerised form of compound II crystallised out of solution when the hydrogenation was performed in ethyl acetate, as used by Marshall & Neuberger (1964), but it was shown from the results of the present studies that identical products in 70% yield were obtained when either ethyl acetate or ethanol was used as solvent.

Single spots were located after thin-layer chromatography of GlcNAc-NH_2 and compound II (Plate 4.1). The O-deacetylated glycosylamine had the same mobility on thin-layer silica plates as D-glucosamine, but the brown colour developed much less rapidly than it did with D-glucosamine after spraying with alkaline silver nitrate. When cellulose plates were sprayed with the same reagent, the spots were faintly visible on a brown background. Hydrogenation of GlcNAc-N_3 was also performed in glacial acetic acid in order to increase the activity of the catalyst (Keenan, Gieseman & Smith, 1954), but the specific rotation of the product was more positive than that des-

Plate 4.1. Thin-layer chromatography of GlcNAc-NH_2

Cellulose plates were developed with ethyl-acetate - propan-1-ol - 0.88 ammonia - water (1 : 7 : 0.1 : 2 by vol)

1 D-Glucosamine (100µg)

2 GlcNAc-NH_2 (200µg)

3 GlcNAc-NH_2 (100µg)

4 GlcNAc-NH_2 (100µg)

Compounds were located with the reagent of Trevelyan, Proctor & Harrison (1950)

Plate 4.2. Crystals of the hydrated and anhydrous forms of GlcNAc-Asn.

GlcNAc-Asn.$3H_2O$ (25 magnification)

GlcNAc-Asn.anh. (160 magnification)

Table 4.1. The physical constants of GlcNAc-Asn as reported in the literature

State of hydration	m.p.(°C) (decomp.)	Specific rotation in water $[\alpha]_D$	temp.(°C)	c.	Ref.
Anhydrous	250	+24.9	22	1.0	1
	264-266	+23.6	24	1.0	2
	255-258	+23.6	24	1.5	3
	257-260	+22.4	30	6.7	4
	254-257(a)	+27	30	4.89	5
	259-261	+26.0	26	0.84	10
Monohydrate	222-223	+18.6	21	1.0	6
	220(b)	+21.4		1.0	7
	219-221	+24	24	1.0	8
Sesquihydrate	230-240	+20.6	23	1.5	9
Trihydrate	215-222	+23.2	24	1.5	2
	211-213	+24.6	26	1.7	10

Ref.

1. Kiyozumi et al (1969).
2. Yamamoto, Miyashita & Tsukamoto (1965).
3. Tsukamoto, Yamamoto & Miyashita (1964).
4. Yamashina, Makino, Ban-I & Kojima (1965).
5. Plummer, Tarentino & Maley (1968).
6. Marshall & Neuberger (1964).
7. Micheel, Tanaka & Romer (1964).
8. Bolton, Hough & Khan (1966).
9. Yoshimura, Hashimoto & Ando (1967).
10. Present studies.

(a) The state of hydration was not recorded, but the infrared spectrum of the compound was that of the anhydrous form.

(b) A further melting point at 252°C was also found.

cribed above, and by thin-layer chromatography the presence of three compounds was shown, one with the same mobility as GlcNAc.

Compound III was also prepared for n.m.r. studies. Purification of the product from the reaction mixture was difficult when the coupling reagent dicylohexylcarbodiimide was used. Therefore, the related water-soluble reagent 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide was used, and the product was washed with dilute aqueous HCl before crystallisation.

Properties of GlcNAc-Asn and related compounds.

A variety of melting points has been recorded for this compound (Table 4.1). It was confirmed in the present studies that a form with a higher melting point readily crystallised from aqueous ethanol at 60°C, while a product with a lower melting point crystallised out at +4°C. These findings agree with those of Yamamoto, Miyashita & Tsukamoto (1965). In a series of experiments in which crystallisation was performed at different temperatures, the transition point between the two forms was found to lie between 10°C and 24°C. But the situation is not clear-cut because on one occasion the form with a higher melting point crystallised out at 4°C, possibly due to the presence of seed crystals.

Examination of the low and high melting forms under the microscope showed that they had different crystalline forms (Plate 4.2). The low melting form had chisel shaped ends, the flat edges of which were orthogonal, and large crystals (approx. 1mm diameter) could be grown. The high

Table 4.2. **Elemental analyses of samples of GlcNAc-Asn (anhydrous) and GlcNAc-Asn (trihydrate) that had been dried at different temperatures.**

Formulae	Temp. of drying (°C)	Found C	Found H	Found N	Calc. C	Calc. H	Calc. N
1. $C_{12}H_{21}O_8N_3 \cdot 3H_2O$	24	37.20	6.76	10.73	37.01	6.94	10.79
2. $C_{12}H_{21}O_8N_3 \cdot 2.75H_2O$	64	37.48	6.68	10.52	37.50	6.93	10.92
3. $C_{12}H_{21}O_8N_3 \cdot 1H_2O$	100	40.36	6.30	12.8	40.79	6.56	11.89
4. $C_{12}H_{21}O_8N_3 \cdot 0.7H_2O$	130	41.34	6.19	11.73	41.42	6.49	12.08
5. $C_{12}H_{21}O_8N_3$ (anhydrous form)	64	42.89	6.21	12.32	42.98	6.31	12.53

1-4 Recrystallised at 4°C.

5 Recrystallised at 60°C.

melting form was obtained as small needles, and attempts to grow large crystals have not yet been successful.

After drying at room temperature, the lower melting form showed an elemental analysis corresponding to a trihydrate, while the high melting form appeared to be anhydrous. After drying the trihydrate for 48 hr at 100°C *in vacuo*, a loss of 9% in weight was recorded. This loss is equivalent to the loss of two molecules of water of crystallisation; this finding contrasts with that reported by Tsukamoto, Yamamoto & Miyashita (1964) who reported no change in weight on heating at temperatures up to 140°C *in vacuo* over P_2O_5.

The elemental analyses, obtained after drying at this temperature, corresponded approximately with a monohydrate. The third molecule of water was not completely removed by drying at 130°C for 24 hr *in vacuo*, a process which resulted in discolouration of the crystals. Elemental analyses are reported in Table 4.2. It was reported by Bolton, Hough & Khan (1966) that the monohydrate lost its water of crystallisation when dried at 100°C.

Determination of the molecular weights of GlcNAc-Asn $3H_2O$ by X-ray crystallographic methods (Table 3.3) confirmed, among other things, that the low melting point form was the trihydrate. Furthermore, in the course of the determination of the three dimensional structure, three molecules of water per molecule of GlcNAc-Asn were located. Determination, by the same method, of the molecular weight of the corresponding compound in which β-D-glucosylamine is linked to L-aspartic acid confirmed that the compound

Table 4.3. Molecular weight determination of GlcNAc-Asn $3H_2O$ by X-ray diffraction methods.

(Performed by Dr. L. Delbaere, Department of Chemical Crystallography, Oxford).

Space group $P2_1$ (2 molecules per unit cell).

Unit cell dimensions:	a = 4.90 Å
	b = 7.70 Å
	c = 24.17 Å
Monoclinic angle	$\angle = 97^{\circ}42'$
Volume of unit cell	V = a x b x c x sin $\angle$
	$= 903.7 \times 10^{-24} cm^3$
Density d	$d = 1.4250 g/cm^3$
Avogadro's number	$N = 6.02225 \times 10^{23} mole^{-1}$
	$d = \frac{2 \times \text{Mol. wt.}}{V \times N}$
Mol. wt.	= 387.8
Calculated for $C_{12}H_{21}O_8N_3 \cdot 3H_2O$; Mol. wt.	= 389.3

Table 4.4. Molecular weight determination of Glc-Asn H_2O by X-ray diffraction methods.

Space group $P2_1$ (2 molecules per unit cell).

Unit cell dimensions:	a = 4.94 Å
	b = 8.08 Å
	c = 16.68 Å
Monoclinic angle	$\angle = 96^{\circ}10'$
Density	$d = 1.5737 g/cm^3$
Mol. wt.	= 313.7
Calculated for $C_{10}H_{18}O_8N_2 \cdot H_2O$; Mol. wt.	= 312.2

is the monohydrate (Table 4.4). Crystallisation of Glc-Asn at high temperatures (60°C) did not produce a crystalline form with the properties described by Coutsogeorgopoulos & Zervas (1961) for an anhydrous form.

The infrared spectra of the two forms of GlcNAc-Asn differed considerably (Fig. 4.3a) and agreed with those published elsewhere (see Marshall & Neuberger, 1972). The most notable differences were in the amino-acid I and amide I band regions (1600-1700cm^{-1}) and at 1300cm^{-1} (see Table 4.5). The spectra given by the trihydrated material after extensive drying (either at 100°C for 2 days <u>in vacuo</u> or under these same conditions followed by drying at 140°C for 18 hr) was not greatly changed (Fig. 4.4). It is still very markedly different from that of the anhydrous form.

The spectra of L-asparagine monohydrate and of the anhydrous form, also differ quite extensively (Fig. 4.3b). The latter was obtained after drying the monohydrate at 120°C (Pelouze, 1833). The anhydrous form was the only derivative examined in the present studies that possessed an absorption frequency at 2100cm^{-1}, characteristic of many amino acids (Greenstein & Winitz, 1961).

Possible assignments of a number of the absorption frequencies observed in the spectra of these, and a number of related compounds, are given in Table 4.5. It might appear that differences between the hydrated and anhydrous forms are due to differences in hydrogen-bonding or some conformational changes affecting the amino-acid group. An intense absorption peak at 1305cm^{-1} that is

Table 4.5. Infrared frequencies (above 1300cm^{-1}) exhibited by GlcNAc-Asn. $3H_2O$, GlcNAc-Asn (anh.), and related compounds, in KBr discs

	Free N-H	H-bonded O-H or N-H	$-NH_3$ def'n	Amino acid I COO^- str.	Amino acid II N-H def'n	Amino acid III $-COO^-$ str.	Amide I -CO str.	Amide II -NH def'n	Amide III C-N str.	$-CH_2$ def'n
GlcNAc-Asn. $3H_2O$		3380 s		1625 s	1500 w	1395 m	1665 s	1558 s	1324 w	1364 w
							1650 s	1550 sh		1350 w
GlcNAc-Asn. anh.		3350 s		1618 w	1512 w	1395 w	1657 s	1555 s	1305 s	
Glc-Asn. H_2O		3430 s		1615 m.	1513 w	1396 w	1660 s	1564 s	1310 s	1375 w
		3350 s								1356 w
GlcNAc-NHAc.	3466 s	3350 s					1672 s	1550 s	1320 m	
							1660 sh	1540 sh		
Glc-NHAc.	3500 s	3320 s					1665 s	1575 m	1320 m	
		3170 s								
GlcNAc.	3480 s	3340 s					1636 s	1552 m	1300 m	
L-Asn. H_2O.	3420 s	3130 s		1645 s	1540 s	1412 m	p1682 s	p1638 sh	1442 s	1372 s
										1322 m
L-Asn. anh.	3380 s	3080 s	2100 w	1618 s	1510 s	1406 s	p1680 s	p1645 s	1432 w	1362 m
										1342 m
2-Amino butyric acid (from data of Koegel et al (1955))			2110 w	1580 s	1510 s	1405 s				1350 m
										1322 s

s - Strong intensity bands. m - medium intensity bands. w - weak intensity bands. sh- shoulders.

Fig 4.3a The infrared spectra of GlcNAc-Asn.3H$_2$O ..... and GlcNAc-Asn, anhydrous ——

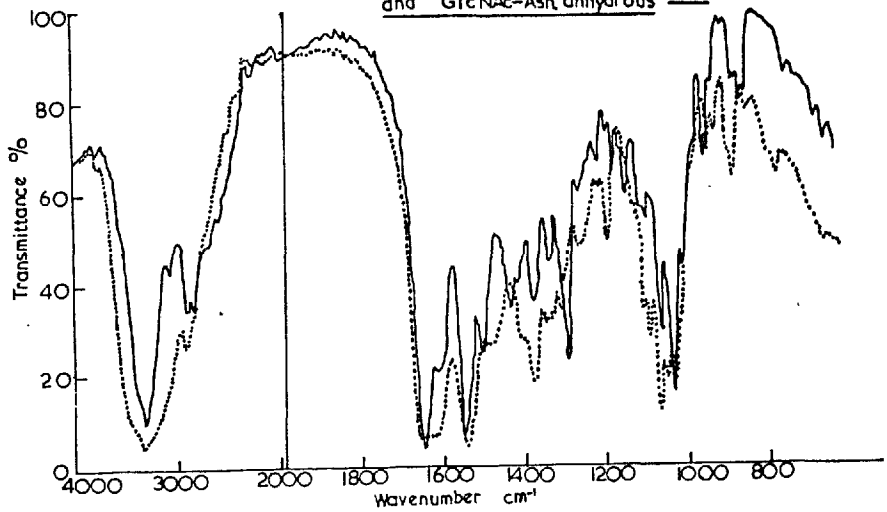


Fig 4.3b The infrared spectra of L-Asparagine.H$_2$O ..... and L-Asparagine. anhydrous ——

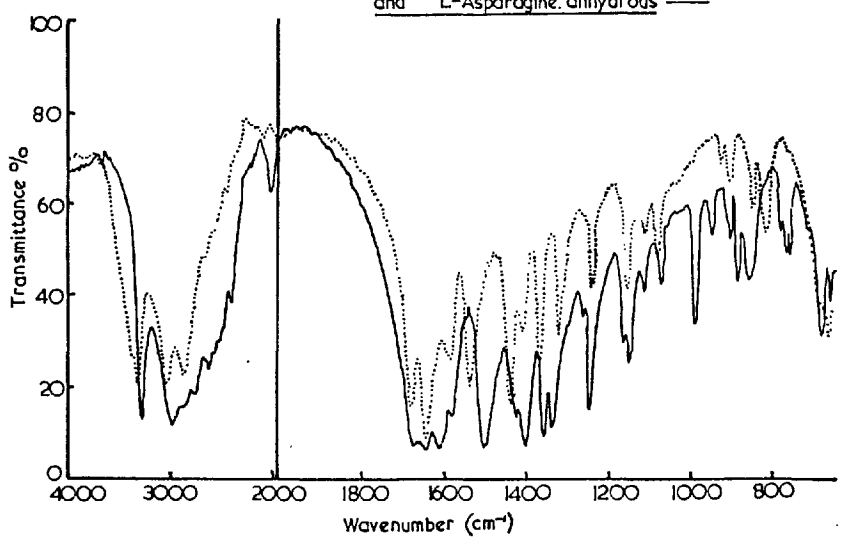


Fig.4.4a INFRARED SPECTRA OF GlcNAc-Asn.3H$_2$O DRIED AT 100°C FOR 48 hr

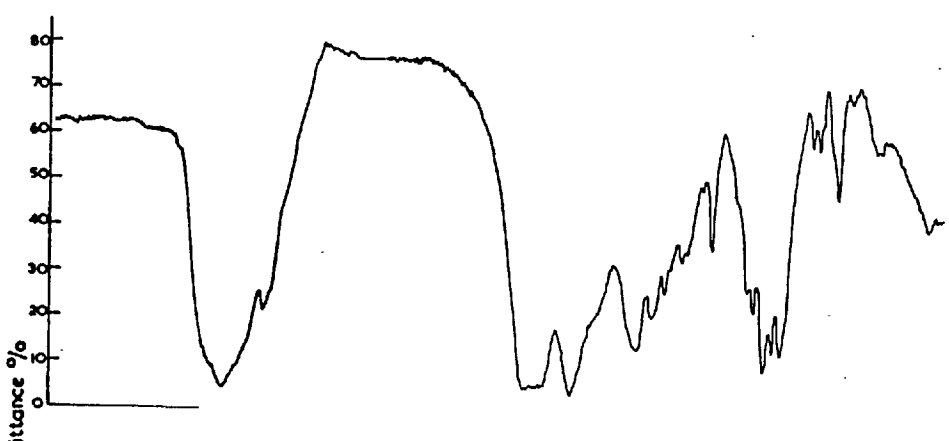


Fig.4.4b INFRARED SPECTRA OF GlcNAc-Asn.3H$_2$O DRIED AT 140°C FOR 18 hr

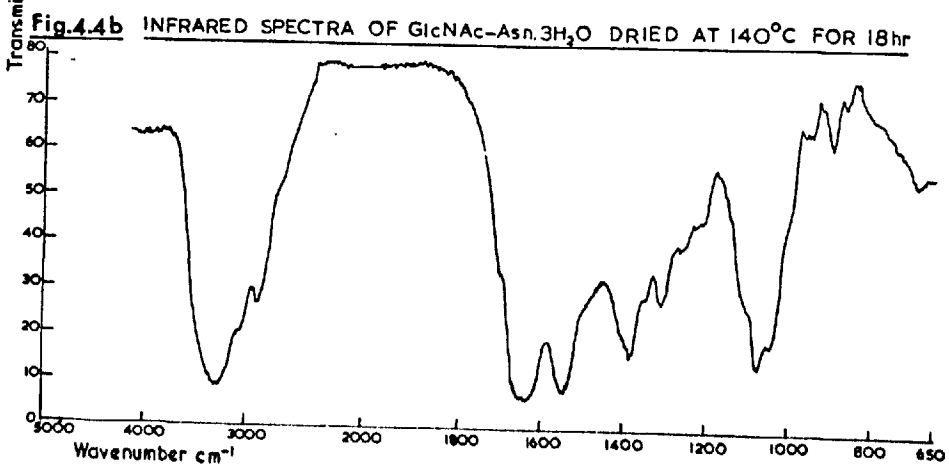


Table 4.6. Molar optical rotations of GlcNAc-Asn in water at various wavelengths and temperatures.

Positive molar rotations (degrees) at various temperatures (t)

nm	6.5°C	18°C	26°C	31°C	41°C	51°C	61°C	72°C	79°C
589	92.9	91.0	88.4	87.0	83.9	78.5	75.7	72.4	69.6
578	98.5	95.2	92.9	92.2	87.0	83.0	79.5	75.2	73.1
546	114	111	107	106	101	96.2	92.2	87.5	84.4
436	216	209	203	200	192	183	175	167	161
365	391	378	368	362	349	334	320	305	282

Change of specific rotation with temperature:- $\frac{d[\alpha]_D}{dt} = -0.1(^\circ \text{per} ^\circ C)$

found in GlcNAc-Asn in its high melting crystalline form only (i.e., in the anhydrous form), is also possessed by Glc-Asn H_2O.

It is interesting that the amide I band exhibited by N-acetyl-β-D-glucopyranosylamine absorbs at higher wavelengths (1665cm^{-1}) than that of N-acetyl-D-glucosamine (1636cm^{-1}). This may reflect the electron-withdrawing properties of the ring oxygen adjacent to the C_1-amide group in the former compound.

There is variation in the values of $[\alpha]_D$ which have been reported for GlcNAc-Asn, but these were measured at various temperatures (Table 4.1). The optical rotation of the compound decreases as the temperature is increased (Table 4.6) in a similar manner to that observed for L-asparagine (Clough, 1915).

On paper, or on thin-layer plates, both GlcNAc-Asn and Glc-Asn stain brown after treatment with ninhydrin in acetone containing 2% pyridine, followed by heating at 80-100°C (Plate 4.3). This brown colour has been observed previously (Marks, Marshall & Neuberger, 1963; Yamamoto, Miyashita & Tsukamoto, 1965) and is also given by glycopeptides that contain the glycosylated L-asparagine residue in an N-terminal position (see for example, Fletcher, Marks, Marshall & Neuberger, 1963; Pollitt & Pretty, 1972 and also page 99).

The molar colour yields of GlcNAc-Asn on the autoanalysers were found to be lower than those of most other amino acids and depended on the autoanalysis system used. The molar colour yield of 0.21 reported previously for

Plate 4.3. Thin layer chromatography of GlcNAc-Asn.

Cellulose plates were developed with phenol-water (4 : 1 w/v). Compounds were located by spraying with acetone containing ninhydrin (1%) and pyridine (2% v/v), followed by heating at 80°C

1 & 3 = GlcNAc-Asn (80µg)

2 & 4 = aspartic acid (20µg)

Plate 4.4. Paper electrophoresis of GlcNAc-Asn.

Electrophoresis was performed at pH 1.85 and 32V/cm for 1 hr. Compounds were located with the ninhydrin reagent described above.

1 = Aspartic acid (25µg)

2 = GlcNAc-Asn (100µg)

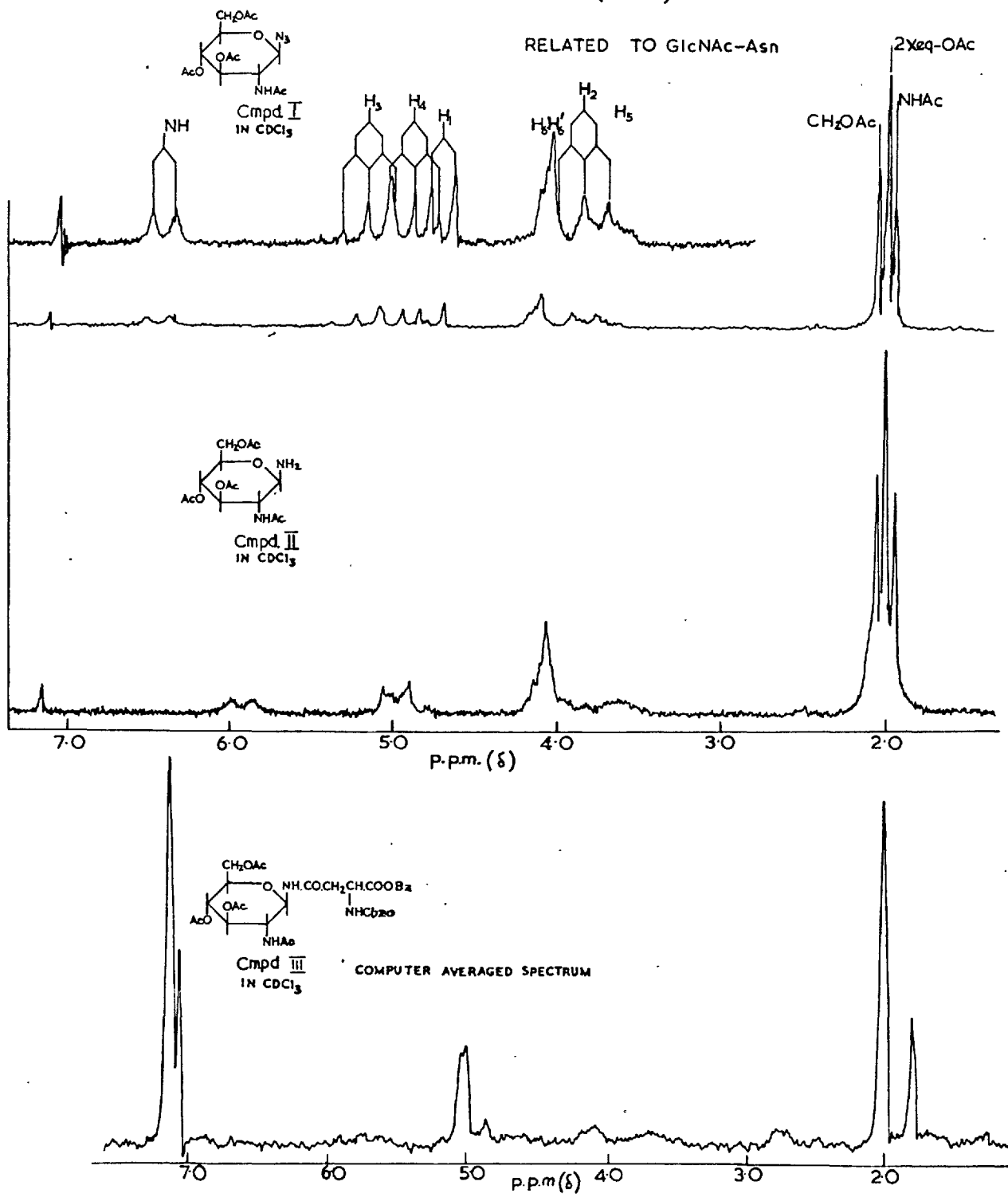
the Technicon system (Marshall, 1969) agreed with that measured in the present studies. On the Locarte analyser a relative value of 0.41 was found (see Experimental section). A colour yield of 0.74 with respect to aspartic acid has been reported for glycopeptides from ribonuclease B containing L-aspartic acid as the only amino acid (Plummer, Tarentino & Maley, 1968), but the analyser system used was not stated.

Nuclear magnetic resonance of a derivative of GlcNAc-Asn and of related compounds.

The n.m.r. spectrum of compound I (see Fig. 4.2) in saturated solution in deuterochloroform is shown (Fig. 4.5). The chemical shifts were measured with respect to the resonance of tetra-methyl silane included as an internal standard. The spectrum is similar to that described by Kiyozumi et al., (1970).

The three single peaks in the region of δ 2.0 p.p.m. may be assigned to the methyl protons of acetoxy groups. The peak at δ 1.98 may be assigned to the N-acetoxy group as this group is known to resonate in the region δ1.90 p.p.m.-δ1.96 p.p.m. (Richardson & McLauchlan, 1962). The two other peaks found near 2.0 p.p.m. may be assigned to a combination of either two equatorial O-acetoxy groups (δ1.98 p.p.m.) and one primary O-acetoxy group (δ2.04 p.p.m.) or to one equatorial O-acetoxy group, one primary O-acetoxy group (δ1.98 p.p.m.) and one axial O-acetoxy group (δ2.04 p.p.m.), because it is known that all axial acetoxy groups resonate at lower fields than equatorial groups, and that primary equatorial groups are often at higher

Fig. 4·5 NMR SPECTRA (60 MHz) OF COMPOUNDS RELATED TO GlcNAc-Asn



fields than secondary groups (Lemieux, Kullnig, Bernstein & Schneider, 1958).

As the splitting of all the resolved ring protons are of the order of 8-9 c.p.s., the arrangement of any pair of vicinal ring protons must be trans-diaxial. Ring protons in a gauche arrangement would couple by about 2.5-3 c.p.s. (Richardson & McLauchlan, 1962; Hall, Hough, McLauchlan & Pachler, 1962). The O-acetoxy groups must, therefore, be equatorial, and the primary O-acetoxy group resonates at lower field than the secondary groups in this case.

The peaks in the region of δ3.8 p.p.m., δ4.1 p.p.m., δ4.7-5.2 p.p.m. and at δ6.4 p.p.m. have been assigned previously by Kiyozumi et al., (1970) by reference to spectra of related compounds. The assignments are shown in Fig. 4.5. The chemical shifts and coupling constants differ only marginally from those measured previously.

The H_1-doublet (δ 4.87 p.p.m.) resonates at much lower field than does the H_1-doublet (δ 4.43) of methyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (Igarashi & Honda, , 1967). This is due presumably to a lesser shielding of the proton at C_1 induced by the azide group.

The n.m.r. spectrum of compound I is, therefore, consistent with a structure in which all the acetoxy groups are equatorial, and the anomeric centre has a β-configuration.

The spectrum of compound II(see Fig. 4.2) is similar to that of compound I in the region of δ2.0 p.p.m. and δ4.1 p.p.m. (Fig. 4.5). An NH doublet appears at

δ 5.91 p.p.m., but other peaks are not well resolved.

The n.m.r. spectrum of compound III (see Fig. 4.2) in solution in $CDCl_3$ does not show completely resolved ring proton resonances, even after application of computer averaging techniques (Fig. 4.5). This compound was also studied in solution in DMSO-d_6 at 100 MHz by Kiyozumi et al., (1970). The N-acetoxy group gives a peak at higher field (δ 1.80 p.p.m.) than do the N-acetoxy groups of the two previous compounds studied; the corresponding peak in the spectrum reported by Kiyozumi et al., (1970) was at δ 1.68 p.p.m. The peak at δ 2.0 p.p.m., which is three times as large as the peak at δ 1.80 p.p.m., may be assigned to the three O-acetoxy groups, which resonate at equivalent field strengths in the spectrum of compound III. The two equally intense peaks at δ 5.0 p.p.m. may be assigned to the methylene protons of the benzyl and benzyloxycarbonyl groups (Markley, Meadows & Jardetzky, 1957), and those at δ 7.03 and δ 7.1 p.p.m. to the aromatic protons. Other regions of resonance are observed at δ 2.7 p.p.m., δ 3.8 p.p.m., δ 4.05 p.p.m. and δ 4.8 p.p.m. after computer averaging. The resonance at δ 2.7 p.p.m. may be assigned to the Cβ protons of the L-asparagine residue (Roberts & Jardetzky, 1970). The other regions of resonance are at the same field strengths as those observed for the ring protons of the compound I.

Preparation of N-acetyl-glycosylamines.

An improved yield of the α-D-galactopyranosyl-ammonia complex was obtained by treating D-galactose with ammonia

Table 4.7. Physical constants of glycosylamines

	Melting point (°C)		Specific rotation (in H_2O)					
	Found	Lit.	Found			Lit.		
			$[\alpha]_D$	temp.	c	$[\alpha]_D$	temp.	c
β-D-glucopyranosylamine	131-133 (decomp.)	125-127	+20.7	24	1.4	+20.8	20	2
β-D-mannopyranosylamine	95-97	93-94	-11.3	25	1.2	-11.6	20	2
β-D-galactopyranosylamine	133-136 (decomp.)	134-136	+63.8	22	2.3	+62.2	20	2
α-D-galactopyranosylamine-ammonia complex	108-110	107-109	+134	22	2.0	+138	20	2
α-L-arabinopyranosylamine	124-126	124-125	+83.3	24	2.8	+86.3	20	2
β-D-xylopyranosylamine	129-131	128-129	-18.1	20	1.0	-19.6	20	2

Table 4.8. Physical constants of peracetylated glycosylamines

	Melting Point (°C)		Specific Rotation (in $CHCl_3$)						Elemental Analysis					
	Found	Lit.	Found			Lit.[a]			Found			Calc.		
			$[\alpha]_D$	temp.	c	$[\alpha]_D$	temp.	c	C	H	N	C	H	N
N-Acetyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamine	162-164	163-164	+18.4	24	2.0	+17.4	20	2	49.12	5.99	3.71	49.35	5.95	3.60
N-Acetyl-2,3,4,6-tetra-O-acetyl-β-D-mannopyranosylamine	189-191	188-189	-17.6	25	0.8	-16.5	20	2	49.46	5.96	3.59	49.35	5.95	3.60
N-Acetyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranosylamine	164-166 Phase change at 150°C	173-274	+36.8	24	0.7	+34.7	20	2	49.56	6.00	3.52	49.35	5.95	3.60
N-Acetyl-2,3,4,6-tetra-O-acetyl-α-D-galactopyranosylamine	173-174	172-173	+122	24	2.2	+117.4	20	2	49.53	6.04	3.76	49.35	5.95	3.60
N-Acetyl-2,3,4-tri-O-acetyl-α-L-arabinopyranosylamine	179-180	177-178	+89.8	26	1.6	+89.6	20	2	49.38	6.10	4.32	49.21	6.03	4.42
N-Acetyl-2,3,4-tri-O-acetyl-β-D-xylopyranosylamine	171-173	172-173	+28.7	25	1.6	+28.5	20	2	49.27	5.94	4.55	49.21	6.03	4.42

(a) Literature values are those reported by Isbell and Frush (1958).

Table 4.9. Physical constants of N-acetylglycosylamines

	Melting points (°C)		Specific Rotation (in water)						Elemental Analyses					
	Found	Lit.	Found			Lit.[a]			Found			Calc.		
			$[\alpha]_D$	temp.	C	$[\alpha]_D$	temp.	C	C	H	N	C	H	N
N-Acetyl-β-D-glucopyranosylamine	253 (decomp.)	260 (decomp.)	-23.3	24	1.5	-22.8	20	2	43.67	6.86	6.56	43.43	6.84	6.33
N-Acetyl-β-D-mannopyranosylamine monohydrate[b]	202-204	203-204	-47.6	25	1.0	-47.4	20	2	40.22	7.23	6.06	40.16	7.16	5.86
N-Acetyl-β-D-galactopyranosylamine	235 (decomp.)	233	+ 8.6	24	0.4	+ 9.8	20	2	43.59	7.03	6.50	43.43	6.84	6.33
N-Acetyl-α-D-galactopyranosylamine	179-180	179-180	+194	24	1.6	+194.9	20	2	43.63	6.96	6.63	43.43	6.84	6.33
N-Acetyl-α-L-arabinopyranosylamine	226-227 (decomp.)	222-224	+67.1	25	0.7	+69.7	20	4	44.09	6.93	7.42	43.97	6.85	7.33
N-Acetyl-β-D-xylopyranosylamine	217-218 (decomp.)	213-214	-0.8	24	1.2	- 0.7	20	2	43.89	6.69	7.58	43.97	6.85	7.33

a Literature values are those reported by Isbell and Frush (1958).

b All samples were dried at 64°C before analysis except for this derivative of D-mannose, which was dried at room temperature.

at 0°C, rather than at room temperature as described by Frush & Isbell (1951).

Neither the method described by the above authors, nor that described by Sánchez & Del Pino (1965) for the preparation of β-D-galactosylamine was found to yield crystalline material with the specific rotations quoted by these workers. The procedure described in the Experimental section was satisfactory. The physical constants described in Tables 4.7-4.9 agreed with those reported previously (Isbell & Frush, 1958).

EXPERIMENTAL.

L-Aspartic acid, D-glucosamine hydrochloride, D-glucose, D-galactose and L-arabinose were obtained from British Drug Houses; D-xylose was obtained from Koch-Light Laboratories.

Methanol and ethanol were refluxed over magnesium methoxide and ethoxide respectively, and redistilled. iso-Propanol was dried over calcium oxide, and redistilled. Acetic anhydride and pyridine were purified as described in Chapter 3. Benzylalcohol was redistilled under a pressure of about 7mmHg (b.p. 89-91°C). Formamide was left in the presence of anhydrous sodium sulphate, and redistilled under a pressure of about 7mmHg (b.p. 81-83°C). Ethyl acetate and light petroleum (b.p. 60-80°C) were dried over anhydrous sodium sulphate.

Adams' catalyst (platinum oxide) was obtained from Johnson, Matthey & Co., Ltd., Hatton Garden, London, and palladium black was prepared from palladium chloride by

the method of Wieland (1912).

Dowex 1-X2 (200-400 mesh; Cl^- form), Dowex 50-X8 (40-80 mesh; H^+ form) and Dowex 50-X4 (200-400 mesh; H^+ form) were obtained from Sigma Chemical Co. Ltd.

Melting points were determined on a Kofler hot-stage apparatus by raising the temperature at a rate of 2°C per min. Elemental analyses were performed by Mrs. D. Butterworth at the National Physical Laboratory, Teddington, Middlesex. Samples that were sent for elemental analysis were dried at 64°C over solid phosphorus pentoxide in vacuo, unless stated otherwise. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter in a 1dm path-length cell at the temperatures stated. Thin-layer chromatography was performed at room temperature by upwards elution on layers (0.25mm thickness) of silica gel or cellulose. The atmosphere of the chromatography tank was pre-saturated with that of the solvent unless otherwise stated. Nuclear magnetic resonance spectra were obtained on a Varian A 60A at 60MH at the Department of Physical Chemistry, Oxford, on compounds in solutions in $CDCl_3$.

The synthesis of 4-N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn).

The preparation and separation of 1-benzyl-N-benzyloxycarbonyl-L-aspartate ester.

N-Benzyloxycarbonyl-L-aspartic acid was prepared from L-aspartic acid (78g; 0.6 mole) by reaction with benzyloxycarbonyl chloride in the presence of sodium bicarbonate by the general method for preparing benzyloxycarbonyl

derivatives described by Greenstein & Winitz (1961). After crystallising from ethyl acetate and light petroleum, b.p. 60-80°C, N-benzyloxycarbonyl-L-aspartic acid (110g; 0.4 mole) was obtained in 66% yield, m.p. 114-116°C, $[\alpha]_D^{20}$ + 10.9 (c 8.0 in acetic acid). Bergmann & Zervas (1932) report m.p. 116°C, $[\alpha]_D^{18}$ + 9.6 (c 7.5 in acetic acid).

N-Benzyloxycarbonyl-L-aspartic anhydride was prepared by the method of Miller, Behrens & du Vigneaud (1941) from N-benzyloxycarbonyl-L-aspartic acid (100g; 0.35 mole). The product was recrystallised from a mixture of warm ether and light petroleum (b.p. 60-80°C) to yield the anhydride (66g; 0.25 mole) in 70% yield, m.p. 108-109°C, $[\alpha]_D^{18}$ - 37.4° (c 4.0 in acetic acid). Le Quesne & Young (1952) report m.p. 109-111°C.

N-Benzyloxycarbonyl-L-aspartic anhydride (10g; 38 mmoles) was heated with freshly distilled benzyl alcohol (6ml; 59 mmoles) at 100°C for 4 hr in a sealed flask. The contents of the flask were then placed under vacuum in a rotary evaporator at 70°C for 20 min, and the residual oil was diluted with 25ml of methanol — water (4 : 1 v/v). The solution was then placed on a column containing Dowex 1 resin* and eluted at room temperature by an increasing

* Dowex 1-X2 (200-400 mesh; 250g) was suspended in water in a glass column and washed with 2N-NaOH until the eluate was free of chloride ions; followed by 2N-acetic acid (1.5 l) for a further 24 hr. The resin was then removed, resuspended in methanol — water (1 v/v) and repacked into a column measuring 52 x 2.7cm at room temperature.

concentration of acetic acid in a mixture of methanol and water delivered from two vessels arranged to give a linear increase in acetic acid concentration. The first vessel contained 1 l of methanol - water (4 : 1 w/v) and the second contained 1 l of methanol - acetic acid - water (80 : 18 : 2, by vol). The flow rate (18ml/hr) was controlled by a pump, and fractions (10.2ml) were collected on an LKB fraction collector. The absorbance at 258nm of a sample taken from each fraction and diluted 100-fold with methanol - water (4 : 1 v/v), was measured. After 151 fractions had been collected, elution was continued with methanol - formic acid - water (40 : 9 : 1, by vol) until a total number of 188 fractions had been collected. The elution profile is shown in Fig. 4.1. The fractions containing material that absorbed at 258nm were examined by thin-layer chromatography on silica gel F_{254}, together with authentic compounds. The spots were located by examination under ultra-violet light. This procedure showed that peak A (Fractions 15-36; Fig. 4.1) contained benzyl alcohol (R_f 0.29 in chloroform) and a small amount of an unidentified, ultra-violet absorbing substance (R_f 0.18 in chloroform). The first part of peak A (Fractions 15-24) contained, in addition, the 1,4-dibenzyl ester of N-benzyloxycarbonyl-L-aspartic acid [R_f 0.64 in chloroform, and R_f 0.98 in chloroform - methanol (9 : 1 v/v)]. Peaks B_1 and B_2 (Fractions 90-121) both contained 1-benzyl-N-benzyloxycarbonyl-L-aspartate [R_f 0.66 in chloroform - methanol (9 : 1 v/v)], while peak C (Fractions 175-188) contained 4-benzyl-N-benzyloxycarbonyl-L-aspartate

[R_f 0.48 in chloroform-methanol (9 : 1 v/v)]. The values for ϵ_{255} for the 1- and 4-monobenzyl esters were 420 l $mole^{-1}cm^{-1}$, and the absorbance measurements at 255nm showed that yields of 45% (in peak B_1) of the 1-benzyl ester and 16% of the 4-benzyl ester had been eluted from the column.

Left at room temperature for several days, Fractions 15-23 produced needles which were filtered off, and recrystallised from ethyl acetate by the addition of light petroleum (b.p. 60-80°C) to give the dibenzyl ester (1.3g; 2.9 mmoles) in 8% yield, m.p. 64-65°C, $[\alpha]_D^{22}$ - 2.1° (c 5 in acetic acid). Berger & Katchalski (1951) report m.p. 66.5°C, $[\alpha]_D^{25}$ - 2.5° (c 1 in acetic acid).

Methanol was removed from Fractions 97-116 (Fig. 4.1) on the rotary evaporator, and the residual solution was acidified to pH 2 by the addition of conc. HCl. The oil that had separated was extracted into ethyl acetate (3 x 25ml) and the solution was dried over anhydrous sodium sulphate. Addition of light petroleum (b.p. 60-80°C) yielded the 1-benzyl ester in the form of rectangular blades (5.4g; 14.5 mmoles) in 33% yield, m.p. 83-85°C, $[\alpha]_D^{23}$ - 10.6° (c 4 in acetic acid) (Found: C, 63.93; H, 3.31; N, 4.12. Calc. for $C_{19}H_{19}O_6N$: C, 63.86; H, 5.36; N, 3.92%). Bergmann, Zervas & Salzmann (1933) report m.p. 84-85°C, $[\alpha]_D^{21}$ - 10.6° (c 5.2 in acetic acid).

Fractions 175-188 were treated in the same way to yield the 4-benzyl ester as needles (1.6g; 43 mmoles) in 11% yield, m.p. 107-109°C, $[\alpha]_D^{25}$ + 11.6° (c 7.4 in

acetic acid. Berger & Katchalski (1951) report m.p. 108°C, $[\alpha]_D^{25}$ + 12.1° (c 10 in acetic acid).

The preparation of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy α-D-glucopyranosyl chloride (α-acetochloroglucosamine).

D-Glucosamine hydrochloride (largely α ; 175g; 0.8 mole) was converted to β-D-glucosamine according to the method of Westphal & Holzmann (1942) to yield 130g (0.73 mole; 90% yield), m.p. 129-131°C, although lower melting points have been recorded (see Gottschalk, 1966).

β-D-Glucosamine (130g; 0.73 mole) was acetylated with pyridine (677ml) and acetic anhydride (466ml), to yield the peracetylated derivative (130g; 0.33 mole) in 45% yield, m.p. 186-187°C, $[\alpha]_D^{20}$ + 2° (c 1 in chloroform). Hudson & Dale (1916) report m.p. 187-189°C, $[\alpha]_D^{20}$ + 1.2° (c 1 in chloroform). The α-chloro compound was prepared from 92g (0.24 mole) of the above compound by the method of Leaback & Walker (1957). The product (55g; 0.15 mole), m.p. 125-127°C $[\alpha]_D^{20}$ + 119.4° (c 3 in chloroform) was obtained in 63% yield. Leaback & Walker (1957) report m.p. 133-134°C, $[\alpha]_D^{18}$ + 118° (c 1 in chloroform).

Preparation of 2-acetamido-2-deoxy-β-D-glucopyranosyl azide (GlcNAc-N_3).

α-Acetochloroglucosamine (55g; 0.15 mole) reacted with sodium azide in formamide as described by Yamamoto, Miyashita & Tsukamoto (1965). Compound I (Fig. 4.2) was obtained in 49% yield (27g; 0.075 mole), m.p. 167-169°C, $[\alpha]_D^{20}$ - 44.6° (c 2 in chloroform) after crystallising from warm ethyl acetate by the addition of light petroleum

(b.p. 60-80°C) until the solution was turbid. Micheel & Wulff (1956) report m.p. 161°C, $[\alpha]_D$ - 43°. Bolton, Hough & Khan (1966) report m.p. 166-168°C, $[\alpha]_D$ - 60° (c 2 in chloroform). Marshall & Neuberger (1964) report m.p. 167-168°C, $[\alpha]_D^{22}$ - 55° (c 0.84 in chloroform, and also report the production of an azide having $[\alpha]_D$ + 26.6° (c 2.4 in chloroform) from the mother liquors. The specific rotation reported here agrees with the value reported by Micheel & Wulff (1956). The azide was examined by t.l.c. on silica gel F_{256}. Only one spot (R_f 0.21) was found on a plate which had been developed with benzene-methanol (4 : 1 v/v), and only one spot (R_f 0.71) on a plate which had been developed with chloroform-methanol (1 : 1 v/v). The plates were inspected both under the ultra-violet light and after treatment with iodine vapour.

A further quantity of the crude, acetylated azide (4g) was prepared from α-acetochloroglucosamine (5g), and dissolved in warm ethyl acetate (30ml). Light petroleum (b.p. 60-80°C) (3ml) was added, and the solution left at room temperature overnight. Crystals (2.6g) were filtered off, yielding the azide (2.6g), $[\alpha]_D^{24}$ - 45.1 (c 2 in chloroform). Then light petroleum (b.p. 60-80°C) (10ml) was added to the mother liquor, and further crystalline azide was filtered off after 24 hr to yield 0.35g, $[\alpha]_D^{26}$ - 42.5°(c 2 in chloroform). A further quantity of light petroleum (b.p. 60-80°C) was added to the mother liquors, and crystals (0.1g), $[\alpha]_D^{26}$ - 33.9, were again filtered off. No more crystalline material could be obtained from the mother liquors.

The acetylated azide (15g; 40 mmoles) was O-deacetylated in the presence of 700ml of a 1% (w/v) solution of magnesium methoxide in methanol at 0°C by the method of Whitaker, Tate & Bishop (1962). Magnesium ions were removed by shaking with 2 x 350ml of Dowex 50-X8 (40-80 mesh; H^+ form). The crude material obtained by evaporating the solvent was recrystallised twice from iso-propanol at room temperature to give GlcNAc-N_3 (8g; 32 mmoles) in 81% yield, m.p. 142-144°C (decomp), $[\alpha]_D^{25}$ - 25.8° (c 1.0 in water), ϵ_{272} 36.2 (c 0.14 in H_2O). Kiyozumi et al., (1970) report m.p. 143-145°C (decomp), $[\alpha]_D^{16}$ -27.5° (c 2.8 in water). The recrystallised product (1.1g), in a small amount of water, was placed on a column of Dowex 1-X2 (200-400 mesh; OH^- form) which had the dimensions of 87 x 2.5mm, and was eluted with de-ionised water at a flow rate of 17ml/hr at room temperature (Austin, Hardy, Buchanan & Baddiley, 1963). Fractions (7ml) were collected and assayed on the polarimeter. Only one peak, which possessed negative rotation, was eluted from the column. The material was at maximum concentration at an elution volume of 1014ml. Water was removed on the rotary evaporator and the residue of O-deacetylated azide was crystallised from iso-propanol to yield 0.7g, $[\alpha]_D^{26}$ - 25.7° (c 1.0 in water).

The preparation of 2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NH_2).

The O-deacetylated azide (1.7g; 7 mmoles) was hydrogenated in the presence of Adams' catalyst as described by Kiyozumi et al., (1971) to yield GlcNAc-NH_2 in

79% yield (1.2g; 5.5 mmoles) which melted initially between 87-89°C, reset above 130°C, and re-melted with decomposition between 170°C and 180°C. The specific rotation was $[\alpha]_D^{20}$ - 6.7° (c 1.7 in water) when measured immediately, and $[\alpha]_D^{20}$ - 0.7° (c 1.7 in water) after 3 days. The sample that was sent for analysis was dried at room temperature. (Found: C, 42.97; H, 7.48; N, 10.84. Calc. for $C_8H_{16}O_5N_2 + \frac{1}{2}H_2O$: C, 42.75; H, 7.40; N, 12.47%). Kiyozumi et al., (1971) report m.p. 146-147°C (decomp.), $[\alpha]_D^{16}$ - 5.3° (c 2.4 in water) after 42 hr. The nitrogen analysis of the present preparation is low, and this may be due to the presence of small amounts of the diglycosylamine which could be produced during crystallisation, but no evidence of this was found by thin-layer chromatography.

Small amounts (2μl, 1μl and 0.5μl) of a fresh 10% (w/v) aqueous solution of GlcNAc-NH_2, and also 1μl of a 10% solution of D-glucosamine were examined by thin-layer chromatography, both on silica gel H and on cellulose plates by development with ethyl acetate-propan-1-ol-water-0.88 ammonia (1 : 7 : 2 ; 0.1, by vol). The plates were inspected by spraying with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950). One spot developed on silica (R_f 0.21; R_{GlcN} 1.0), or on cellulose (R_f 0.34; R_{GlcN} 1.0).

The preparation of 4-N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (GlcNAc-Asn).

2-Acetamido-2-deoxy-β-D-glucopyranosylamine (0.66g; 3 mmole) was coupled to 1-benzyl-N-benzyloxycarbonyl-L-

aspartate (0.83g; 2.3 mmole) in 15.7ml pyridine — water (4 : 1 v/v) with dicyclohexylcarbodiimide (0.61g) at room temperature for 12 hr. The reaction mixture was stirred magnetically. One drop of glacial acetic acid was then added and the mixture stirred for another 10 min. Insoluble material was filtered off, and the solvent was removed on a rotary evaporator at about 60°C to yield 10g of crude material. A small quantity was washed with water, followed by ethyl acetate, and recrystallised from warm ethanol, yielding 1-benzyl-2-N-benzyloxycarbonyl-4-N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine, m.p. 207-210°C (decomp.), $[\alpha]_D^{36}$ + 10.0 (c 0.5 in acetone—water; 80 : 20 v/v) (Found: C, 57.52; H, 6.09; N, 7.62. $C_{27}H_{33}O_{10}N_3$ requires C, 57.95; H, 5.94; N, 7.51%).

The crude material (0.85g; 1.5 mmole) was hydrogenated in the presence of palladium black (prepared from about 1g palladium chloride) in methanol (130ml) by passing hydrogen over the surface of the stirred solution for 2 hr. The solution was filtered with the aid of Fullers' Earth, and the filtrate evaporated on the rotary evaporator.

The product was examined by t.l.c. on cellulose plates (0.25mm), by developing with phenol—water (4 : 1 w/v). The plates were run immediately after the solvent had been placed in the tank. The plates were dried (60°C overnight), sprayed with 1% ninhydrin in acetone which contained 2% (v/v) pyridine, and heated at 80-100°C. A blue spot (R_{Asp} 1.0) and a brown spot (R_f 0.30) appeared. The substance giving rise to the brown spot was separated from that giving rise to the blue spot by chromatography

on Dowex 1-X2 (200-400 mesh; acetate form; 1 x 5cm). The former was eluted by water (125ml) and the latter remained on the column. The eluate was evaporated to a small volume and a large excess of ethanol (to turbidity) added. The yield was 380mg (1.1 mmole), 73% yield, or an overall yield of 55% from the protected aspartic acid derivative (m.p. 254-259°C, decomp.).

The properties of GlcNAc-Asn.

Some of the material (16mg) was dissolved in 0.1ml water and placed in a water bath at 60°C. Ethanol (3ml) at 60°C was gradually added as the solution was shaken. After 30 min, the white solid that had separated was dried *in vacuo* over conc. sulphuric acid at room temperature, m.p. 259-261°C (decomp.). A sample was dried at 64°C over P_2O_5 for about 24 hr *in vacuo*, m.p. 259-261°C (decomp.) $[\alpha]_D^{26}$ + 26.0° (*c* 0.84 in water), $[\alpha]_{365}^{26}$ + 109.7° (*c* 1.6 in water), $[\alpha]_{365}^{26}$ + 63.6° (*c* 0.4 in 0.2*N*-NaOH) (Found: C, 42.89; H, 6.21; N, 12.32. Calc. for $C_{12}H_{21}O_8N_3$: C, 42.98; H, 6.31; N, 12.53%).

A further sample (16mg) was dissolved in water (0.1ml) and cooled to +4°C. Ethanol (about 1ml) cooled to +4°C was added and the solution left at this temperature until crystals had appeared. These were filtered off and dried over P_2O_5 at room temperature *in vacuo*, m.p. 211-213°C (decomp.) $[\alpha]_D^{26}$ + 24.6° (*c* 1 in water) (Found: C, 37.20; H, 6.76; N, 10.73. Calc. for $C_{12}H_{21}O_8N_3\ 3H_2O$: C, 37.01; H, 6.94; N, 10.79%).

A sample that had crystallised at +4°C was placed in

a small weighing bottle, dried at room temperature over P_2O_5 *in vacuo*, weighed, dried at 100°C for 12 hr over P_2O_5 *in vacuo*, and re-weighed. The loss in weight was 8.98%. The loss in weight calculated for loss of two of the three molecules of water of crystallisation was 9.25%. Drying at 100°C for a further 6 hr registered no further loss in weight. The compound had m.p. 210-213°C (decomp.).

Separate samples of the trihydrate were also dried at 64°C, 100°C or 130°C over P_2O_5 *in vacuo* for 24 hr, and sent for elemental analyses, which are described in Table 4.2. Drying at temperatures of 150°C led to discolouration of the compound.

Samples of the trihydrate were recrystallised at 60°C. The product had the same melting point as that recorded for the anhydrous compound. One recrystallisation at room temperature resulted in a mixture of the two forms, as was evident from microscopic examination and, on another occasion, recrystallisation at +4°C produced the anhydrous form.

The optical rotations of a solution of the trihydrate (*c* 1.6 in water) were measured at 589, 578, 546, 436 and 365nm in a water-jacketed cell of 1dm path-length at various temperatures between 65° and 79°C. The molecular weight of the trihydrate was determined from unit cell and density measurements made during the course of X-ray diffraction studies.

The infrared spectra of the two forms of GlcNAc-Asn pressed into discs in the presence of KBr (1mg/100mg KBr)

were examined. The infrared spectra of the trihydrate previously dried *in vacuo* over phosphorous pentoxide at 100°C for 2 days, and again at 140°C for 20 hr, were also obtained.

For comparison, the infrared spectra of the monohydrate and anhydrous forms of L-asparagine in the form of KBr discs were measured. The anhydrous form was obtained by drying the monohydrate at 110°C over phosphorus pentoxide for 20 hr.

Crystals of the trihydrate, and of the anhydrous form, were photographed under a light microscope at magnifications of 25 and 160 respectively. The photographic plates represented an overall magnification of 50 and 300 respectively (Plate 4.2).

Aqueous solutions of both the anhydrous and trihydrated forms of GlcNAc-Asn were subjected to electrophoresis and chromatography. Small amounts (80 g) were examined by thin-layer chromatography on plates of cellulose and silica HF_{256} by developing with phenol — water (4 : 1 w/v) as described above. One spot stained brown with the spray reagent containing ninhydrin (1%) and pyridine (2% v/v) in acetone on each plate (R_f 0.38; R_{Asp} 2.4 on cellulose and R_f 0.11 on silica). A sample (80 g) was also subjected to ascending paper chromatography on Whatman No. 1 paper with phenol — water (4 : 1 w/v). One elongated spot (R_f 0.38; R_{Asp} 2.4) stained with the same reagent.

Electrophoresis on Whatman 3MM paper in the presence of formic acid — acetic acid — water (7 : 5 : 240, by

vol) at pH 1.85 at a potential of 31V/cm for 1 hr gave one brown spot as a result of dipping through the same ninhydrin reagent (Plate 4.4). The compound ran 8.4cm towards the cathode (R_{Asp} 0.80).

Preparation of 1-benzyl-2-N-benzyloxycarbonyl-4-N-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl-L-asparagine (Compound III, Fig. 4.2).

1-Benzyl-N-benzyloxycarbonyl-L-asparagine (0.51g; 1.3 mmole) was added to 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine (0.47g; 1.4 mmole) together with 0.3g 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide in methylene chloride (12ml). The solution was stirred magnetically for 19 hr, by which time a precipitate had formed. The suspension was shaken with a few drops of added glacial acetic acid, and methylene chloride was removed on the rotary evaporator. The solid was dissolved in chloroform (20ml) (all was soluble), and extracted successively with water (3 x 10ml), 1N-HCl (3 x 10ml), water (3 x 10ml), saturated $NaHCO_3$ and water. The residue was recrystallised from boiling ethanol giving the product (Compound III) (0.35g; 0.51 mole) in 39% yield, m.p. 210-215°C (decomp.) $[\alpha]_D^{26}$ + 8.0°(c 1 in chloroform) Lit. Marshall & Neuberger (1966) report 220-221°C (decomp.), $[\alpha]_D^{22}$ + 8.2° (c 0.98 in chloroform).

Preparation of 1-N-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NHAc).

Hydrogenation of compound I (2g; 5.4 mmoles) in ethyl acetate in the presence of Adams' catalyst (Marshall & Neuberger, 1964) led to the production of the O-acetylated glycosylamine (compound II: Fig. 4.2) (1.3g; 3.8 mmoles)

in 70% yield, m.p. 230-234°C (decomp.) and phase change at 140°C, $[\alpha]_D^{22}$ - 19.9° (c 0.85 in chloroform) (Found: C, 48.70; H, 6.36; N, 8.05. Calc. for $C_{14}H_{20}O_8N_4$: C, 48.55; H, 6.40; N, 8.09%). Thin-layer chromatography on silica gel GF_{256} in chloroform — methanol (15 : 1 v/v) gave one spot (R_f 0.18) staining purple with the ninhydrin-pyridine reagent and yellow with a reagent containing 2% 4-aminobenzoic acid, 5% sulphosalicylic acid and 1% stannous chloride in acetic acid — water (4 : 1), followed by heating to 90°C. Marshall & Neuberger (1964) report m.p. 151°C and also 233-240°C (decomp.), $[\alpha]_D^{20}$ values from - 19.6° to - 23.3° (c 1.5 in chloroform). Bolton, Hough & Khan (1966) report m.p. 150°C (decomp.), $[\alpha]_D^{23}$ - 25.5° (c 2 in chloroform).

The hydrogenation was also performed with ethanol as solvent, as described by Bolton, Hough & Khan (1966). The product was obtained in similar yield with identical physical constants.

The fully O-acetylated glycosylamine (0.36g; 1 mmole) was N-acetylated as described by Yamamoto, Miyashita & Tsukamoto (1965) to give the peracetylated derivative (0.32g; 0.8 mmole) in 80% yield, m.p. 238-239°C, $[\alpha]_D^{32}$ + 7.1° (c 0.85 in chloroform) (Found: C, 49.54; H, 6.11; N, 7.28. Calc. for $C_{16}H_{23}O_9N_2$: C, 49.48; H, 6.23; N, 7.21%). Baer (1958) reports m.p. 236-237°C, $[\alpha]_D^{24}$ + 12°; Bolton, Hough & Khan (1966) report m.p. 236-237°C, $[\alpha]_D^{24}$ + 18.5° (c 2 in chloroform) and Yamamoto, Miyashita & Tsukamoto (1965) report m.p. 241°C, $[\alpha]_D$ + 22.8° (in pyridine).

O-Deacetylation was performed in a 1% solution of

magnesium methoxide in methanol as described by Yamamoto, Miyashita & Tsukamoto (1965). The peracetylated derivative (0.32g; 0.83 mmole) gave the N-acetylglycosylamine (0.16g; 0.68 mmole) a 74% yield, m.p. 255-258°C (decomp.), $[\alpha]_D^{20}$ + 27.8° (c 1 in water) (Found: C, 46.00; H, 7.13; N, 10.71. Calc. for $C_{10}H_{18}O_6N_2$: C, 45.80; H, 6.92; N, 10.68%). Baer (1958) reports m.p. 209°C, $[\alpha]_D$ + 28° (in water) and Bolton, Hough & Khan (1966) report m.p. 232-233°C, $[\alpha]_D^{19}$ - 24° (c 2 in water). Yamamoto, Miyashita & Tsukamoto (1965) report (for the monohydrate) m.p. 240-243°C, $[\alpha]_D^{19}$ + 43.7° (in water). There is a wide divergence in the physical properties quoted for this compound, but it is likely that the value reported by Bolton, Hough & Khan (1966) is incorrect. The values found here agree with those quoted by Baer (1958) who prepared the compound by the action of ketene on the glycosylamine.

The preparation of 4-N-(β-D-glucopyranosyl)-L-asparagine (Glc-Asn).

D-Glucose (160g; 0.89 mole) was converted to β-D-glucosylamine by treating it as a suspension in methanol (400ml) with ammonia in the catalytic presence of ammonium chloride (4g) by the method of Isbell & Frush (1958). After recrystallisation, a yield of 77g (0.43 mole) in 48% yield was obtained, m.p. 131-133°C (decomp.), initial $[\alpha]_D^{24}$ + 20.7° (c 1.4 in water), after 24 hr $[\alpha]_D^{24}$ + 22.2° (c 1.4 in water). Isbell & Frush (1958) report m.p. 125-127°C, $[\alpha]_D^{20}$ + 20.8° (c 2 in water).

1-Benzyl-N-benzyloxycarbonyl-L-aspartate (1.42g; 4 mmoles) and β-D-glucosylamine (0.72g; 4 mmole) were dissolved in

pyridine – water (4 : 1 v/v), and a solution of dicyclohexylcarbodiimide was added to give a final volume of 28.2ml. The solution was stirred at room temperature; a precipitate appeared after a few minutes. After 4 hr a few drops of acetic acid were added, the precipitate was filtered off, and the solvent removed from the filtrate on the rotary evaporator at about 60°C. The residue was taken up in ethanol and treated with Norit. The ethanol was removed, leaving 1.8g of crude material. Some of the residue (0.35g) was dissolved in dry methanol, and palladium black prepared from about 0.5g of palladium chloride was added. The solution was hydrogenated for 2 hr. Water (10ml) and a little Fullers' Earth were added, and the catalyst was filtered off. The solvent was removed from the filtrate and the residue was taken up in a small amount of water, and placed on a column of Dowex 1-X2 (200-400 mesh; 1 x 6cm) in the acetate form. The substance was eluted with water (20ml) treated with Norit, and recrystallised at +4°C from about 0.3ml water by the addition of ethanol to turbidity. The product was obtained as needles (80mg; 0.26 mmole) in a yield of 37%, m.p. 212-215°C (decomp.) $[\alpha]_D^{22}$ – 16.2° (c 1 in water) (Found: C, 38.67; H, 6.20; N, 8.68. Calc. for $C_{10}H_{18}O_8N_2 \cdot H_2O$: C, 38.48; H, 6.46; N, 8.97%). Marks & Neuberger* report (for the monohydrate) m.p. 203-204°C (decomp.), $[\alpha]_D^{20}$ – 17.3° (c 0.55 in water); Coutsogeorgopoulos & Zervas (1961) report (for the anhydrous form) m.p. 253°C (decomp.), $[\alpha]_D^{16}$ – 16.5° (in water).

* (1964)

Thin-layer chromatography on plates of cellulose in phenol — water (4 : 1 w/v) without presaturation of the tank atmosphere, followed by spraying with the ninhydrin-pyridine reagent, gave one brown spot, R_f 0.12, R_{Asp} 0.63. About 0.5 mole of the compound gave one peak on the Locarte autoanalyser after 46 min, with a molar colour yield 0.563 with respect to L-valine.

The preparation of N-acetyl-β-D-glucopyranosylamine (Glc-NHAc) N-acetyl-β-D-mannopyranosylamine (Man-NHAc) N-acetyl-β-D-galactopyranosylamine (βGal-NHAc) N-acetyl-α-D-galactopyranosylamine (αGal-NHAc) N-acetyl-α-L-arabinopyranosylamine (Ara-NHAc) and N-acetyl-β-D-xylopyranosylamine (Xyl-NHAc).

Most of these glycosylamines were prepared from the free sugars by reaction with ammonia as described above for β-D-glucopyranosylamine. Isbell & Frush (1958) prepared the α-D-galactopyranosylamine-ammonia complex at room temperature, but it was found that better yields were obtained when the preparation was performed at 0°C.

A mutarotation constant (k_m = 9.2 x $10^{3}$$min^{-1}$ at 22°C) for the initial change in rotation of the complex in solution was calculated by plotting $k_m = \frac{1}{t} \log \frac{\alpha_o - \alpha_\infty}{\alpha_t - \alpha_\infty}$ This value is larger than that found by Frush & Isbell (1951) (k_m = 4.8 x $10^{-3}$$min^{-1}$ at 20°C).

β-D-Galactosylamine was prepared from the crude α-D-galactosylamine-ammonia complex (4g) by dissolving it in 6ml 3% (v/v) 0.88 ammonia in water at room temperature. This solution was left at room temperature for 30 min, and then 30ml cold methanol, presaturated with ammonia at +4°C, was added. After allowing the preparation to stand

at +4°C for 20 hr, the crystals that had been formed were filtered off and discarded. A further 20 ml cold methanol saturated with ammonia was added, and the solution left overnight. The crystals that formed were filtered off and dried over solid NaOH in the presence of ammonia vapour in a desiccator. The white solid obtained weighed 1.8g, m.p. 133-135°C, $[\alpha]_D^{26}$ + 62.9° (c 2.3 in water). Isbell & Frush (1958) report m.p. 134-136°C, $[\alpha]_D^{20}$ + 62.2° (c in water).

The physical constants of the glycosylamines prepared are shown in Table 4.7.

The glycosylamines were fully N,O-acetylated in acetic anhydride in pyridine as described by Isbell & Frush (1958), and the products were then O-deacetylated in 1% magnesium methoxide in methanol (Whitaker, Tate & Bishop, 1962). The magnesium ions were removed by Dowex 50W-X8 (40-80 mesh; H^+ form) and the products recrystallised several times from the solvents described by Isbell & Frush (1958). The physical constants of the peracetylated and N-acetylglycosylamines are displayed in Tables 4.8 and 4.9.

CHAPTER 5.

THE STEREOCHEMISTRY OF SOME N-ACYLGLYCOSYLAMINES.

Some glycoproteins exhibit specific biological functions, which reside wholly or in part within the carbohydrate prosthetic groups. It is probable that the relative conformations of the carbohydrate and polypeptide residues are important in these functions. There are a number of examples which can be mentioned in support of this view. Firstly, the chondromucoprotein of various connective tissues requires an intact polypeptide chain in order that it may entrap water molecules in an ordered structure so that the cartilage is rigid (Thomas, 1956). In a similar manner, certain glycoproteins in the serum of a certain species of Antarctic fish bring about changes in the structure of the water in the serum so that the freezing point of the latter is markedly depressed (De Vries, Komatsu & Feeney, 1970). Both carbohydrate and polypeptide moieties contribute to this activity.

Many of serum proteins and also protein hormones in man appear to depend on their carbohydrate moieties for their catabolism. These prosthetic groups contain as non-reducing terminal moieties the structure sialyl $\rightarrow$ Gal. Removal of the sialyl residues from many of these macromolecules exposes a terminal galactose residue which is recognized by the plasma membrane of the hepatocytes. It would seem, however, that the environment of these D-galactose residues is highly specific since, for example, asialotransferrin which has an exposed D-galactose residue

is not catabolised by the process (Morell et al., 1971; Pricer & Ashwell, 1971).

The relationship between the antigenicity of the type specific factor of Shigella flexneri and the stereochemistry of the carbohydrate moieties of these factors has been emphasised (Simmons, 1971). It is also suggested that antisera to red cell membranes are evoked when the carbohydrate moieties on these surfaces "are presented in a conformationally favourable form". (Hardoe, 1971).

All these examples point to the need for an understanding of the conformation of glycopeptides and glycoproteins. It is clear from studies of the chiroptical properties of oligosaccharides derived from the soluble blood group substances (see below) that the former have definite preferred conformations, the relationship of which to those occurring in the whole glycoproteins are unknown. Certainly there are highly organized structures in polysaccharide gels (Rees, 1969).

The present studies have been aimed at elucidating the structure in the neighbourhood of carbohydrate-peptide linkages of the GlcNAc-Asn type. A need was clearly evident for a knowledge of the stereochemistry of GlcNAc-Asn in the crystalline form, and Dr. J. Delbaere kindly carried out X-ray crystallographic studies. However, it was desirable to ascertain the structure of the compound in an aqueous environment. Chiroptical methods were used for examining this. Finally, it was felt necessary to see whether a protein environment might

modify the structure of GlcNAc-Asn, and Professor D. Phillips was approached in order to see if it was feasible to examine, by X-ray crystallographic methods, lysozyme to which was bound the compound in question. Drs. L. Johnson and C. Beddell carried out these studies and allowed me to participate. Dr. D. Barry carried out an analysis to obtain the best fit of the data to a stereochemical representation of the model by methods developed for a computer.

SECTION 1. CHIROPTICAL PROPERTIES OF 4-N-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-L-ASPARAGINE AND RELATED COMPOUNDS.

The results obtained in optical rotary and circular dichroism measurements of a number of compounds will be presented. Some of the data have been briefly described (Austen & Marshall, 1970) and have now been interpreted in terms of a rule of symmetry, based on the "one electron" theory of optical rotation (Condon, Altar & Eyring, 1937). The basis for this rule is discussed later in this Chapter.

The units employed in ORD and CD studies are the following.

The specific rotation $[\alpha]$ of a solution is defined as:

$$[\alpha]_{\lambda} = \frac{\alpha}{l.c.}$$

where α(in degrees) is the rotation of plane polarized light, l is the optical path-length of the solution (in decimeters) and c is the concentration of the substance (g/ml).

Molecular rotation $[M]$ will be calculated in the studies to be described to allow comparison of different

compounds on a mole to mole basis

$$[M] = \frac{[\alpha] \text{ x mol. wt.}}{100} \text{ (deg. cm}^2\text{/dmole)}$$

Circular dichroism is often expressed in terms of molecular ellipticity $[\theta]$, a related phenomenon. Plane polarized light passing through optically active material may become elliptically polarized, and some CD instruments measure the ellipticity directly. It is related to the differential dichroic absorption ($\Delta\epsilon$) by the relationship:

$$\Delta\epsilon = \frac{[\theta]}{3,300} \quad \text{(Moscowitz, 1960)}$$

The molecular ellipticity $[\theta]$ is calculated from the measured angle (ϕ) as follows:

$$[\theta] = \frac{\phi \text{ x mol. wt.}}{c \text{ x } l \text{ x } 100} \quad \text{(deg. cm}^2\text{/dmole}^{-1}\text{)}$$

$[\theta_o]$ is the molecular ellipticity at the maximum or minimum of a Cotton effect.

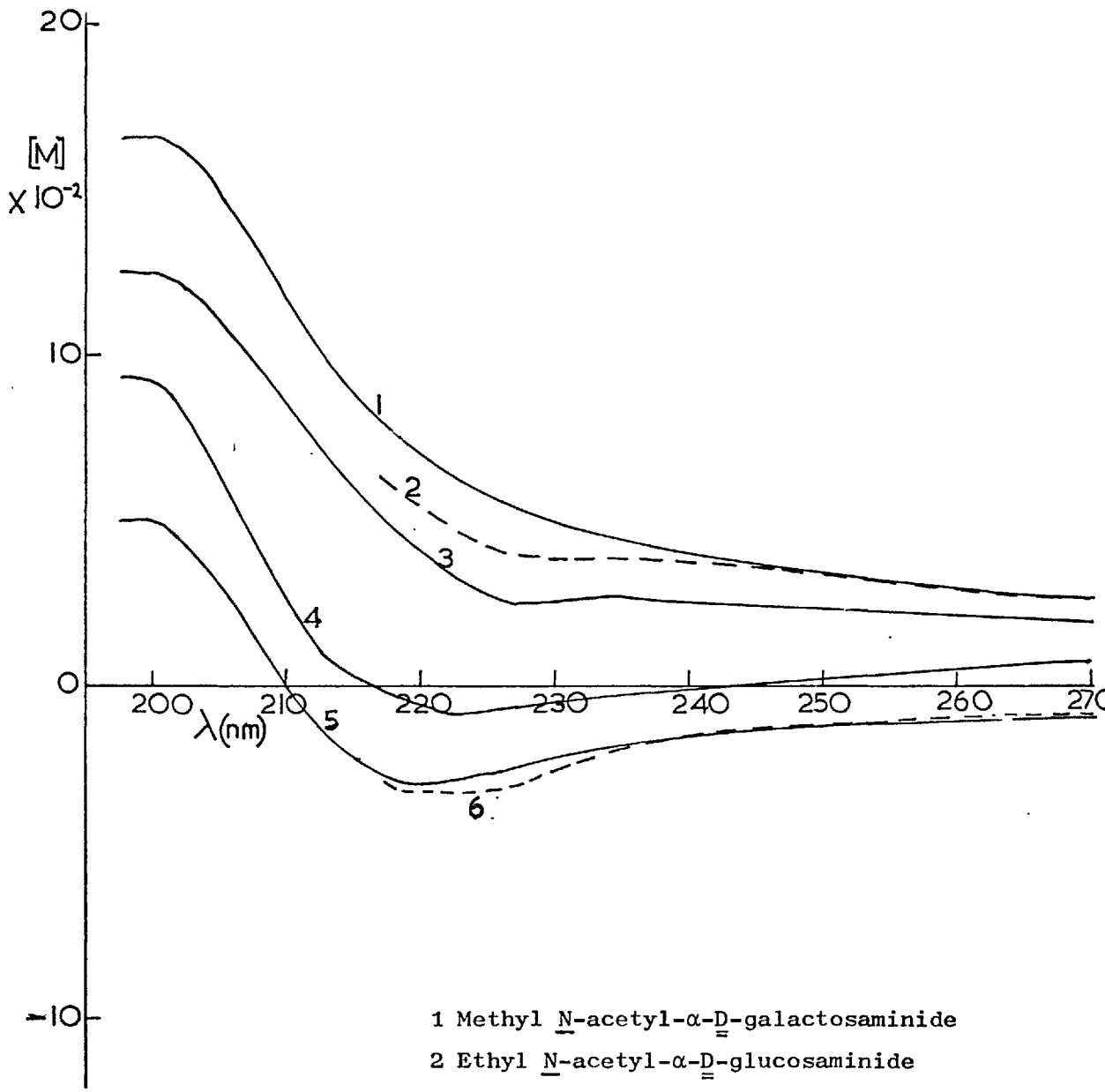
RESULTS.

The chiroptical properties of N-acetyl-D-glycosaminides.

The ORD curves of aqueous solutions of the methyl α- and β-glycosides of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine (Fig. 5.1) were measured, and confirmed those measured previously (Listowsky, Avigad & Englard, 1968; Beychok & Kabat, 1965). The N-acetyl-D-glucosaminides and methyl N-acetyl-β-D-galactosaminide show small troughs at wavelengths between 220 and 230nm, and the rotations

Fig. 5.1. The ORD of aqueous solutions of α- and β- glycosaminides of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine.

ORD curves were measured on Bendix I and II instruments



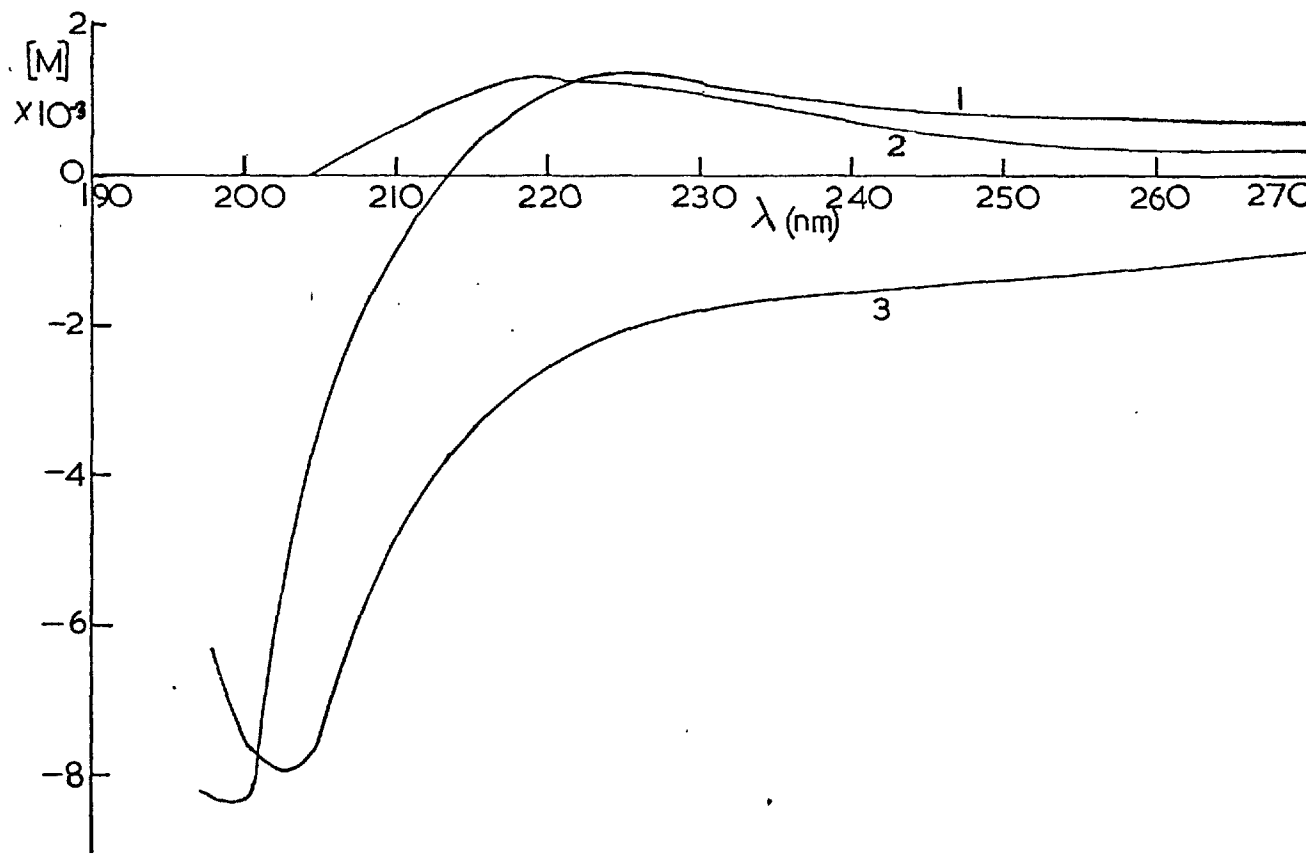
1 Methyl N-acetyl-α-D-galactosaminide
2 Ethyl N-acetyl-α-D-glucosaminide
3 Methyl N-acetyl-α-D-glucosaminide
4 Methyl N-acetyl-β-D-galactosaminide
5 Methyl N-acetyl-β-D-glucosaminide
6 Ethyl N-acetyl-β-D-glucosaminide

increase to more positive values at lower wavelengths. Increase in rotation ceases by about 200nm, suggesting that peaks occur at this wavelength. Penetration to lower wavelengths was limited by the range of the instrument used.

Mutarotated N-acetyl-D-mannosamine and methyl N-acetyl-α-D-mannosaminide show peaks at 225nm and 220nm in their ORD spectra, while methyl N-acetyl-α- and β-D-mannosaminides show large troughs at 200 and 203nm (Fig. 5.2). The ORD curves are similar to those reported previously from 350 to 210nm (Beychok, Ashwell & Kabat, 1971). The shapes of the ORD curves of the N-acetyl-D-glycosaminides are suggestive of two overlapping Cotton effects of opposite sign (see Fig. 5.3, which is discussed in some detail later).

The Cotton effects are resolved more effectively in the CD spectra (Figs. 5.4-5.6). Methyl N-acetyl-β-D-glucosaminide has a negative CD minimum at 207.5nm ($[\theta_o]$ =-6,100 deg. cm^2/dmole). Previous workers (Kabat, Lloyd & Beychok, 1969) state this minimum to be at 215nm, and the reason for the difference is unknown, but report a similar value for $[\theta_o]$. Methyl N-acetyl-β-D-galactosaminide exhibits a negative minimum at 211nm, while methyl N-acetyl-β-D-mannosaminide shows a positive maximum at 217nm. At lower wavelengths, the ellipticity changes sign. A definite negative minimum is observed with methyl N-acetyl-β-D-mannosaminide. Inflections in the CD curves at about 190nm are exhibited by the other N-acetyl-D-glycosaminides.

Fig. 5.2. The ORD of aqueous solutions of methyl N-acetyl-α- and β- D-mannosaminides and mutarotated N-acetyl-D-mannosamine



1 Methyl N-acetyl-α-D-mannosaminide

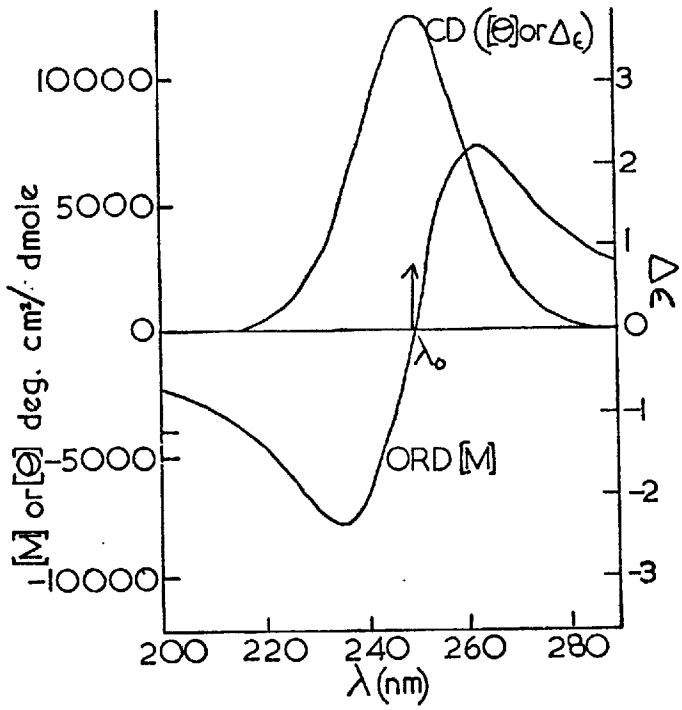
2 Mutarotated N-acetyl-D-mannosamine

3 Methyl N-acetyl-β-D-mannosaminide

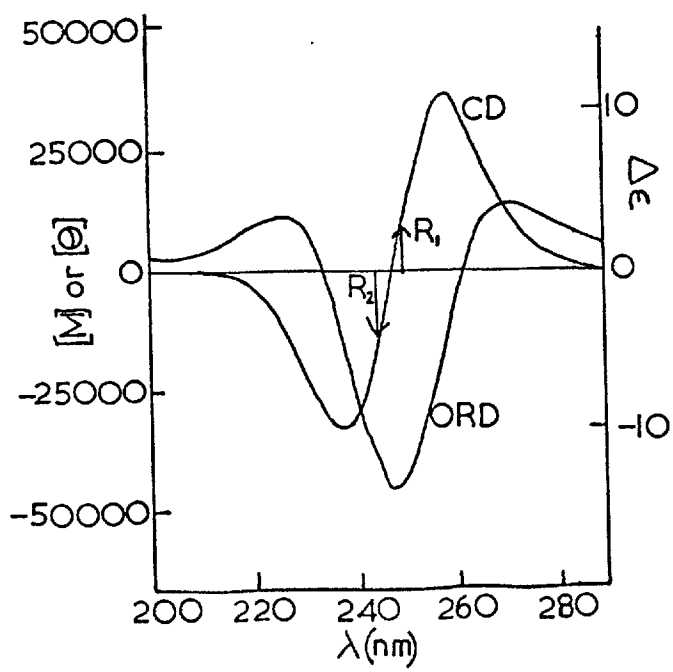
Measured on the Bendix II instrument

Fig. 5.3. Cotton effects exhibited by hypothetical disymmetric molecules containing one or two chromophores.

The CD and ORD curves given by one isolated optically-active chromophore.



The CD and ORD curves given by two optically-active chromophores of equal, but opposite, rotational strength, separated by 5nm.



CD of N-acetyl-D-glycosaminides

Measured on Cary 61

Fig.5.4. CD of an aqueous solution of methyl N-acetyl-β-D-glucosaminide.

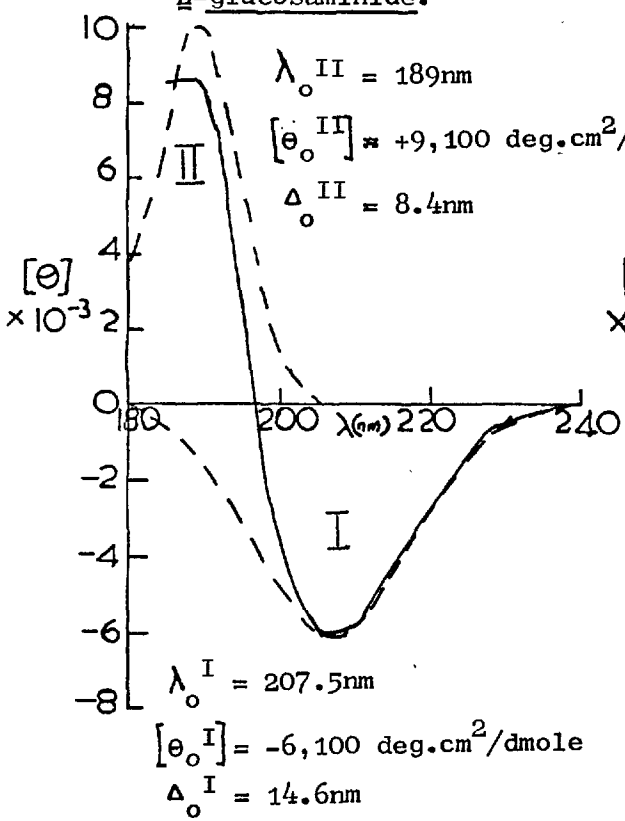


Fig.5.5. CD of an aqueous solution of methyl N-acetyl-β-D-galactosaminide.

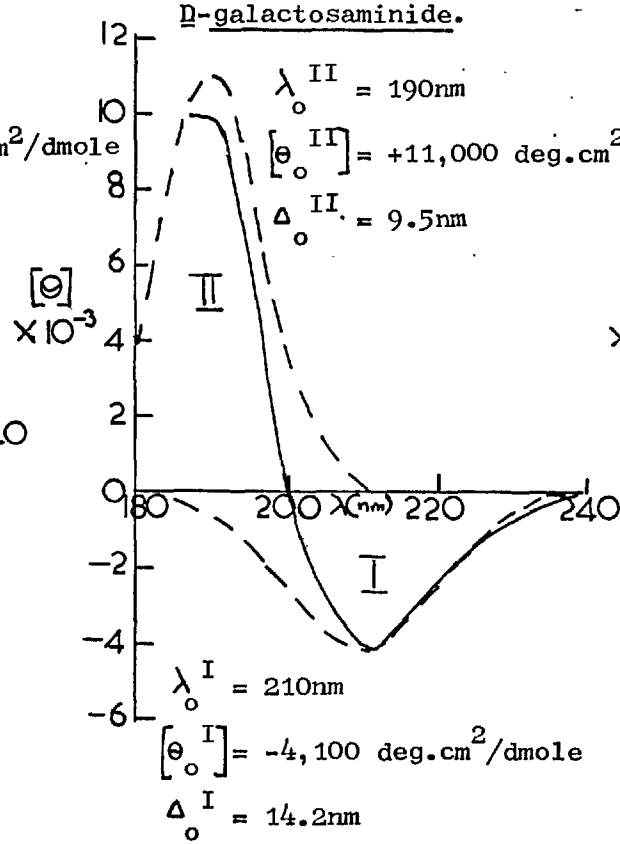
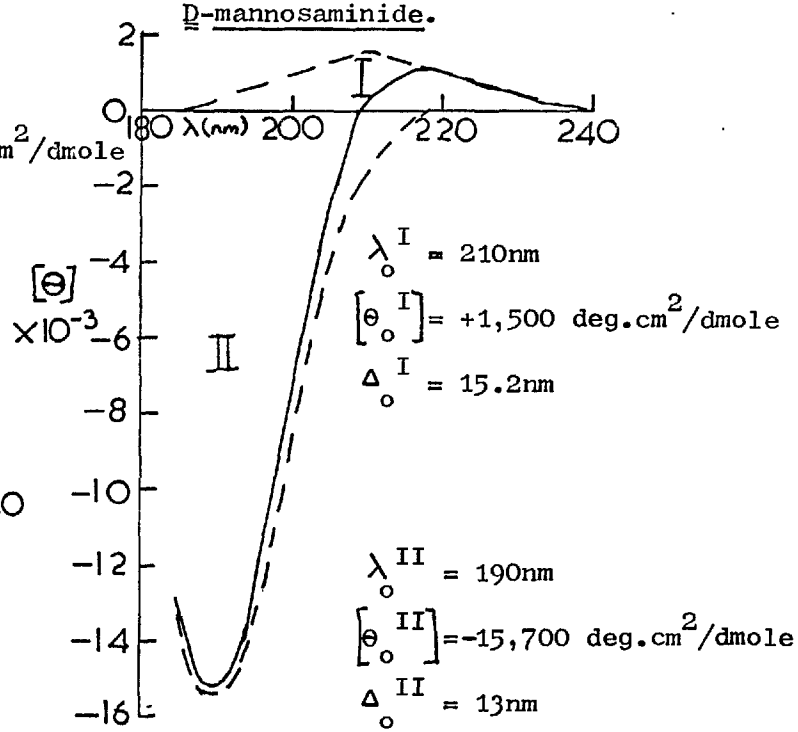


Fig. 5.6. CD of an aqueous solution of methyl N-acetyl-β-D-mannosaminide.



The observed CD curves have been resolved into two Gaussian bands of opposite signs. Values for the wavelength of the extrema, λ_o, the extreme ellipticity $[\theta_o]$, and the half-band width Δ_o, of these bands are recorded in Figs. 5.4-5.6. This approach is discussed on page 352.

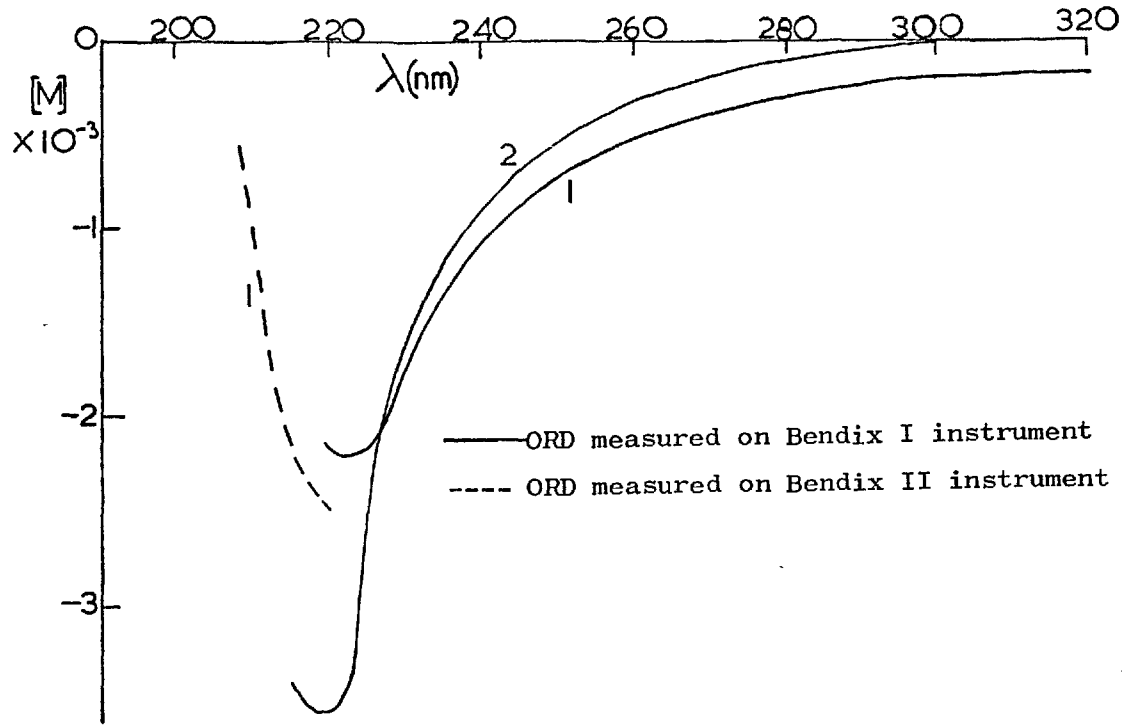
Reversal of the signs of the Cotton effects of the N-acetyl-D-mannosaminides compared to those of the N-acetyl-D-glucosaminides and galactosaminides is not wholly unexpected because of the spacial relationships of the chromophoric acetamido groups to the sugar rings.

The ORD of some glycosylamines.

Aqueous solutions of α-L-arabinopyranosylamine, β-D-glucopyranosylamine and β-D-galactopyranosylamine were prepared and immediately examined on the Bendix I instrument. The compounds show positive plain curves from 600 to 220nm. α-D-galactopyranosylamine mutarotated to less positive rotations at all wavelengths. Positive plain curves for mutarotated solutions of D-glucose were observed (Pace, Tanford & Davidson, 1964; Listowsky, Avigad & Englard, 1965). These workers found a general correlation between the ORD and the conformation of aldoses and aldosides. The curves for mutarotated D-galactose and L-fucose were found to be more complicated, presumably due to the presence of furanose and open chain forms.

The ORD of GlcNAc-NH_2 is similar to that of methyl N-acetyl-β-D-glucosaminide (Fig. 5.7) and shows a trough at 224nm, with an [M] value of about -2,300 (deg. cm^2/dmole). After storage of the aqueous solution under aseptic

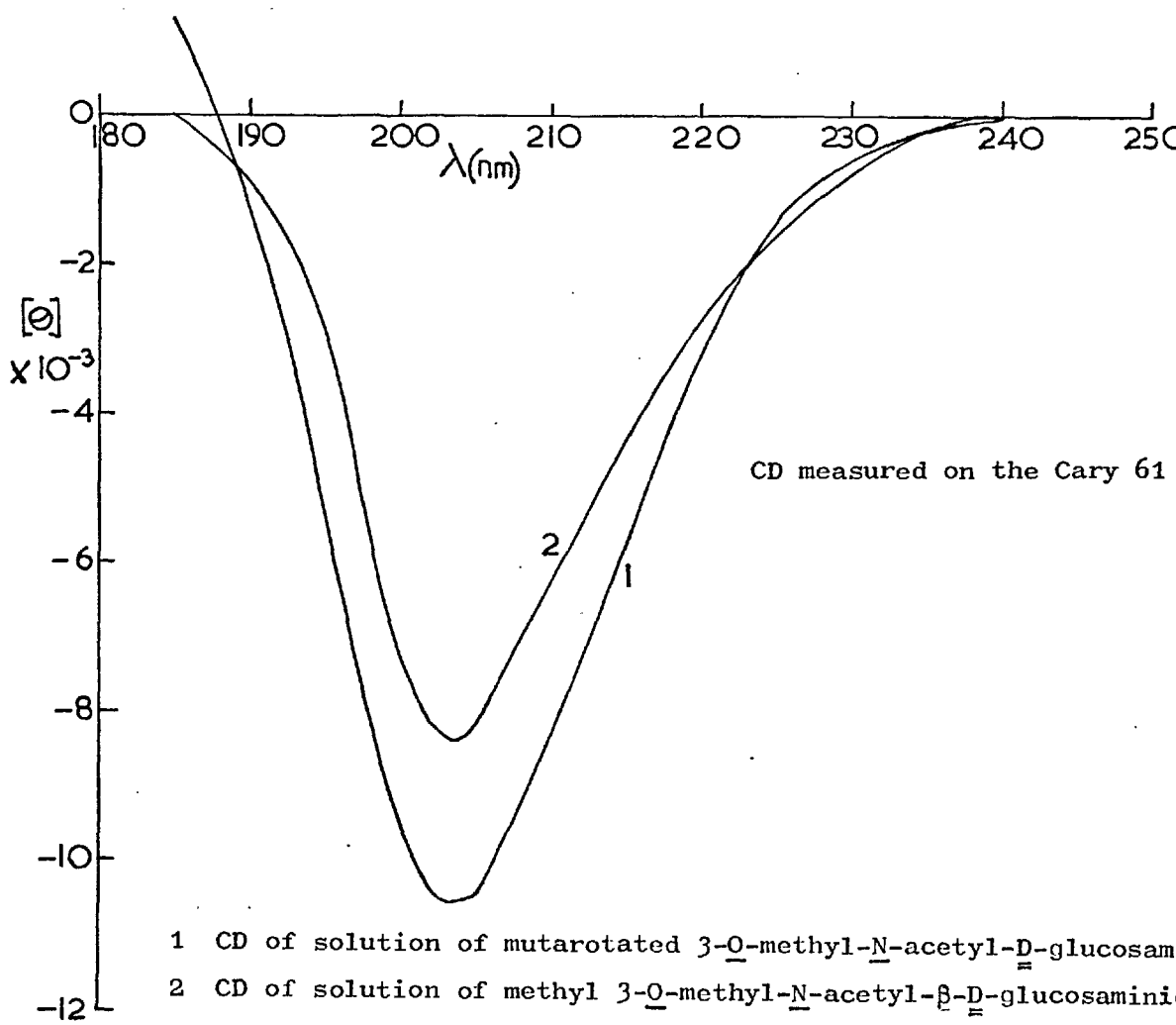
Fig. 5.7. The ORD of aqueous solutions of GlcNAc-NH_2



1 The ORD exhibited by a fresh solution of GlcNAc-NH_2

2 The ORD exhibited by a solution of mutarotated GlcNAc-NH_2

Fig. 5.8. The CD of aqueous solutions of 3-O-methyl-N-acetyl-D-glucosamine.



1 CD of solution of mutarotated 3-O-methyl-N-acetyl-D-glucosamine

2 CD of solution of methyl 3-O-methyl-N-acetyl-β-D-glucosaminide

conditions for four weeks at +4°C, the molecular rotation of the trough becomes more negative ([M] -3,300 deg. cm^2/dmole), whereas the rotations at higher wavelength become more positive. Because the molecular rotation at the first trough (220nm) of a mutarotated solution of N-acetyl-D-glucosamine is only -1,100 deg. cm^2/dmole, it may be seen that the changes in the ORD of GlcNAc-NH_2 suggest that the mutarotation of GlcNAc-NH_2 is not caused simply by anomerisation, or by hydrolysis to yield N-acetyl-D-glucosamine. This problem has been discussed in Chapter 3.

The CD of 2-acetamido-2-deoxy-3-O-methyl-D-glucopyranose.

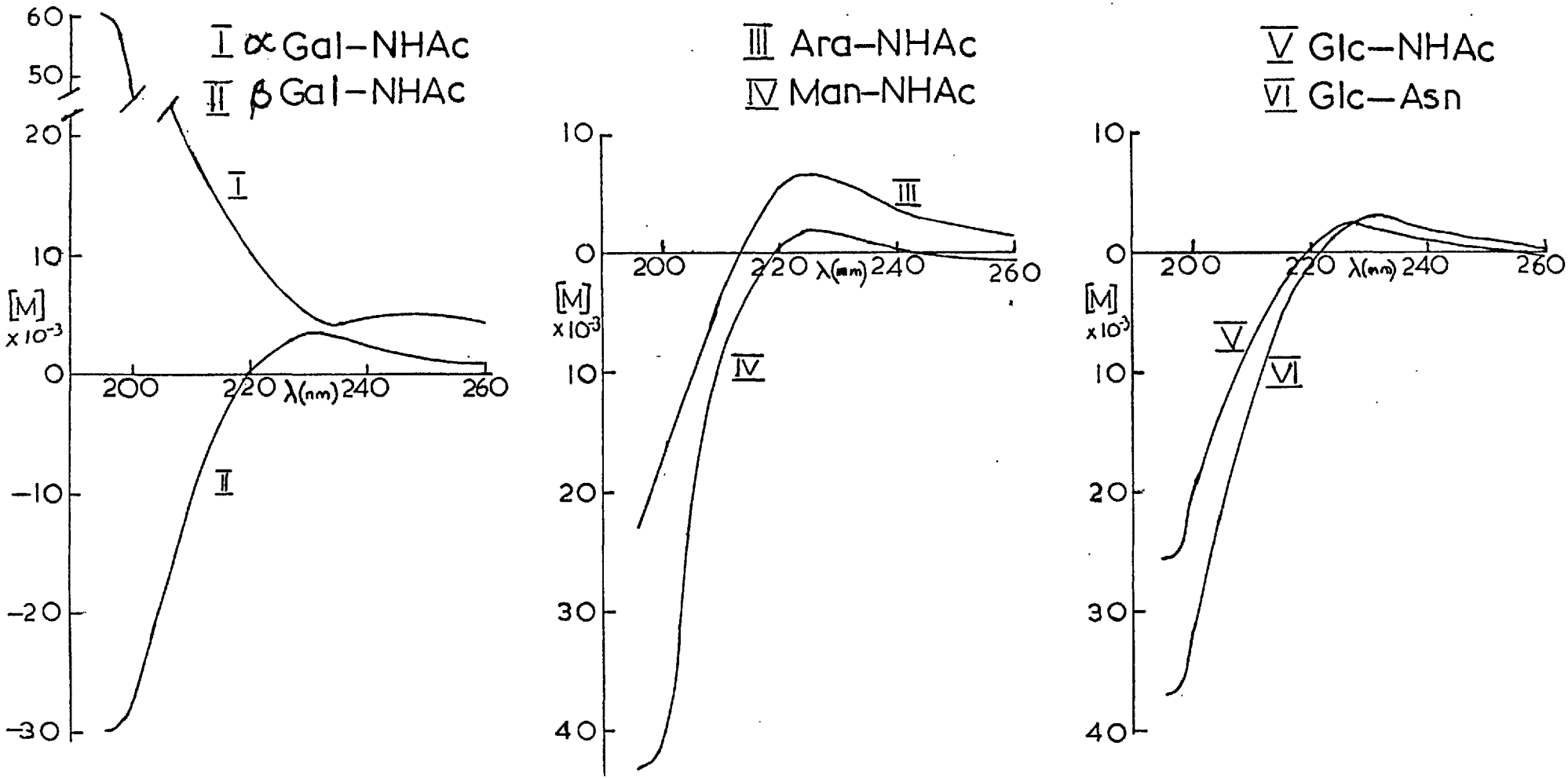
The CD curves of mutarotated 2-acetamido-2-deoxy-3-O-methyl-D-glucopyranose and its methyl β-glycoside (Fig. 5.8) have their first negative minima at lower wavelengths (204nm) than the wavelength of the minimum exhibited by methyl N-acetyl-β-D-glucosaminide.

The rotational strengths of the transitions responsible for these minima are greater than those responsible for the first extrema of the methyl glycosaminides of N-acetyl-D-glucosamine or N-acetyl-D-galactosamine and, in contrast to these latter N-acetyl-D-glycosaminides, no positive maxima are visible between 185nm and 200nm.

The chiroptical properties of N-acylglycosylamines bearing acetamido groups at the C_1 positions only.

More marked anomalous dispersions are seen in the ORD curves of the N-acylglycosylamines (Fig. 5.9) than in those of any of the N-acetyl-D-glycosaminides. The overall shape of the ORD curve of N-acetyl-α-D-galactosylamine

Fig. 5.9. ORD curves of N-acylglycosylamines (Bendix II) in aqueous solution



is, however, similar to that of methyl N-acetyl-β-D-galactosaminide (Fig. 5.1), while the shape of the ORD curve of N-acetyl-β-D-galactosylamine is similar to that of methyl N-acetyl-α-D-mannosaminide. The ORD curves of Ara-NHAc, Man-NHAc, Glc-NHAc and Glc-Asn have similar shapes to the ORD curve of N-acetyl-β-D-galactosylamine and show peaks at wavelengths between 231nm (Glc-Asn) and 224nm (Ara-NHAc). Inflections are observed at wavelengths below 200nm in all these ORD curves except in that of Ara-NHAc. The presence of these inflections suggests that troughs of negative Cotton effects are present at wavelengths just below 195nm, the wavelength limit of the instrument.

The characteristic shapes of the ORD curves of the N-acylglycosylamines studied here would be expected if these compounds give rise to first Cotton effects at about 210nm which are superimposed on much larger Cotton effects of opposite sign at lower wavelength (see Fig. 5.3). Not unexpectedly the Cotton effects may be seen more clearly in the CD spectra (Figs. 5.10-5.13).

The CD curves for the N-acylglycosylamines all have first extrema at 212nm and inflections at wavelengths approaching 185nm. The latter suggests that there is present in each case a further extremum at about 185nm of opposite sign to the first. α-Gal-NHAc gives rise to a negative minimum at 212nm, and positive ellipticities at shorter wavelengths, while the other N-acylglycosylamines have positive maxima at 212nm and negative ellipticities at shorter wavelengths.

Fig. 5.10. The CD of aqueous solutions of N-acylhexosylamines.

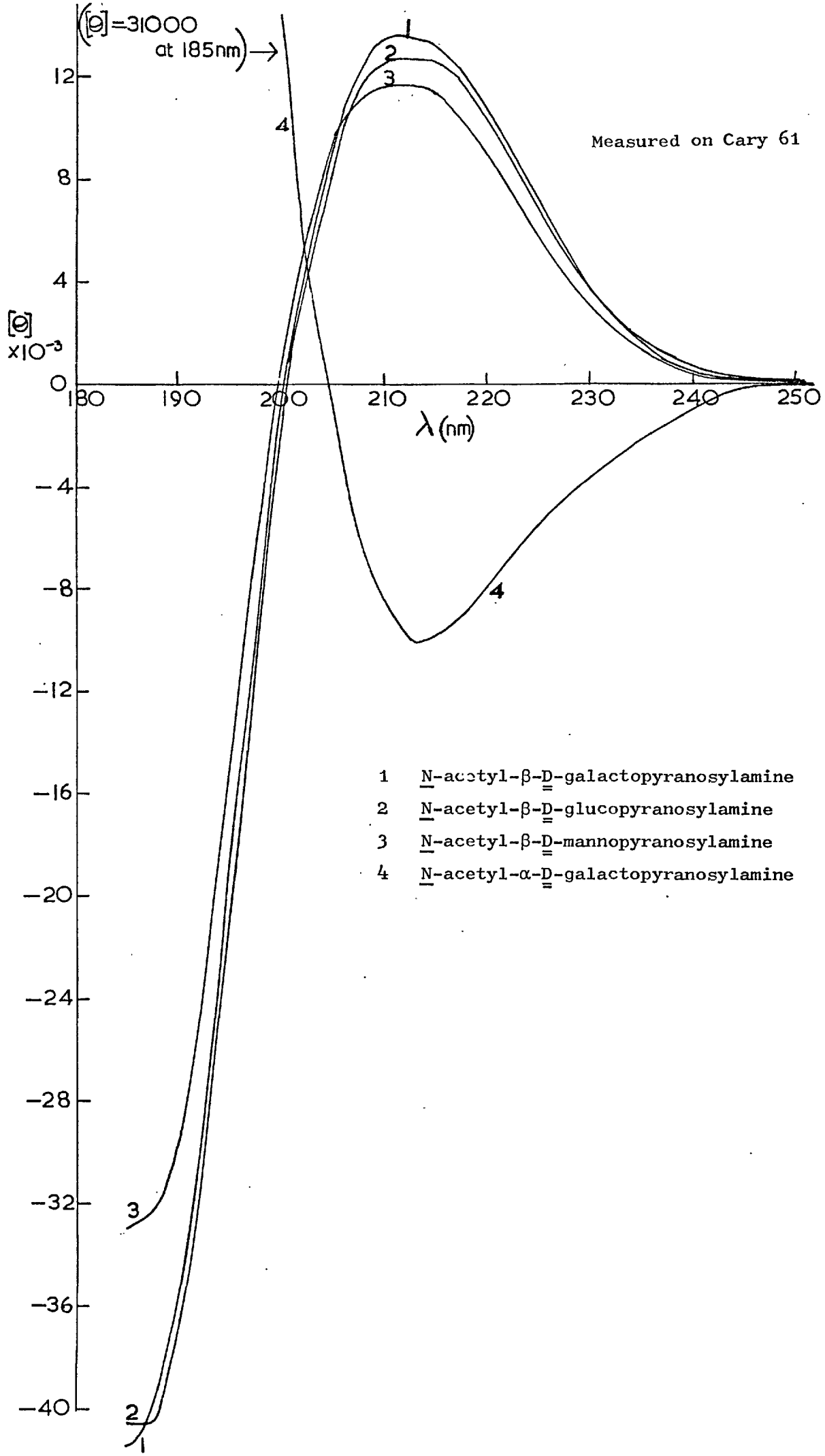


Fig. 5.11. The CD of aqueous solutions of N-acylpentosylamines.

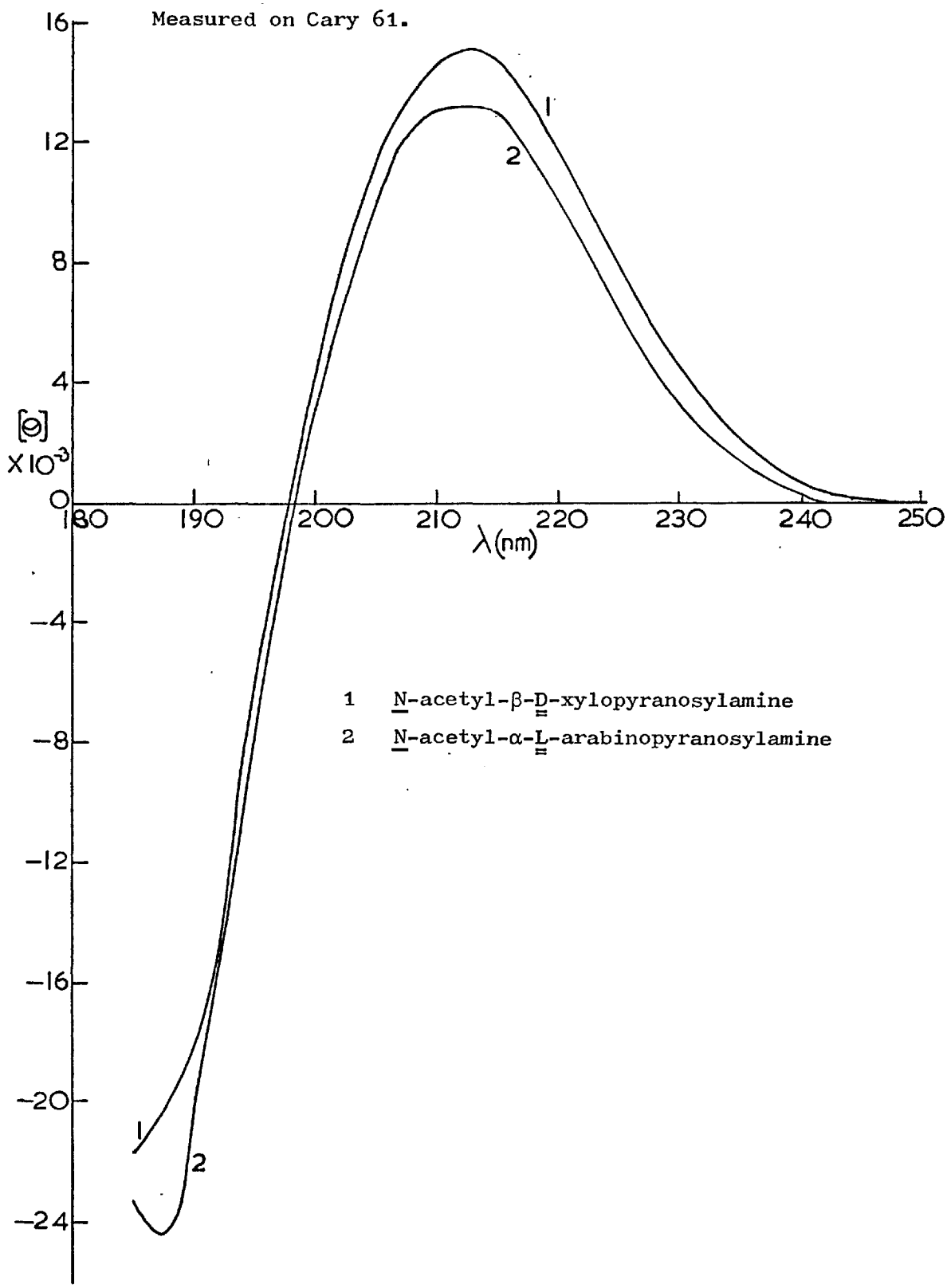
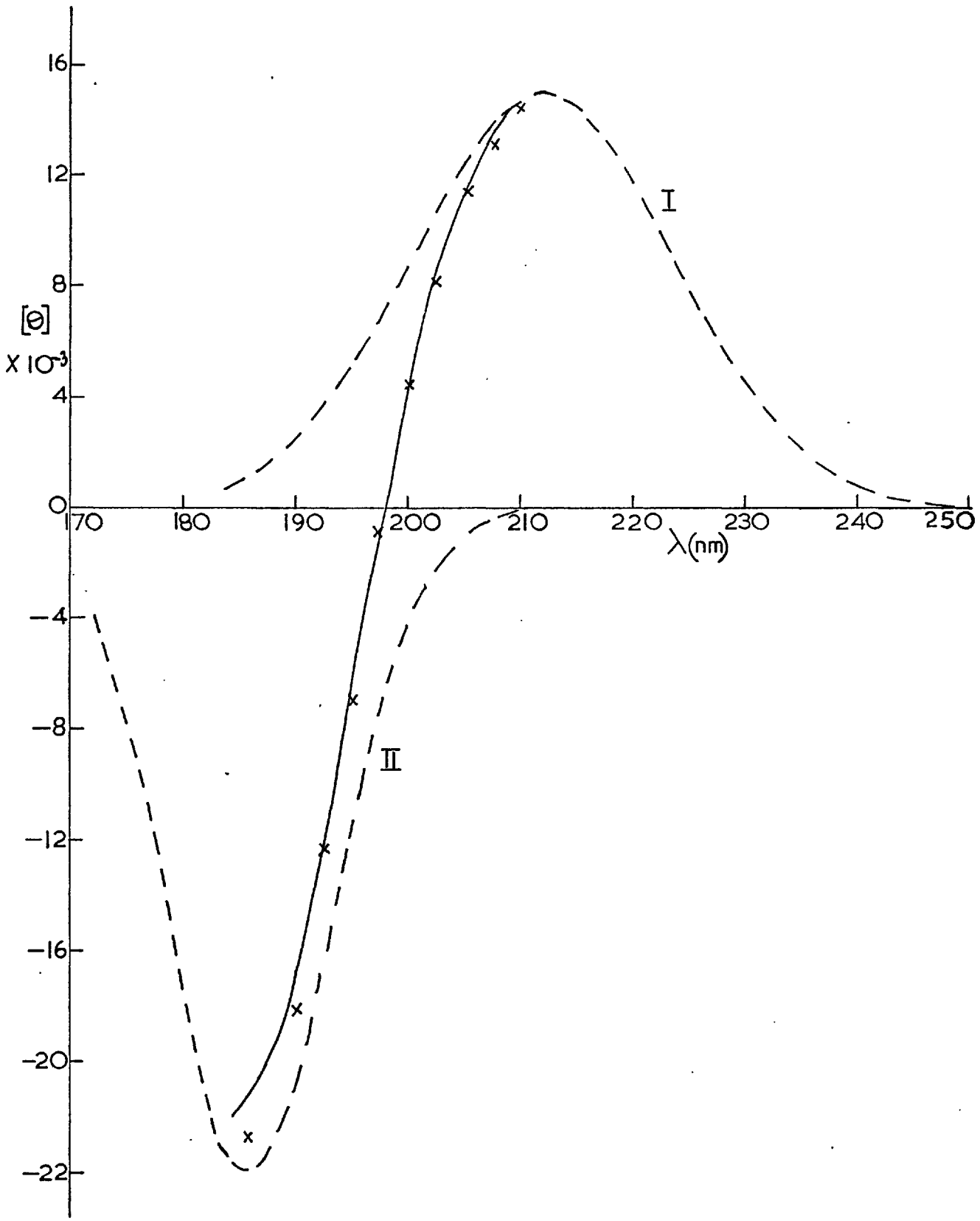


Fig. 5.12. Resolution of the CD curve of N-acetyl-β-D-xylopyranosylamine into two Gaussian bands of opposite rotational strength



—— Experimentally determined CD curve

---- Gaussian bands generated for the parameters listed in Table 5.1.

X—X Summation of the Gaussian bands generated for transitions I and II

From a comparison of the ORD and CD of the α- and β-forms of N-acetylgalactosylamine, it is apparent that changing the configuration at the carbon atom bearing the acetamido group changes the signs of the Cotton effects.

Comparison of the ORD and CD properties of these N-acylglycosylamines with those of the N-acetyl-D-glycosaminides shows that an equatorial acetamido group at C_1 gives rise to Cotton effects with mid-points near 210nm and near 185nm which are each of opposite sign to the Cotton effects of an equatorial acetamido group at C_2. Similarly, the Cotton effects of an axially orientated acetamido group at C_1 are of opposite sign to those of an axially orientated acetamido group at C_2.

The CD curves of the N-acylglycosylamines were each resolved into two Gaussian bands of opposite sign, separated by about 26nm. The first band (transition I) is centred at 212nm, and the second near 186nm (transition II), and an example of the correspondence obtained between the theoretical CD curve generated by a summation of the two bands and the observed CD curve is shown in Fig. 5.12. It is found that in all cases a good fit to the longer wavelength of the curve is obtained by choosing, as parameters for the Gaussian band at 212nm, the value of $[\theta_o]$, and the half-band width, actually observed in the CD curve at this wavelength. This demonstrates that there is no extensive overlap of CD bands at this wavelength. A satisfactory correspondence of the lower half of the curve to a Gaussian band is obtained by choosing the parameters listed in Table 5.1.

Table 5.1. **The wavelengths (λ_o), molecular ellipticities ($[\theta_o]$), half-band widths (Δ_o) and rotational strengths (R) of resolved CD bands**[a] The significance of these parameters is discussed on page 352.

Compound	λ_o^I(nm)	$[\theta_o^I]$ deg.cm^2/dmole	Δ_o^I(nm)	R x 10^{40} c.g.s. units	λ_o^{II}(nm)	$[\theta_o^{II}]$ deg.cm^2/dmole	Δ_o^{II}(nm)	R x 10^{40} c.g.s. units
Me β-GlcNAc	207.5	- 6,100	14.6	- 5.3	189	+ 9,100	8.4	+ 5.0
Me β-GalNAc	210	- 4,100	14.2	- 3.4	190	+11,000	9.5	+ 6.8
Me β-ManNAc	210	+ 1,500	15.2	+ 1.3	190	+15,700	13	-13.1
Me 3-O-Me-β-GlcNAc	204	- 8,400	14.6	- 7.4				
3-O-Me-GlcNAc[b]	204	-10,500	14.7	- 9.3				
Glc-NHAc	212	+12,700	16.3	+12.0	186	-41,500	10.5	-28
β-Gal-NHAc	212	+13,600	16.2	+12.8	186	-44,000	10.5	-31
Man-NHAc	212	+11,700	15.4	+10.5	185	-33,000	11.5	-25
Xyl-NHAc	212	+15,000	16.3	+14.0	185	-24,000	10.5	-21
Ara-NHAc	212	+13,200	15.5	+11.9	185	-26,000	11	-19
α-Gal-NHAc	213	-10,100	16.3	-13.0	185	+31,000	13	+27
GlcNAc-NHAc	213	+ 7,500	16.0	+ 7.0	195	+57,900	11	+40
GlcNAc-Asn	213	+10,500	17.3	+10.6	195	+59,500	9	+34

(a) The band at longer wavelength is termed transition I in all cases, and the parameters corresponding to this are termed λ_o^I, $[\theta_o^I]$ and Δ_o^I. The terms λ_o^{II}, $[\theta_o^{II}]$ and Δ_o^{II} are used as parameters for transition II, the band at shorter wavelengths. (b) This is for a mutarotated aqueous solution.

There is no guarantee that the resolution of the results into two overlapping Gaussian bands corresponds with the number of transitions which occur. This approach has, however, been taken by others to examine the results obtained with disymmetric molecules (see page 361). The method is sufficiently sensitive to eliminate the possibility of further transitions, at wavelengths close to those being considered, leading to large Cotton effects.

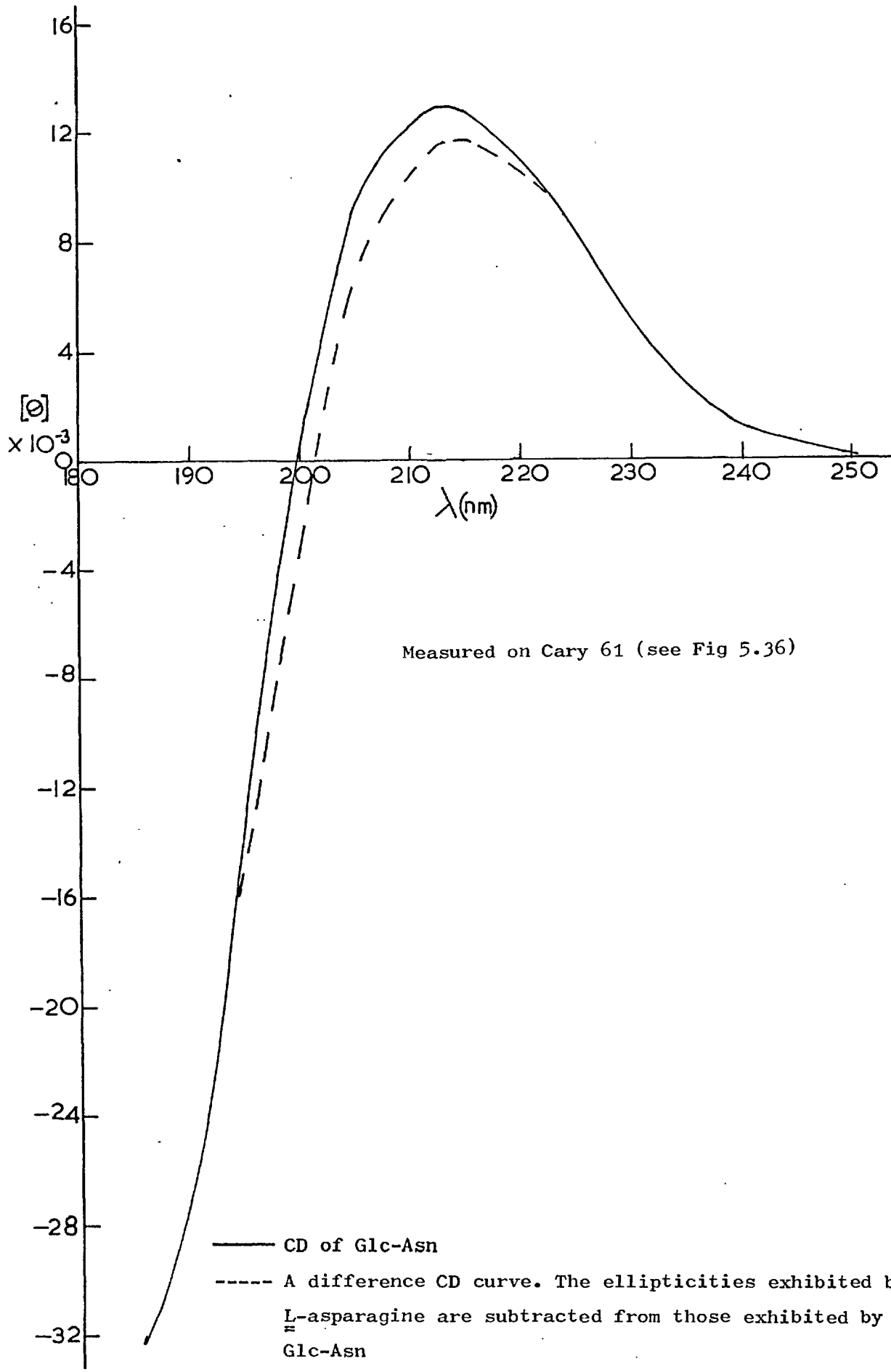
The Δ_o values for transition I in the CD spectra of the Nacylglycosylamines are all of the order of 15.4nm while the values for transition II are about 10.9nm.

The CD curve of Glc-Asn (Fig. 5.13) is closely similar to that of Glc-NHAc, suggesting that the amino acid residue itself contributes very little to the chiroptical properties of this molecule between 250 and 185nm. This view is substantiated by an examination of the difference CD curve (Fig. 5.13) for the $[\theta]$ values of Glc-Asn and L-asparagine at various wavelengths. In this difference curve, the positive maximum is seen to be displaced to longer wavelengths (215nm), but the difference in molecular ellipticity between this and the native curve is small. The chiroptical properties of L-asparagine are discussed below.

The chiroptical properties of L-asparagine, GlcNAc-Asn, GlcNAc-NHAc and GlcNAc-N_3.

The ORD curve of aqueous solutions of GlcNAc-NHAc shows a positive shoulder at 227nm with a much larger peak ($[M]$ = 13,600 deg. cm^2/dmole) at 204nm. There is in addition an inversion point at 197nm with a change to highly negative

Fig. 5.13. The CD of an aqueous solution of Glc-Asn



Measured on Cary 61 (see Fig 5.36)

——— CD of Glc-Asn

----- A difference CD curve. The ellipticities exhibited by L-asparagine are subtracted from those exhibited by Glc-Asn

rotations at lower wavelengths. Results obtained on various types of instruments are shown (Fig. 5.14) and these data are indicative of the agreement which was obtained. The limitations in penetration into the ultraviolet exhibited by the various instruments are also indicated.

The ORD curve of the model linkage compound (GlcNAc-Asn; Fig. 5.15) is similar to that of GlcNAc-NHAc, but possesses a more pronounced shoulder at 227nm. Here again it may be seen by a comparison of these curves that the amino acid residue makes little contribution to the ORD curve. This is emphasised by the following consideration also. A difference curve constructed for the $[M]$ values of GlcNAc-Asn minus those of L-asparagine (Fig. 5.15) is little different from the native curve.

The molecular rotation of L-asparagine at 210nm has a value of only +800 deg. cm^2/dmole) (Fig. 5.16). The first extremum of a positive Cotton effect is visible at this wavelength and an inversion point occurs at 204nm. The CD curve of L-asparagine (Fig. 5.16) shows a positive maximum at 202.5nm, in agreement with the ORD data. A maximum has been observed in the CD spectra of aqueous solutions of L-asparagine at 202nm by other workers (Fowden, Scopes & Thomas, 1971). Their quoted value for the maximum ellipticity at this wavelength (+2,840 deg. cm^2/dmole) agrees with that measured here (+2,900 deg. cm^2/dmole). The Cotton effect was assigned to an $n \rightarrow \pi^*$ transition in the carboxyl group.

The CD curve of GlcNAc-NHAc (Fig. 5.17) shows a very

Fig. 5.14. The ORD, measured on various instruments, of aqueous solutions of GlcNAc-NHAc and Glc-NHAc.

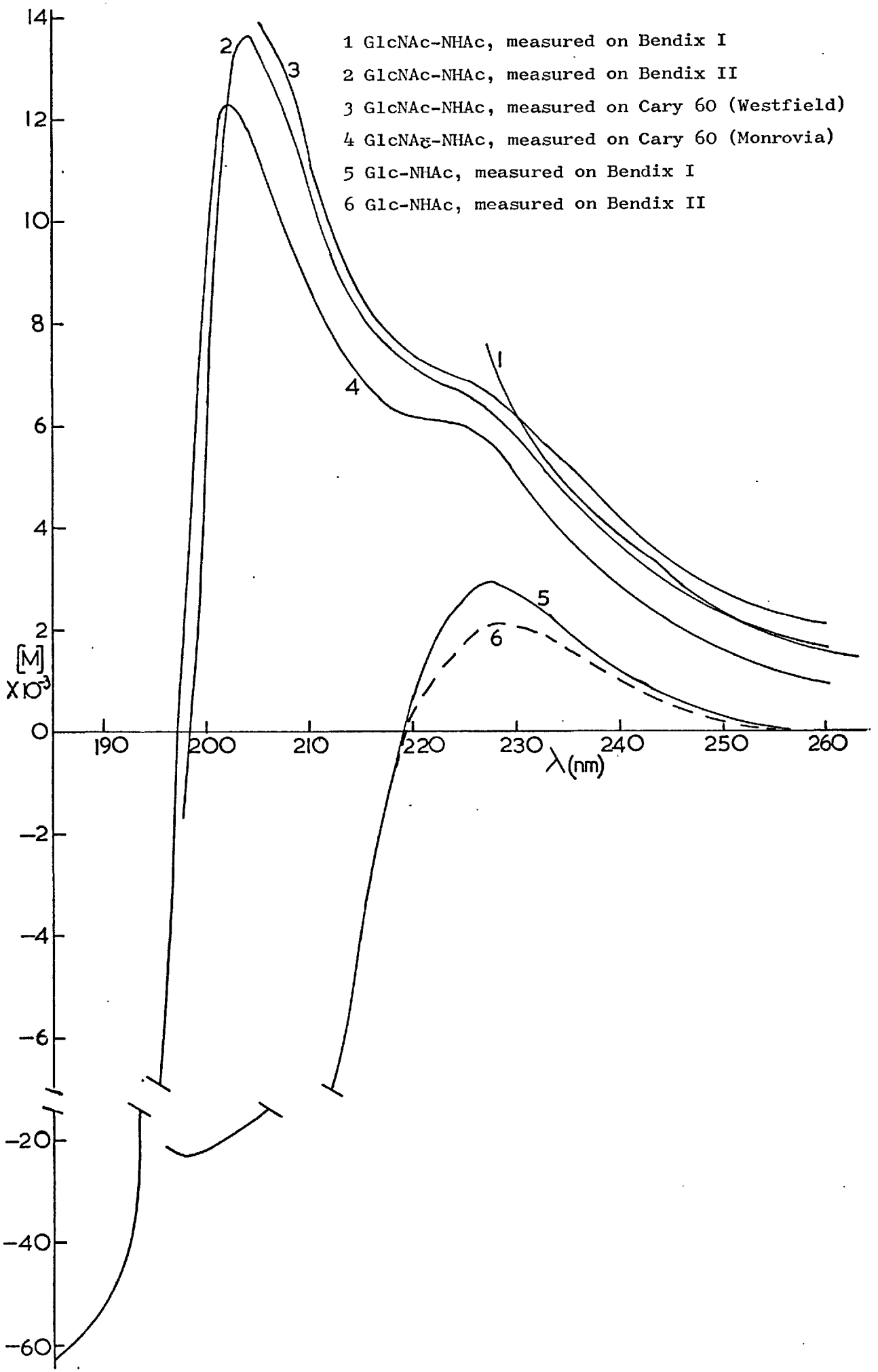
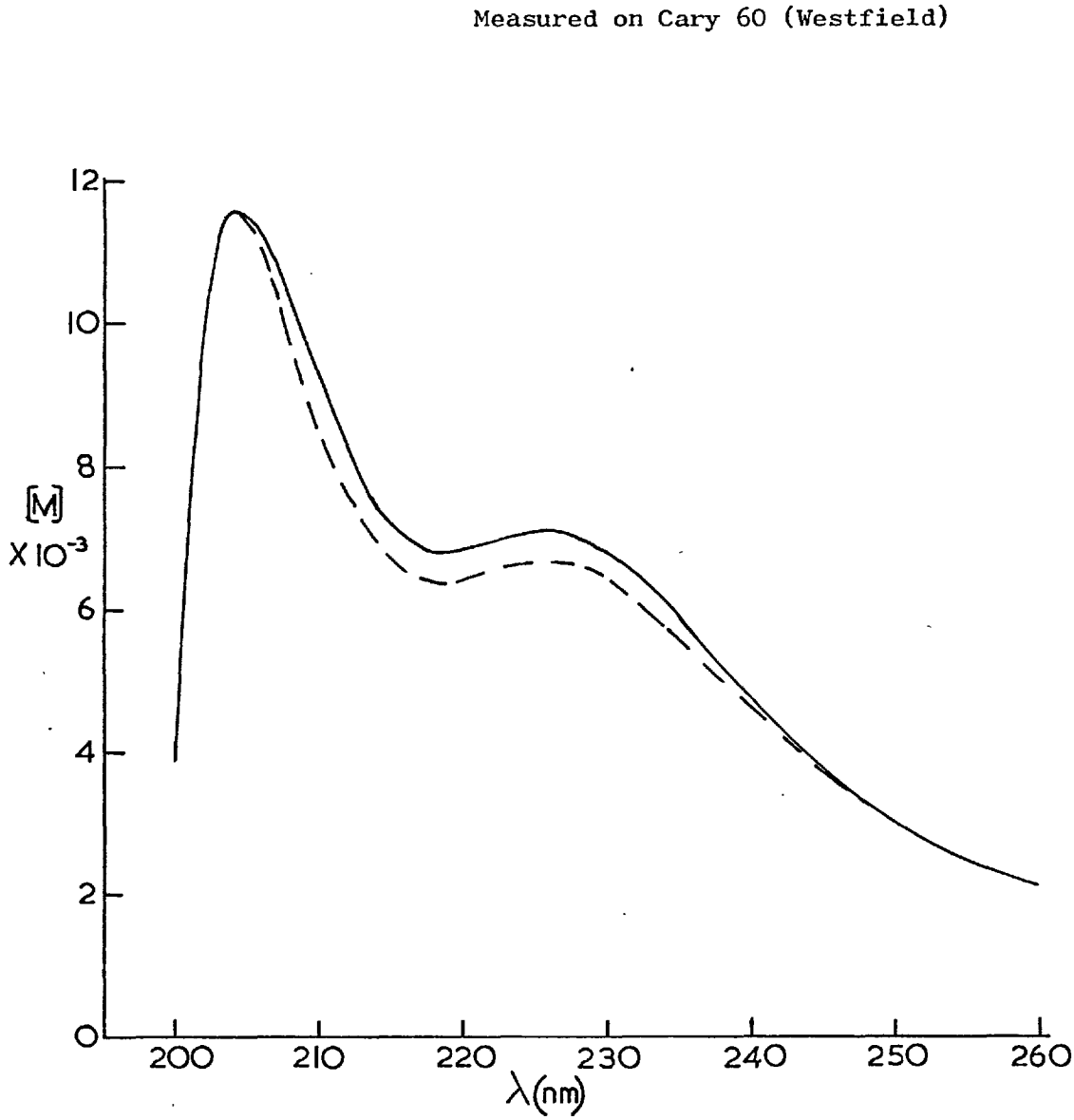


Fig. 5.15. The ORD of an aqueous solution of the model linkage compound, GlcNAc-Asn.

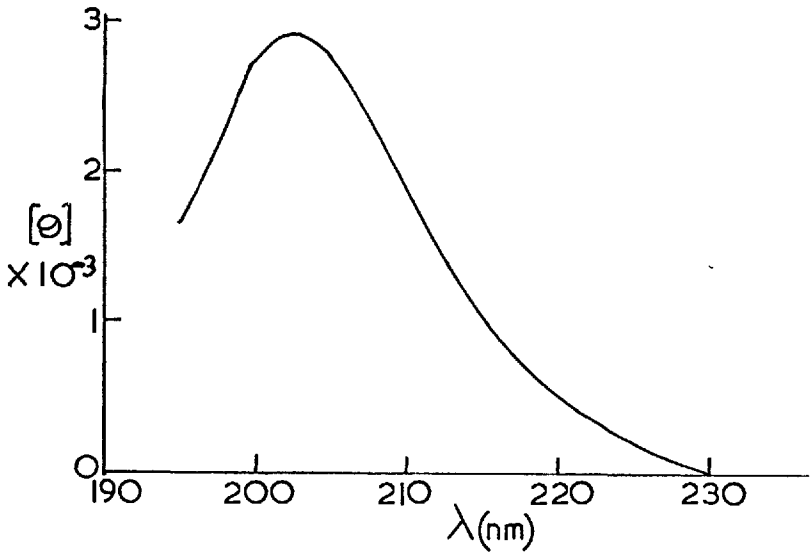


——— ORD of GlcNAc-Asn

— — — Difference ORD curve. The molecular rotations given by L-asparagine are subtracted from those given by GlcNAc-Asn.

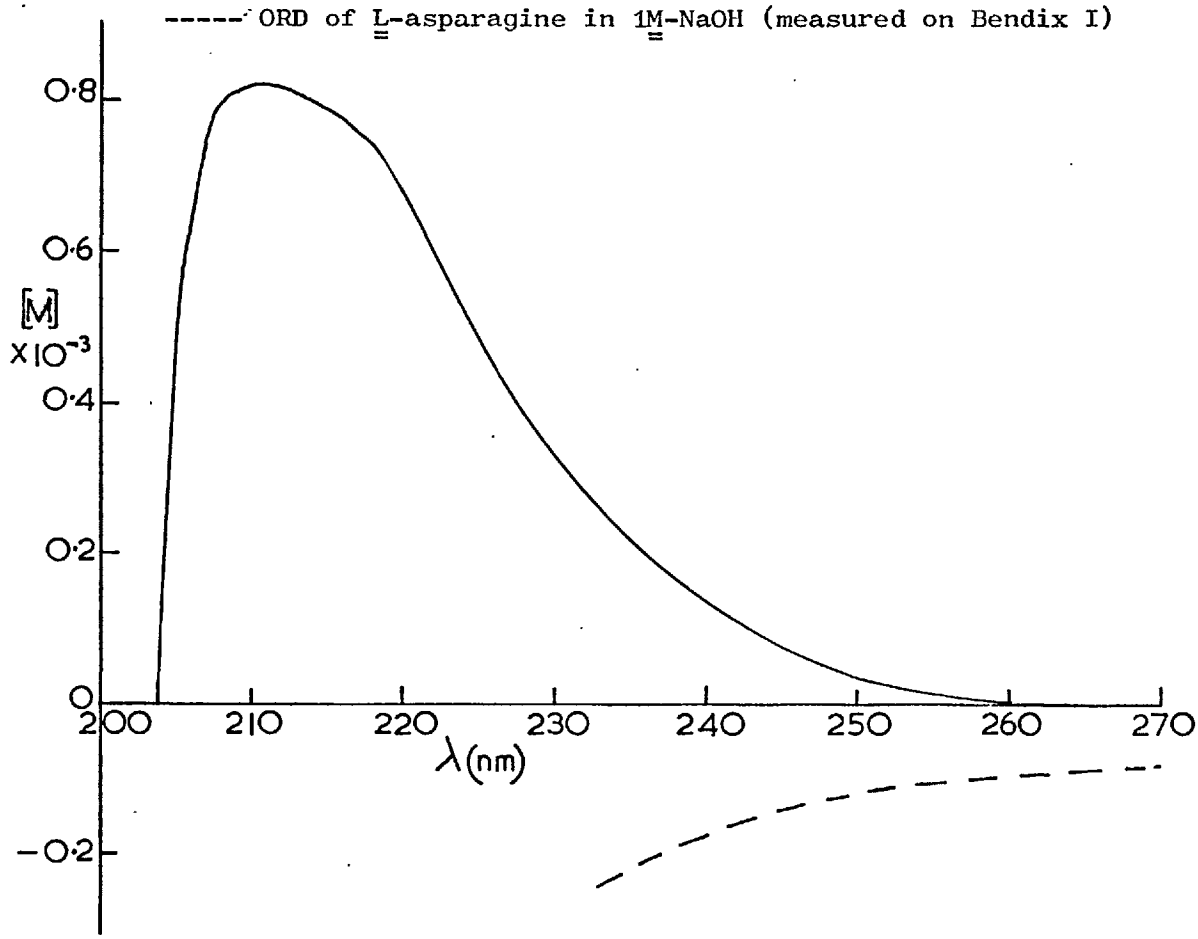
Fig. 5.16. The ORD and CD of solutions of L-asparagine in water and in 1M-NaOH.

CD of L-asparagine (measured on Cary 61)



——— ORD of L-asparagine in water (measured on Bendix I and II)

----- ORD of L-asparagine in 1M-NaOH (measured on Bendix I)



large, positive band at 195nm with a shoulder at longer wavelengths (210-220nm). Altogether four Cotton effects might be expected, with two optically active bands contributed from each of the acetamido groups at C_1 and C_2. If there is no interference exhibited by the groups on each other, the acetamido group at C_2 might be expected to give rise to a negative peak at about 210nm ($[\Theta_o] \sim -6000$) and a positive peak at about 190nm ($[\Theta_o] \sim 10,000$). Superimposed on this curve would be one given by the acetamido group at C_1 with a positive peak at about 212nm ($[\Theta_o] \sim 12,000$) and a negative one at about 185nm ($[\Theta_o] = -42,000$). The resultant circular dichroism might be expected therefore to look similar to the additive curve obtained for the CD curves of Glc-NHAc and methyl N-acetyl-β-D-glucosaminide, and this is described in Fig. 5.18. An examination of this latter figure and of the CD curve for GlcNAc-NHAc would suggest that the approach for the theoretical analysis is an oversimplified one. On the contrary, it was found to be reasonable to resolve the observed CD curve into two positive Gaussian bands, the first with λ_o at 212.5nm ($[\Theta_o] = +7,500$) and the second at 195nm ($[\Theta_o] = +57,900$; Fig. 5.17). Summation of these theoretical bands leaves a small amount of negative ellipticity, possible reasons for which are discussed later.

The half-band width of the band at 195nm ($\Delta_o = 10.8$) is the same as the half-band widths measured for transition II from the CD curves of the Nacylglycosylamines and N-acetylglycosaminides. It is likely, therefore, that this band is caused by the same type of electronic transition

Fig. 5.17. The CD of an aqueous solution of GlcNAc-NHAc.

The CD curve is resolved into two major Gaussian bands generated for the parameters, listed in Table 5.1, for transitions I and II. Summation of the Gaussian bands I and II leaves a small amount of ellipticity unaccounted for, and this is labelled III.

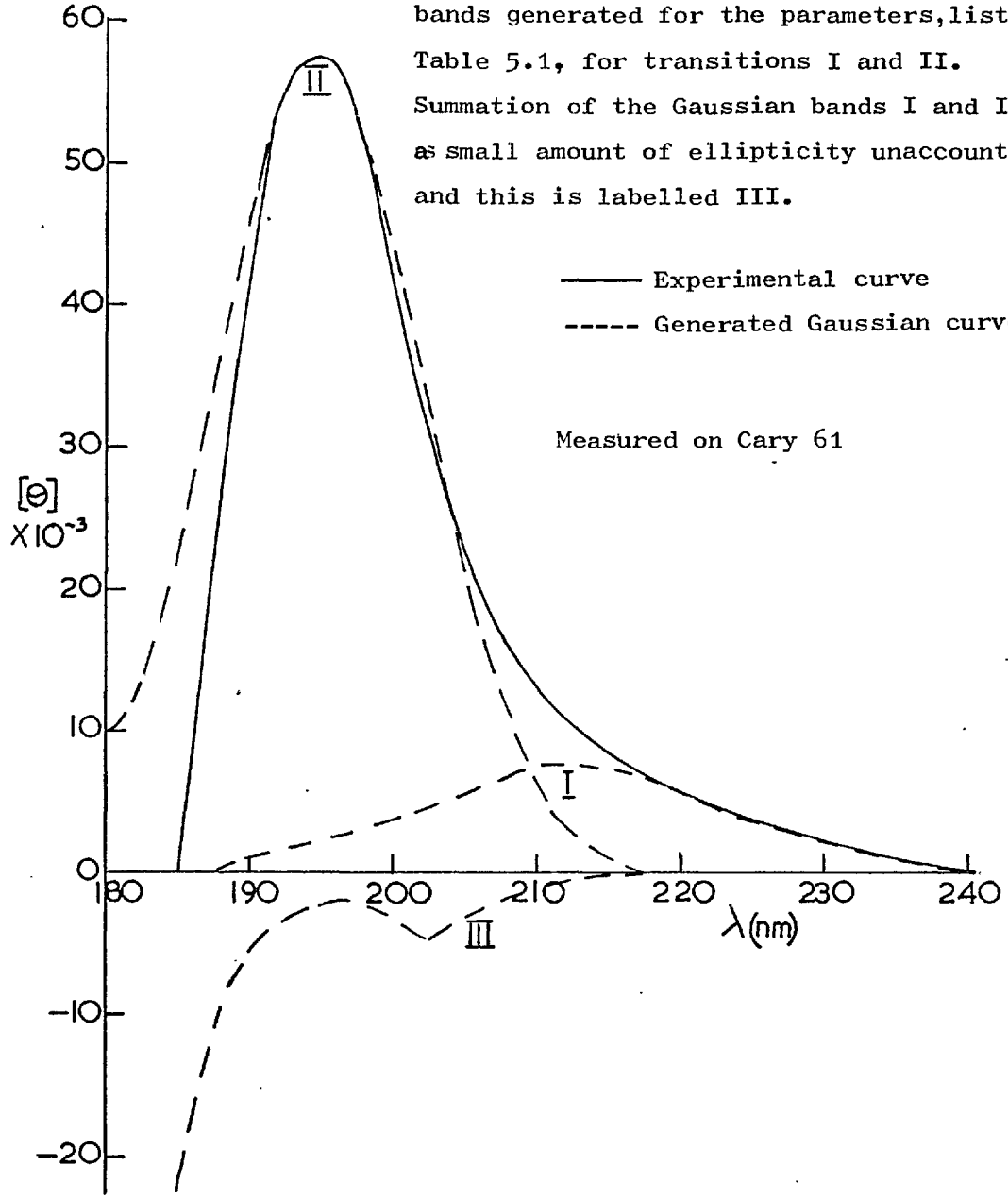
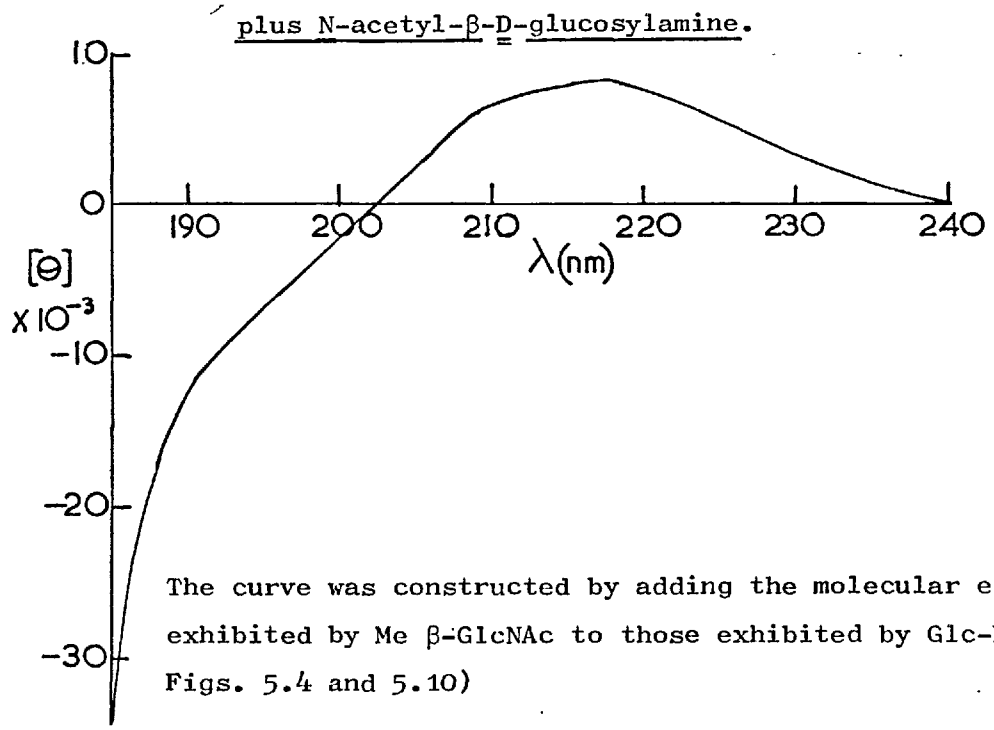


Fig. 5.18. An addition CD curve for methyl N-acetyl-β-D-glucosaminide plus N-acetyl-β-D-glucosylamine.



The curve was constructed by adding the molecular ellipticities exhibited by Me β-GlcNAc to those exhibited by Glc-NHAc (compare Figs. 5.4 and 5.10)

(probably $\pi \rightarrow \pi^*$ which is displaced to longer wavelengths by about 10nm in GlcNAc-NHAc. Half-band widths are characteristic of particular types of transition (Moscowitz, 1961). The positive band resolved at 212nm has the same half-band width as those already observed at this wavelength for the other compounds studied, and therefore may be assigned to the same type of transition as that labelled transition I.

The CD difference curve of GlcNAc-Asn minus L-asparagine may be resolved into two Gaussian bands in a similar way (Fig. 5.19). The parameters of the resolved Gaussian bands are listed in Table 5.1.

2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (GlcNAc-N_3) has a molar ultra-violet absorption maximum of 36 at 272nm. The absorption band is attributable to an $n \rightarrow \pi^*$ transition in the azido group, for which closely similar values have been found from studies with other compounds (Levene & Rothen, 1937; Sheinker, 1951). Aqueous solutions of GlcNAc-N_3 show a CD positive maximum at 270nm (Fig. 5.20), and a positive anomalous dispersion curve with a first extremum at 288nm (Fig. 5.21), but this is only partly resolved. The difference ORD curve constructed for the [M] values of GlcNAc-N_3 minus those of GlcNAc-NH_2 shows a complete positive Cotton effect, with an inversion point at 268nm (Fig. 5.22). β-D-Glucopyranosyl azide also exhibits a positive Cotton effect at about the same wavelength (Paulsen, 1968). Thus it would appear that intro-

Fig. 5.19. The CD of an aqueous solution of the model linkage compound, GlcNAc-Asn

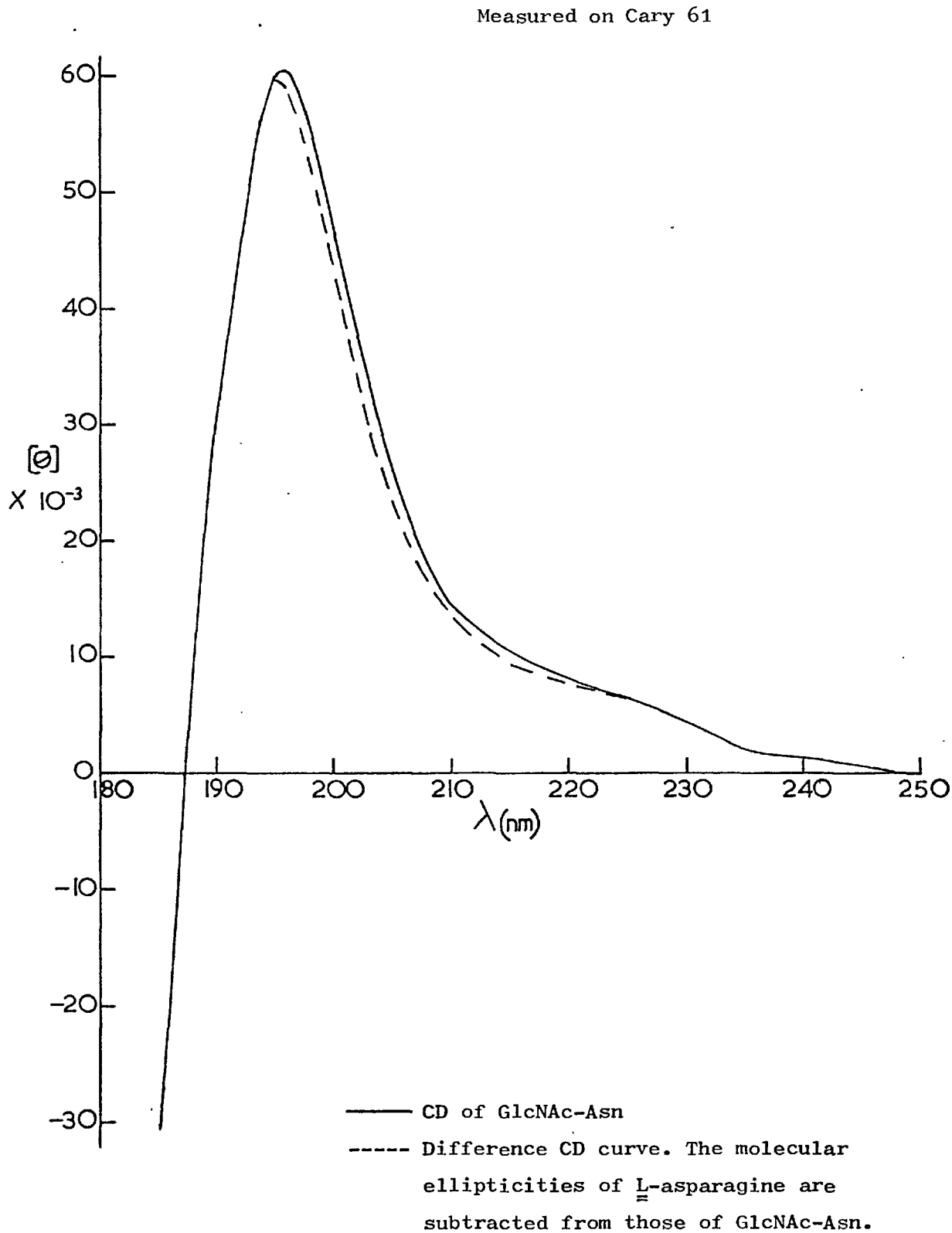


Fig. 5.20. The CD of an aqueous solution of GlcNAc-N_3
Measured on Cary 61

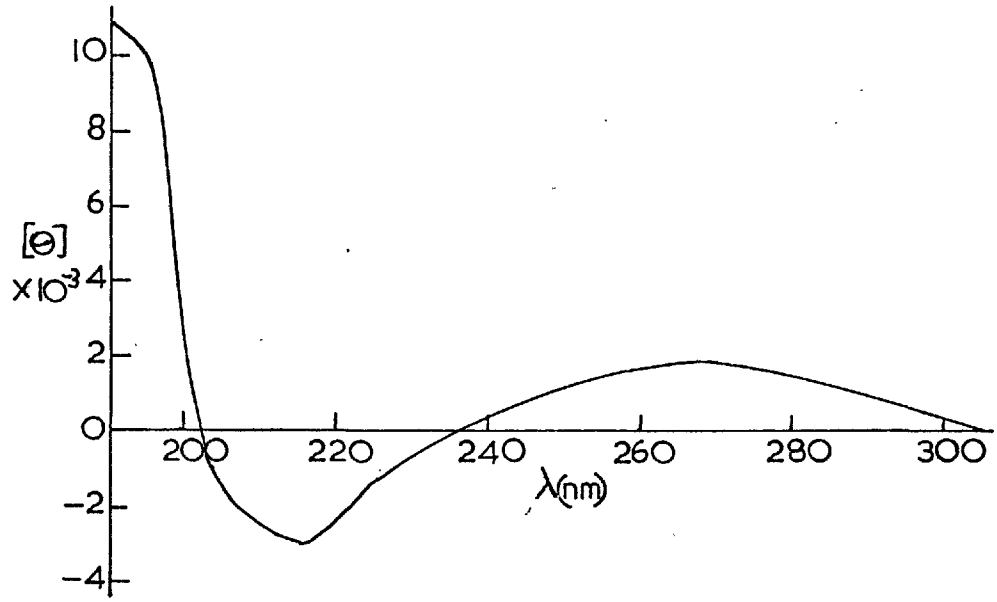


Fig. 5.21. The ORD of aqueous solutions of GlcNAc-N_3 and GlcNAc-NH_2

1 ORD of GlcNAc-N_3 2 ORD of GlcNAc-NH_2

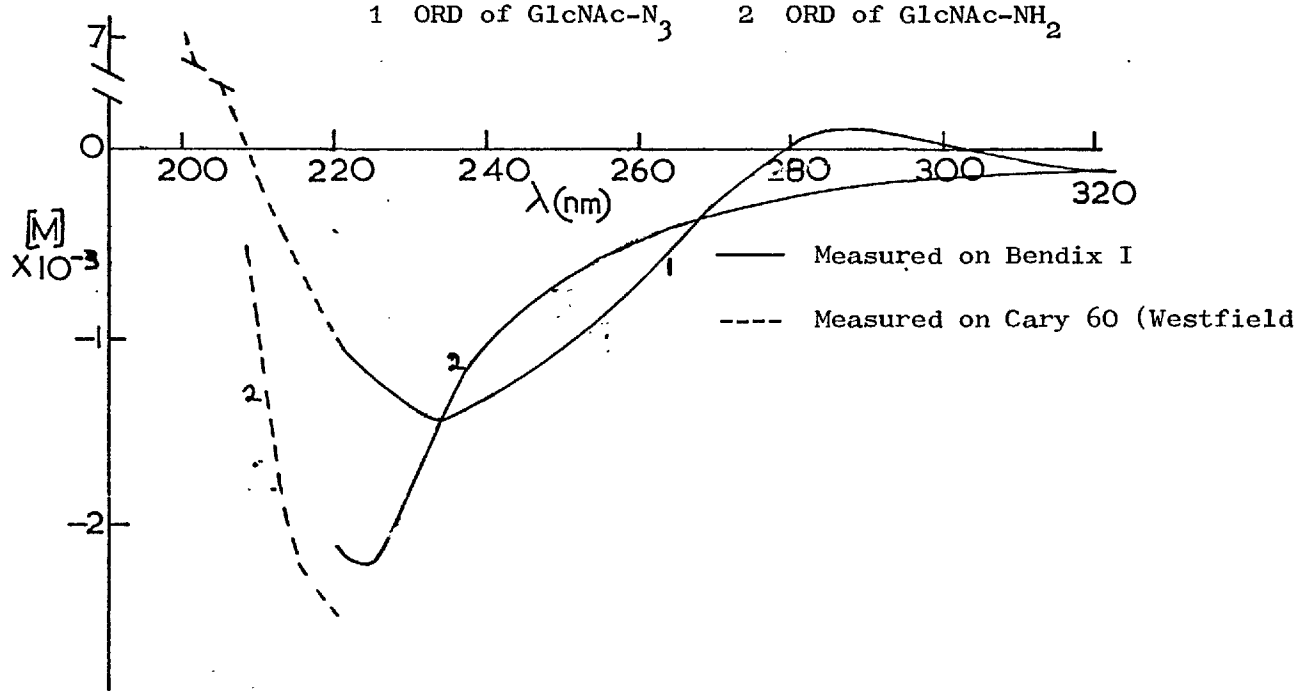
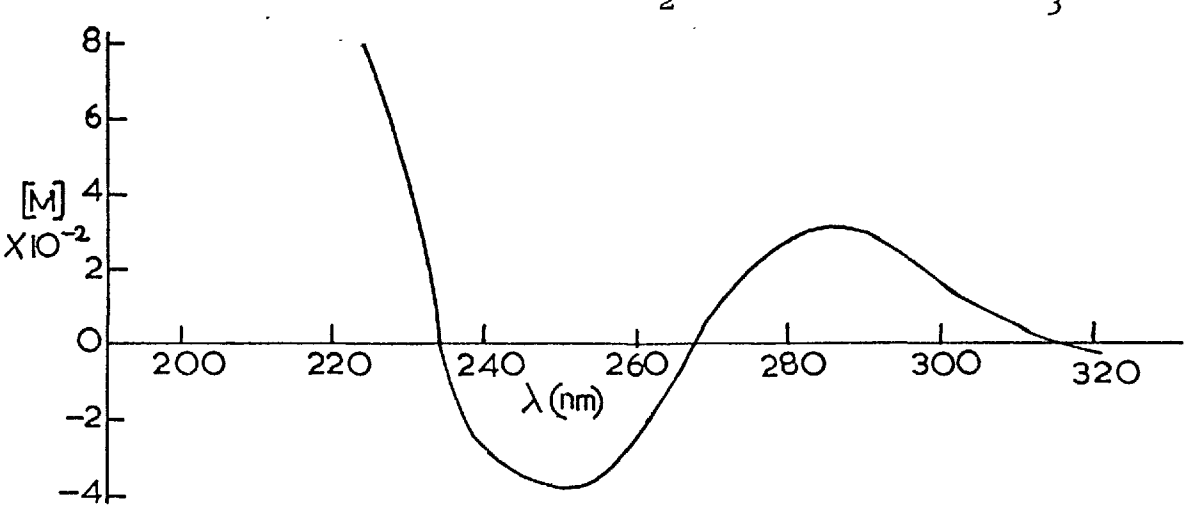


Fig. 5.22. An ORD difference curve of GlcNAc-N_3 minus GlcNAc-NH_2

The curve is constructed by subtracting the molecular rotations of GlcNAc-NH_2 from those of GlcNAc-N_3 (see Fig.5.21)



duction of an acetamido group at C_2 does not alter the sign of the Cotton effect associated with the n — π^* transition in the azido group at C_1.

Chiroptical properties of N-acylglycosylamines in dioxane-water.

Assignments of the types of transitions of groups giving rise to Cotton effects have often been achieved by measurements of elliptical properties of substances in a variety of solvents of different dielectric properties.

The CD curves of solutions of Ara-NHAc, βGal-NHAc, Glc-NHAc and Man-NHAc in dioxane - water (9 : 1 v/v) are recorded in Fig. 5.23. The high absorbance of the solvent limited measurements to wavelengths above 205nm. In all cases, the Cotton effects observed in this solvent occur at longer wavelengths (217nm) than those observed in water. The $[\theta_o]$ values measured in aqueous dioxane are about 80% of those measured in water.

The shoulders in the CD curve of GlcNAc-NHAc in dioxane - water (9 : 1 v/v) and in the ORD curve of GlcNAc-Asn in dioxane - water (2 : 3 v/v) are similarly displaced to longer wavelengths, and the sizes of the Cotton effects are reduced (Fig. 5.24).

Estimation of rotational strengths.

The rotational strength (R) of the kth transition may be calculated from the following expression (Djerassi, 1960):

$$R = \frac{3\,hc}{8\pi^3 N}\sqrt{\pi}\,[\theta_o^{\,k}]\quad\frac{\Delta^k}{\lambda_o^{\,k}}$$

where h is Planck's constant, c is the velocity of light and N is Avogadro's number. The rotational strengths

Fig. 5.23. The CD of solutions of N-acetylglycosylamines in dioxane-water (9 : 1 v/v)

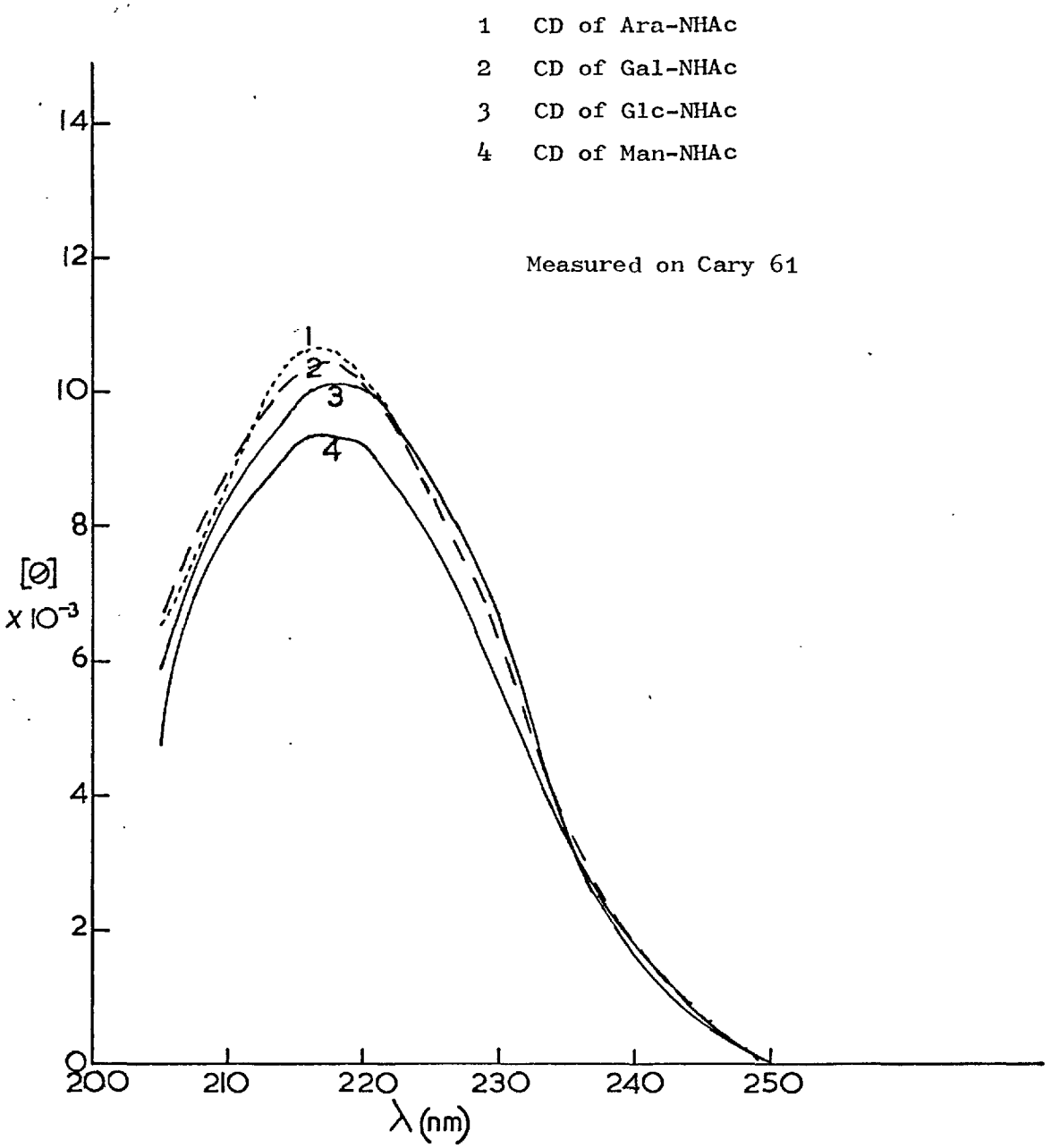
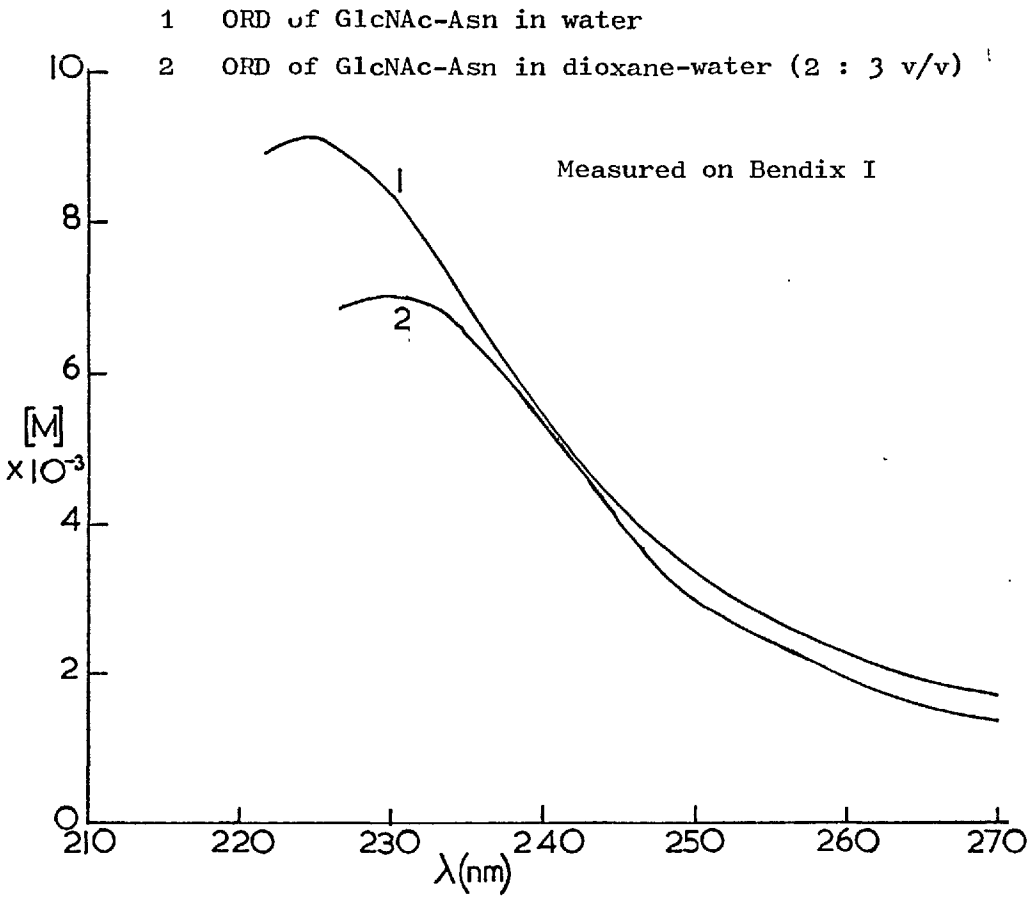
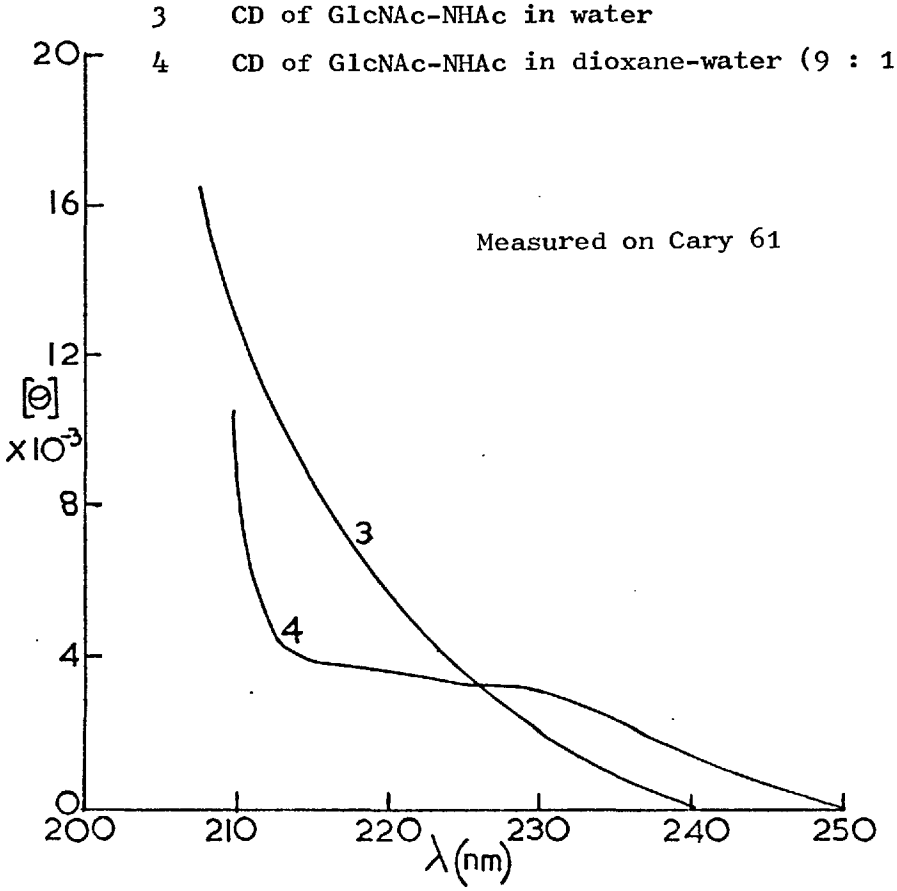


Fig. 5.24. The chiroptical properties of solutions of GlcNAc-Asn and GlcNAc-NHAc in dioxane-water





(in c.g.s. units) of transitions I and II for all compounds studied are listed in Table 5.1. It will be seen that the rotational strengths associated with transitions in the C_1 acetamido group are greatly in excess of those associated with the C_2 acetamido group.

The rotational strengths of the Cotton effects at 195nm exhibited by GlcNAc-NHAc ($R = 40 \times 10^{-40}$ c.g.s. units) and in GlcNAc-Asn ($R = 34 \times 10^{-40}$ c.g.s. units) are of the same order of magnitude as those normally associated with inherently disymmetric chromophores (Mislow, 1967).

DISCUSSION.

It is proposed to consider some general aspects of ORD and CD, and to postpone a consideration of the results obtained until later in the Chapter. The typical shapes of ORD and CD curves generated by a hypothetical molecule containing only one absorption band which is optically active are shown in Fig. 5.3. The characteristic curves are called Cotton effects, after the name of their discoverer (Cotton, 1896). Positive Cotton effects give rise to positive CD bands and ORD curves with peaks at longer wavelengths and troughs at shorter wavelengths (as shown in Fig. 5.3), whereas negative Cotton effects give rise to negative CD bands and ORD curves which have troughs at longer wavelengths.

Circular dichroism curves are generally Gaussian, and correspond to the equation

$$[\theta] = [\theta_o]\, e^{\frac{-(\lambda - \lambda_o)^2}{\Delta_o^2}}$$

where $[\theta_o]$ is the maximum or minimum molecule ellipticity at λ_0 (Fig. 5.3), and Δ_o is the value of half the width of the band at a value for the molar ellipticity equal to $\frac{[\theta_o]}{e}$.

Circular dichroism is directly related to ORD by a well-established mathematical transformation (Kronig, 1926; Kramers, 1927). There is an essential difference between CD and ORD curves. As all molecules absorb at wavelengths below 180nm, disymmetric molecules exhibit non-zero rotations at wavelengths far removed from that at which any chromophore contained in that molecule absorb, giving rise to plain dispersion curves. Consequently, ORD curves near a Cotton effect are superimposed on a "background" rotation, and, for studying the environment of a group associated with one particular electronic transition without interference from neighbouring Cotton effects, CD is the preferred technique. However, when two Cotton effects lie very close to each other, some overlap of both the CD bands and ORD bands are to be expected. The bottom part of Fig. 5.3 shows the CD and ORD curves that would be expected from a hypothetical molecule giving rise to two Cotton effects of opposite and equal sign, which are separated by only 5nm.

Molecular theories of optical rotation and symmetry rules.

Moscowitz (1962) has classified optically active chromophores into two types:-

1. The inherently disymmetric chromophore
2. The inherently symmetric chromophore which is asymmetrically perturbed.

Hexahelicene (Moscowitz, 1961) and twisted biphenyls (Mislow, 1967) are molecules of the first type. These molecules generally possess very marked optical activity. Many organic molecules are of the second type in which symmetrical chromophores absorbing above 185nm are asymmetrically perturbed by chiral centres elsewhere in the molecule.

Optical rotation and circular dichroism may be considered as resulting from the coupling of the magnetic dipole of an electronic transition with the electric dipole moments of all the other electronic transitions in the molecule. In reality, ORD and CD detect only the relative orientations of the transition moments of constituent groups within a molecule and not the relative positions of atoms and bonds. In order to correlate the signs and sizes of the observed Cotton effects with the stereochemistry of the molecule under investigation, semi-emperical rules have been derived. These rules were originally derived from an application of quantum-mechanics to the theories of optical rotation, and from measurements that were made on compounds containing chromophores that give rise to well-defined optically-active transitions.

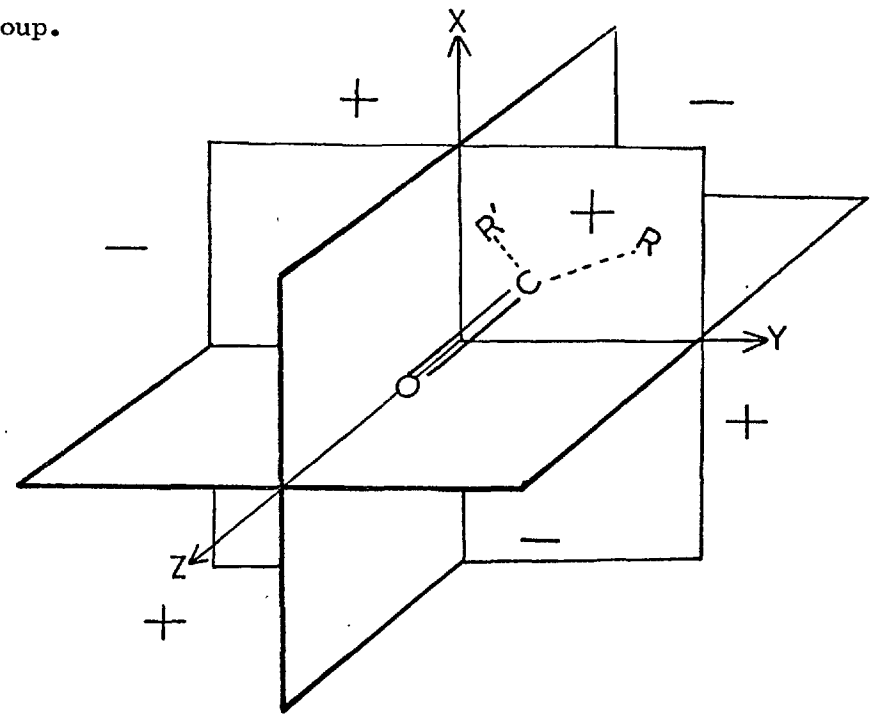
The "one electron" theory of Condon, Altar & Eyring (1937) restricts the model to one which contains only two electronically excited states. In this model, both the magnetic and the electric transitions occur in the same chromophore. The rest of the molecule acts as a perturbing field which breaks down the symmetry of the chromophore and "mixes" the two transitions. This theory was applied

to the carbonyl group of certain disymmetric molecules. The first Cotton effect occurring at approximately 290nm has been associated with an $n \rightarrow \pi^*$ transition, in which an electron is promoted from a lone pair orbital on the oxygen atom to an antibonding π^* orbital, a molecular orbital which belongs to both the carbon and oxygen atoms. By considering the symmetry properties of the orbitals involved, Moffitt et al., (1961) derived the octant rule for ketones. The two natural planes of symmetry of the carbonyl group (ZY and ZX) are perpendicular and these planes and the nodal surface of the π^* orbital (YX) divides up the space around the carbonyl group into octants (Fig. 5.25). The contribution of a perturbing group depends upon the sign of the octant in which it lies. The sign of each octant was established by consideration of the symmetry of the orbitals involved in the transition.

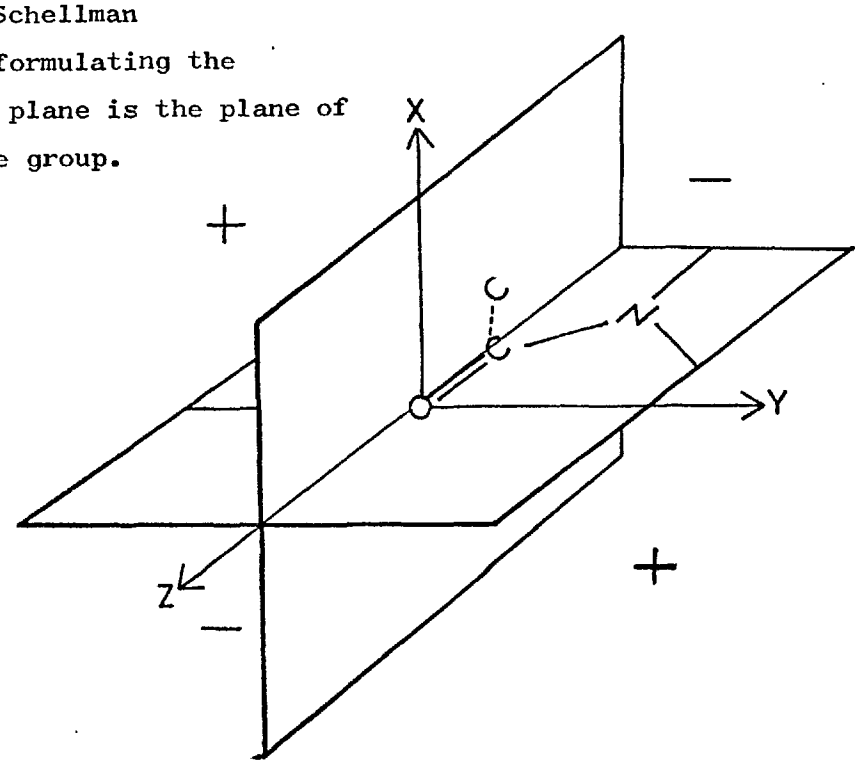
Studies of the chiroptical properties of compounds in which the carbonyl group is held in a rigid framework have verified in many cases the applicability of the octant rule. The rule was developed for derivatives of cyclohexanone, and for bicyclic and polycyclic rigid structures of the trans-decalone type (Djerassi, 1960) and has since been established for many other types of compounds. Extensions of the octant rule have been proposed for unsaturated ketones (Moscowitz, Mislow, Glass & Djerassi, 1962) and a sector rule has been proposed for lactones (Jennings, Klyne & Scopes, 1965). The application of the sector rule to carboxylic acid derivatives has shown that the carboxyl group adopts a preferred conformation, despite the

Fig. 5.25. The division of space around the carbonyl chromophore into octants, and the division of space around the amide chromophore into quadrants.

The geometrical arrangement is that proposed by Moffitt et al (1961) in formulating the octant rule. The sign of the contribution of each atom in the molecule to the rotational strength may be predicted from the sign of the octant (marked in the Figure) in which that atom lies. The ZY plane is the plane of symmetry of the carbonyl group.



The geometrical arrangement is that proposed by Schellman and Oriel (1962) in formulating the quadrant rule. The ZY plane is the plane of symmetry of the amide group.



possibility of free rotation around the C-CO_2H bond (Renwick & Scopes, 1969). The effect on their contribution of distance of the perturbing atoms from the chromophore, predicted according to the octant rule, has not been fully evaluated, but it is clear that the closer the atom is to the chromophoric group, the greater is its contribution (Kuhn & Freudenberg, 1933).

In general, substituent alkyl groups and halogens (except fluorine) follow the ordinary octant rule for saturated carbonyl compounds. However, fluorine substituents appear to obey a reverse octant rule, whereby their contribution to the Cotton effect is opposite to that predicted (Djerassi & Klyne, 1957; Djerassi, Osiecki, Riniker & Riniker, 1958). A reverse octant rule is also found to hold for some ketones substituted with oxirane rings (Djerassi et al., 1965), and hydroxy and acetoxy groups of camphor derivatives appear also to produce reverse octant effects (Bartlett et al., 1970). Reverse octant contributions by nitrogen-containing substituents are discussed by Yamada & Kunieda (1967) and Höhn & Weigang (1968). It would appear, then, that the electronegative characters of substituents are important factors to be considered when attempting to predict the sign and magnitude of a Cotton effect from a geometrical rule.

It is reasonable, therefore, to be cautious when applying a geometrical rule to carbohydrate derivatives and other derivatives which contain oxygen atoms. Beychok & Kabat (1965) have, however, applied the octant rule of

Moffitt et al., (1961) to predict the conformation of the 2-acetamido group of methyl and N-acetyl-α- and β -D-glucosaminides and galactosaminides. From various considerations, including ORD curves, they deduced that in these compounds the plane of the acetamido group is coplanar with C_2, C_5 and C_6, and that the carbonyl oxygen is cis to the C_2 ring proton. The octant rule has also been applied to five-membered sugar lactones (Okuda, Harigaya & Kiyomoto, 1964).

The electronic transitions of amide groups.

Early investigations of the spectra in the gas phase of amide groups showed the existence of an intense band at about 190nm, and a series of less intense bands below 165nm (Hunt & Simpson, 1953). The 190nm band was assigned to a $\pi \longrightarrow \pi^*$ transition (Peterson & Simpson, 1957), and the direction of its transition moment measured in myristamide crystals. A transition at higher wavelengths (220nm) was also detected in myristamide. This transition is not normally resolved in other amides because it is very weak, and was assigned to an $n \rightarrow \pi^*$ transition by analogy with the spectra of carboxyl groups. Recent interpretations of spectra of simple primary amides in the vapour-phase have led to the assignment of four bands in the 220nm - 130nm region (Basch, Robin & Kuebler, 1967). A weak band at 220nm was assigned to an $n \longrightarrow \pi^*$ transition and a second relatively intense absorption band at 195-170nm to a $\pi \longrightarrow \pi^*$ transition, the exact wavelength of which depended on the nature of the substituent group adjacent

to the amide.

Investigations of the ORD of helical polypeptides and proteins in wavelength regions between 250 and 185nm have revealed three optically active bands (Blout, Carver & Schechter, 1967). The first is negative, and is centred between 215 and 225nm. Because of its position, it has been assigned to the weak $n \rightarrow \pi^*$ transition (Schellman & Oriel, 1962). Moffitt (1956) predicted that the $\pi \rightarrow \pi^*$ transition of a peptide group would be split into two transitions separated by about 10nm when that peptide group is involved in helix formation, and the two lower transitions have been assigned to this split transition.

The Cotton effect associated with an $n \rightarrow \pi^*$ transition in an amide group of a substance simpler than a protein or polypeptide, was first observed by Litman & Schellman (1965) in the ORD of L-3-aminopyrrolid-2-one. Decreasing the polarity of the solvent caused the centre of the Cotton effect to shift to longer wavelengths. This bathochromic behaviour would be expected for an $n \rightarrow \pi^*$ transition (McConnell, 1952). Hypsochromic shifts were observed for $\pi \rightarrow \pi^*$ transitions occurring at shorter wavelengths in a number of different types of amides (Nielsen & Schellman, 1967). The chiroptical properties, and the changes of those properties with changes of solvent, of a number of other small, optically active compounds containing amide groups have recently revealed the existence of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions at 190-195nm, and 215-230nm (see for example Goodman, Toniolo & Falcetta, 1969).

Most of these examples contain the relatively rare cis-amides, but trans-amides have the same spectral properties (Nielsen & Schellman, 1967). The wavelengths at which $n \longrightarrow \pi^*$ transitions occur depends on the overall structure and environment of the molecule in which the excited group occurs. For example, the cyclic peptides Gly_5-L-Leu and Gly_4-L-Leu in water exhibit first CD extrema at 214nm and 222nm respectively (Ziegler & Dush, 1971), and the difference in wavelengths of the extrema have been ascribed to the effects of intramolecular hydrogen bonding.

Schellman (Schellman & Oriel, 1962; Schellman, 1968) has discussed in considerable detail the form of symmetry rule which is required for predicting the sign and order of magnitude of the Cotton effect which results from the $n \longrightarrow \pi^*$ of a peptide bond. From a consideration of the planes of symmetry a quadrant rule was proposed. It was shown that the magnitude and sign of the Cotton effect was proportional to $C\left(\frac{q_i X_i Y_i}{r_i^5}\right)$, where X_i, Y_i and r_i are the radial co-ordinates of the ith atom in the geometrical arrangement depicted in Fig. 5.25, q_i is the partial charge of the ith atom, and the coefficient C depends on certain perturbation coefficients and transition moments of the isolated peptide group, and on the effective dielectric constant of the atomic environment. The coefficient C might therefore be expected to be constant for a given series of related compounds. The derivation of the formula depends on the assumption that perturbation of the peptide group arises solely from the electrostatic fields of polar and

charged groups. The use of this rule appears to be more satisfactory than a simple application of the octant rule, because it takes into account the nature of the charge on the perturbing atoms. It is clear from the presence of the $\frac{1}{r^5}$ term that the contribution of a given atom is very dependant upon its distance from the chromophoric group.

The quadrant rule has been applied to studies of the Cotton effects produced by 3-L-aminopyrrolid-2-one (Litman & Schellman, 1965), L-pyrrolid-2-one-5-carboxylic acid, L-α-aminocaprolactam and camphorolactams (Goodman, Toniolo & Falcetta, 1969), and cyclic diketopiperazines (Balasubramanian & Wetlaufer, 1967). In these examples the contributions by the nearest atoms have alone been considered. This type of rule is the one which has now been applied to the $n \rightarrow \pi^*$ transitions exhibited by the N-acylglycosylamines.

Application of symmetry rules to the Cotton effects produced by N-acylglycosylamines.

The Cotton effect produced by all the N-acylglycosylamines in the region of 210nm was displaced bathochromically upon decreasing the polarity of the solvent (Fig. 5.23). As already discussed, this behaviour is typical of an $n \rightarrow \pi^*$ transition. The wavelength at which these first Cotton effects are observed (called transition I) are similar to those that have previously been assigned to $n \rightarrow \pi^*$ transitions in disymmetric molecules containing amide groups (see above). The second Cotton effect (II) is almost certainly due to the $\pi \rightarrow \pi^*$ transition.

These assignments are in general agreement with those made previously (Beychok & Kabat, 1965; Beychok, Ashwell & Kabat, 1971; Listowsky, Avigad & Englard, 1968) to the two Cotton effects found with N-acetylglycosaminides.

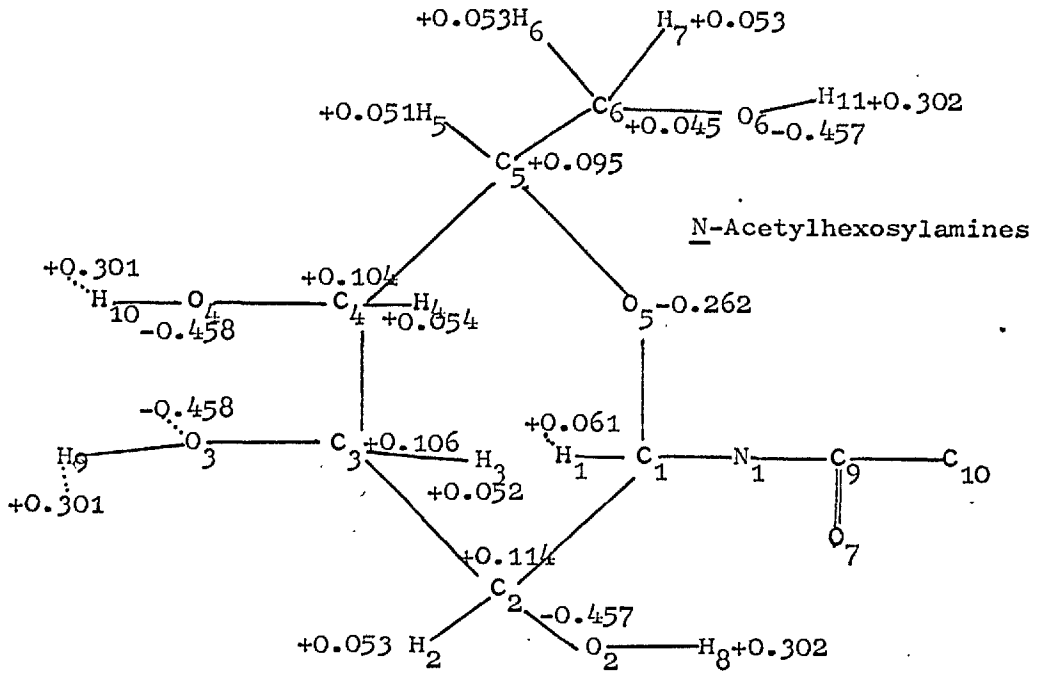
The conformations of the acetamido groups of N-acetylglycosylamines.

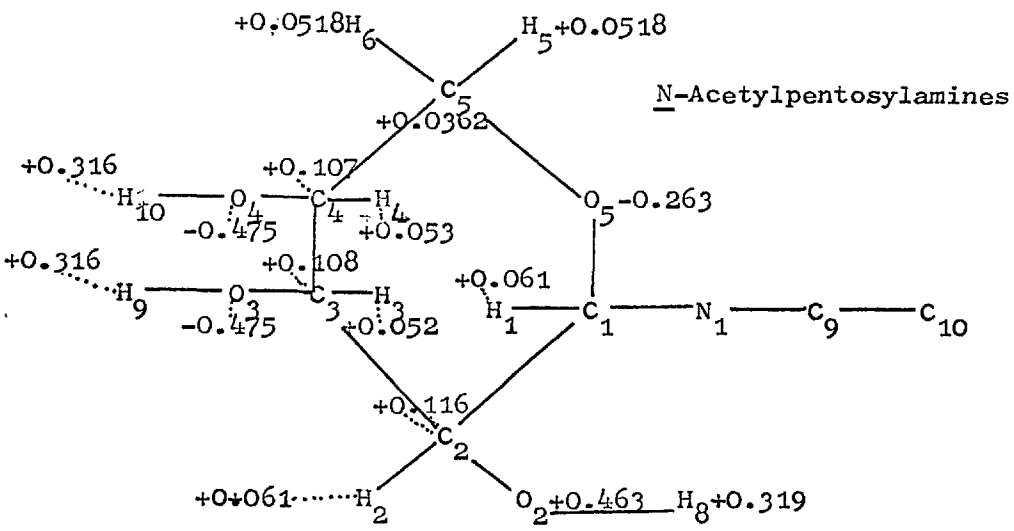
The sizes of the Cotton effects observed in the wavelength region 250nm-185nm, suggest that the acetamido groups of the N-acetylglycosylamines studied here adopt preferred orientations with respect to their neighbouring atoms. In an attempt to establish the preferred conformations, the quadrant rule (Litman & Schellman, 1965) has been applied.

The partial charges on each perturbing atom in the N-acetylhexosylamines (Salinas & Sproviero, 1971) and in the N-acetylpentosylamines (Zhdanov, Minkin, Ostroumov & Dorofeenko, 1968) were derived by quantum mechanical calculations. The values assigned to each atom are shown in Fig. 5.26.

Assessments of the signs and sizes of the Cotton effects have then been made with the following assumptions. Firstly, it has been assumed that the amide group attached to C_1 is planar and trans. Secondly, it is assumed that the compounds all adopt the Cl preferred conformation. Thirdly, the protons attached to the oxygen atoms and, in the case of the N-acetylhexosylamines, C_6 also, have been assumed to have unrestricted rotations or, alternatively, that all the staggered positions are of equal importance. The question then arising concerns the size of the angle

Fig. 5.26. Partial atomic charges used to calculate rotational strengths.





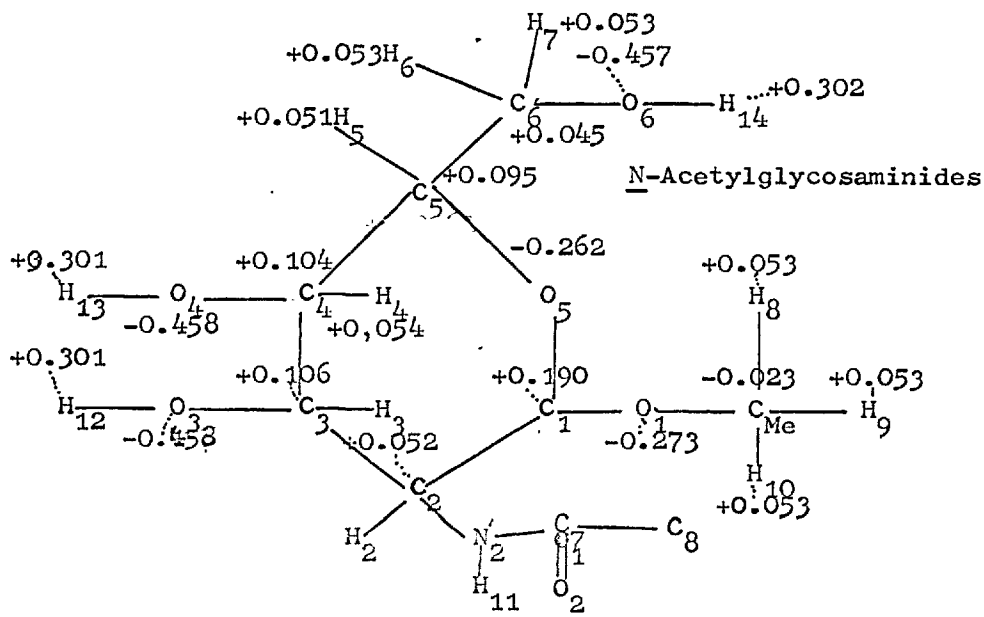


Table 5.2. A calculation by Schellman's procedure of the rotational strength (R) expected for Glc-NHAc with a conformation such that $\tau_1 = +38^\circ$.

The significance of τ_1, and the parameters X, Y, q, r and C, are defined in the text.

Sign of quadrant	Atom	X(Å)	Y(Å)	q(eV)	r(Å)	$r^5 \times 10^{-3}$	$\frac{XYq}{r^5 \times 10^{-3}}$	$\frac{R^{(a)}}{C}$
Right upper (-ve)	O_5	1.20	2.80	-0.262	3.25	0.362	-2.43	+2.43
	C_4	0.75	5.20	+0.104	5.5	5.03	+0.08	-0.08
	O_4	0.75	6.45	-0.458	6.55	12	-0.18	+0.18
	H_{10}	0.75	6.75	+0.301	6.85	15	+0.10	-0.10
	H_4	1.40	5.3	+0.052	6.10	8.45	+0.05	-0.05
	C_5	1.40	4.15	+0.095	4.30	1.47	+0.38	-0.38
	H_5	1.00	4.20	+0.051	4.25	1.39	+0.15	-0.15
	C_6	2.90	4.70	+0.045	5.30	4.18	+0.15	-0.15
	H_6	3.30	4.90	+0.053	5.90	7.15	+0.12	-0.12
	H_7	3.30	4.90	+0.053	5.90	7.15	+0.12	-0.12
	O_6	3.40	4.90	-0.457	6.00	7.78	-0.98	+0.98
	H_{11}	3.40	4.90	+0.302	6.00	7.78	+0.66	-0.64
	H_2	0.20	3.50	+0.053	4.80	2.55	+0.02	-0.02
Right lower (+ve)	H_1	0.6	2.5	+0.06	2.65	0.131	+0.70	+0.70
	C_2	0.55	3.35	+0.114	4.30	1.47	+0.14	+0.14
	O_2	1.75	3.10	-0.457	4.60	2.06	-1.20	-1.20
	H_8	2.15	2.70	+0.302	4.75	2.42	+0.72	+0.72
	C_3	0.55	4.85	+0.106	5.20	3.80	+0.07	+0.07
	O_3	1.15	5.80	-0.458	6.45	11.17	-0.27	-0.27
	H_9	1.10	6.00	+0.301	6.80	14.54	+0.14	+0.14
	H_3	1.30	4.90	+0.052	5.20	3.80	+0.09	+0.09
							Total =	+2.17

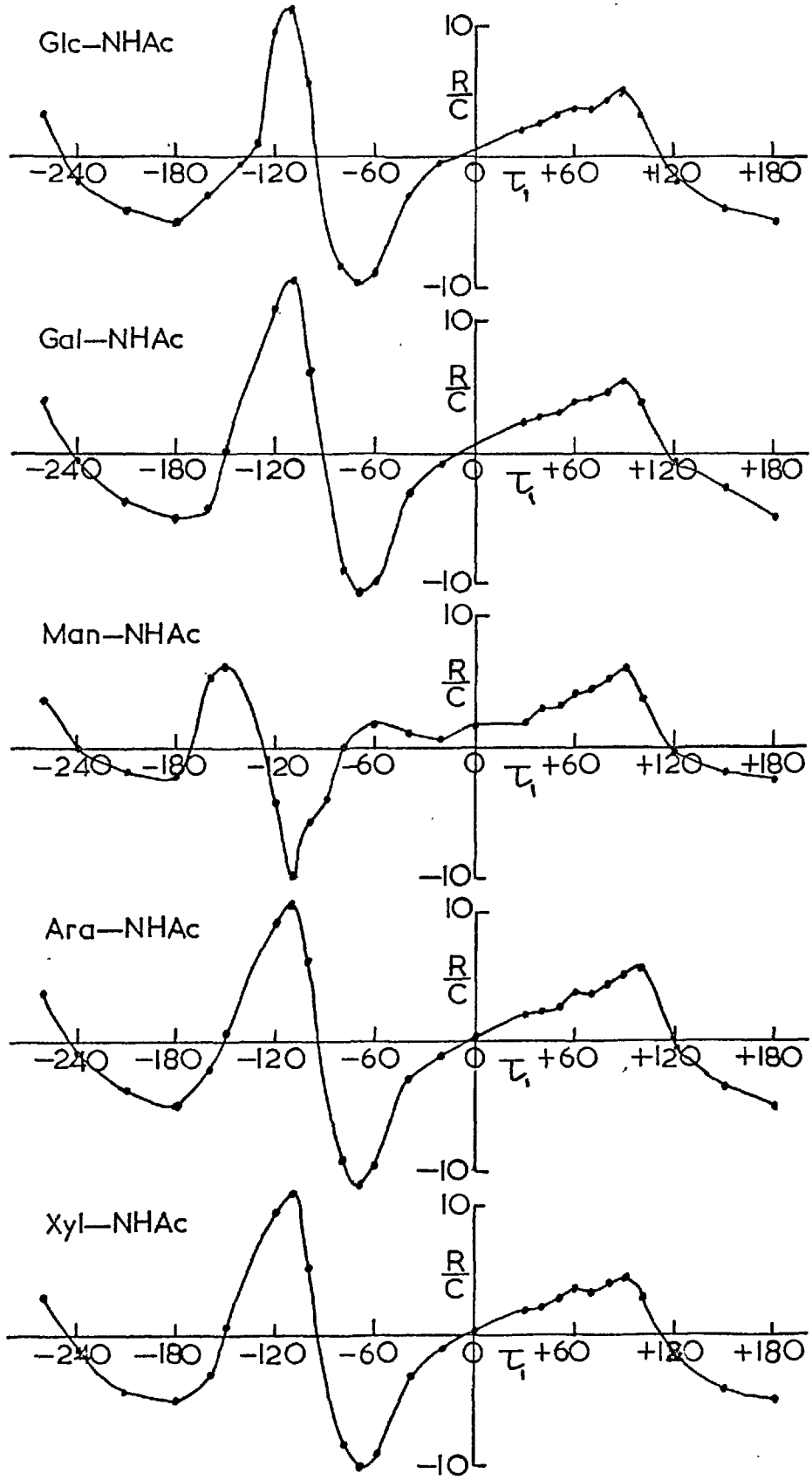
(a) The contribution of each atom to the total rotational strength is calculated in arbitrary units resulting from the application of Schellman's expression.

between the amide plane at C_1 and the sugar ring itself. This torsional angle (τ_i) is defined as $\angle C_9N_1C_1H_1$, where the observer is looking <u>down</u> the bond joining N_1 to C_1, the atoms being numbered in Fig. 5.26. The angle is regarded as zero when the projection of the N_1C_9 and C_1H_1 bonds are superimposed. The angle is positive when the projection of the C_1H_1 bond appears at a position which is clockwise to that of the projection of the N_1C_9 bond. This is the convention adopted by others (Edsall <u>et al</u>., 1966). In the following discussion the use of atomic models, such as the Dreiding type, will be found of considerable value. These models were used in the measurements of X_i, Y_i and r_i from which the calculations were made. It was assumed that the value of C in Schellman's equation was constant from one compound to another and for each conformation considered.

An example of one of these calculations for Glc-NHAc with $\tau_i = +38^\circ$, is shown in Table 5.2. As can be seen from this table, the ring oxygen makes a large positive contribution to the rotational strength in this conformation and does so for values of τ_i between about $+40^\circ$ and $+100^\circ$.

The results of calculations of the rotational strengths of Glc-NHAc, Gal-NHAc, Man-NHAc, Ara-NHAc and Xyl-NHAc for all values of τ_i are recorded in Fig. 5.27. The rotational strengths are in units of a somewhat arbitrary nature, but would be related to those listed in Table 5.1 by the constant factor, C. The rotational strengths determined experimentally for the $n \rightarrow \pi^*$ transitions of all the <u>N</u>-acetylglycosylamines used are positive, and have

Fig. 5.27. Rotational strengths calculated for different conformations, adopted by N-acetylglycosylamines, by Schellman's procedure.



Rotational strengths are calculated for different values of the torsional angle (τ_1) around the C_1N_1 bond. Rotational strengths ($\frac{R}{C}$) are in those units which arise from the application of Schellman's equation (see text)

Table 5.3. The conformations of N-acetylglycosylamines for which rotational strengths are calculated to be positive.

Values of τ_1, that give rise to positive rotational strengths as calculated by Schellman's procedure, are listed.

Glc-NHAc	$-136^\circ \rightarrow -92^\circ$	$-10^\circ \rightarrow +113^\circ$
Man-NHAc	$-169^\circ \rightarrow -130^\circ$	$-80^\circ \rightarrow +180^\circ$
βGal-NHAc	$-150^\circ \rightarrow -92^\circ$	$-12^\circ \rightarrow +115^\circ$
Ara-NHAc	$-152^\circ \rightarrow -92^\circ$	$-7^\circ \rightarrow +117^\circ$
Xyl-NHAc	$-152^\circ \rightarrow -92^\circ$	$-7^\circ \rightarrow +113^\circ$

The rotational strengths relative to those of Glc-NHAc, of N-acetylglycosylamines. The rotational strengths are calculated for conformations in which τ_1 has values from 0° to $+80^\circ$.

τ_1	Glc-NHAc	βGal-NHAc	Man-NHAc	Ara-NHAc	Xyl-NHAc
0	1.0	1.0	2.8	0.50	0.50
+10	1.0	1.2	1.8	1.0	0.92
+20	1.0	1.1	1.3	1.1	0.94
+30	1.0	1.0	1.0	0.92	0.88
+40	1.0	1.1	1.2	1.0	0.92
+50	1.0	1.0	1.2	0.96	0.96
+60	1.0	0.97	1.2	1.1	1.1
+70	1.0	1.1	1.2	1.0	0.92
+80	1.0	1.1	1.2	1.0	0.93

values that lie between +10.5 and +14.0 c.g.s. units (Table 5.1). The values of τ_1, for which the rotational strengths were calculated by the quadrant rule to be positive are listed in Table 5.3. If it is assumed that the conformation of the acetamido group with respect to the sugar ring is the same for each N-acetylglycosylamine, then the most likely τ_1 value might be expected to lie within the limits -136° to -130° or -7° to $+113^\circ$, as only between these limits are all the rotational strengths positive (see also Fig. 5.27).

The permissible limits for the angle can be narrowed still further by considering the minimum distance that the amido oxygen (O_7) may approach the ring oxygen (O_5), or the O_2 oxygen atom. The usually accepted distance needed between two non-bonded oxygen atoms is 2.7 Å (Ramachandran & Sasisekharan, 1968) and considerations of this nature rule out the range of permissible angles for τ_1 from -136° to -130° and $+80^\circ$ to $+113^\circ$.

The rotational strengths calculated from the quadrant rule are expressed relative to those of Glc-NHAc at angles from 0° to $+80^\circ$ (Table 5.3). As the rotational strengths determined from experiment are about the same for each substance in this series of compounds (Table 5.1), the preferred conformation is likely to be that in which the value of τ_1 is somewhere between $+15^\circ$ and $+80^\circ$ (see Table 5.3).

The conformations of the acetamido groups of N-acetylglycosaminides.

The quadrant rule was also applied to methyl N-acetyl-

β-D-glucosaminide and methyl N-acetyl-β-D-galactosaminide. The angle τ_2 ($= \angle C_7N_2C_2H_2$) is now defined in such a way that an observer looks down the bond joining N_2 to C_2, the numbering of atoms being given in Fig. 5.26. When the projections of the bonds N_2C_7 and C_2H_2 are superimposed, τ_2 is said to be zero. When the projection of the bond C_2H_2 is in a position clockwise to that of N_2C_7 the value of τ_2 is positive.

Similar calculations to those described above were carried out for these substances with the partial charges on the atoms used shown in Fig. 5.26. Partial charge values for atoms of the methyl group involved in the glycosidic linkage were taken from the calculations of Del Re (1958) for dimethylether. The glycosidic methyl group could not occupy all structurally possible conformations. The normal limiting distance of approach between the methyl carbon atom and the ring oxygen is 2.8 Å, that between the methyl carbon and C_2 is 3.0 Å and that between the methyl carbon and the amide nitrogen atom would be 2.9 Å (Ramachandran & Sasisekharan, 1968). The limiting positions for the methyl group are those in which the torsion angle ($\angle = C_{Me}O_1C_1H_1$), lies between -38° and $+60^\circ$. Crystallographic studies of glycosides have shown previously that the torsion angle of the methyl group in methyl β-D-maltopyranoside is about $+50^\circ$ (Chu & Jeffrey, 1967) and that in methyl β-D-xylopyranoside is $+12^\circ$ (Brown, Cox & Llewellyan, 1966). An angle of $+30^\circ$ was accepted as a reasonable value for these calculations.

The results of calculation of the rotational strengths

for methyl N-acetyl-β-D-glucosamide and -galactosaminide are shown in Fig. 5.28 for values of τ_2 between +40° and -40°. The rotational strengths are calculated to be negative for values of τ_2 between about +15° and -40°, and those of methyl N-acetyl-β-D-galactosaminide are calculated to be slightly less negative than those of methyl N-acetyl-β-D-glucosaminide. These results agree with the values of the relative rotational strengths of these two compounds measured experimentally (Table 5.1).

As the calculated rotational strengths (Fig. 5.28) are in the same arbitrary units as those shown in Fig. 5.27, the most likely values of τ_2 may be determined from a comparison of the experimentally determined rotational strengths. From Table 5.1, it may be seen that the experimentally determined rotational strengths of methyl N-acetyl-β-D-glucosaminide is about half of that of Glc-NHAc. The values for the calculated rotational strengths (assessed in the arbitrary units used in the ordinate of Figs. 5.27 and 5.28) of methyl N-acetyl-β-D-glucosaminide are therefore between about -1 and -2.5 , corresponding with τ_2 values between 0° and -20°. The preferred conformation of the 2-acetamido group is therefore likely to lie between these limits.

The conformation of GlcNAc-NHAc and GlcNAc-Asn.

Interpretation of the chiroptical properties of GlcNAc-NHAc and GlcNAc-Asn is complicated by the presence in each of them of two chromophores associated with degenerate transitions. However, this type of problem has

been examined previously by several workers in attempts to rationalise the rotational properties of proteins.

Moffitt (1956) first predicted that the $\pi \rightarrow \pi^*$ transition of a peptide bond would be split into two transitions of opposite rotational strengths, separated by about 10nm, were that peptide to be involved in an α-helix. It can be seen from his work that splitting would be expected to occur whenever two peptide groups possess relative conformations such that the planes of the two peptides are not parallel. Experimental verification of the splitting of this transition has come from ultra-violet spectral work (Gratzer, Holzwarth & Doty, 1961; Holzwarth & Doty, 1965), and from the chiroptical properties of small molecules containing two amide groups (Schellman & Nielsen, 1967; Balasubramanian & Wetlaufer, 1967; Bláha & Fric, 1970). The dependence of the magnitude of the splitting upon the values of the conformational angles ϕ and ψ (for a definition see Edsall et al., 1966) for di-peptide systems has been calculated (Bayley, Nielsen & Schellman, 1969).

The large positive Cotton effect observed at 196nm in the CD of GlcNAc-Asn and GlcNAc-NHAc may be the long-wavelength part of a split $\pi \rightarrow \pi^*$ transition of the coupled diamide system. It should be noted that the $\pi \rightarrow \pi^*$ transitions of the N-acetylglycosylamines of β-aspartyl-glucosylamine and of methyl N-acetylglycosaminides are at somewhat shorter wavelengths. The occurrence of splitting of the $\pi \rightarrow \pi^*$ transition is evidence that planes of the amide groups at C_1 and C_2 are not parallel, but in any case this would be highly unlikely.

Fig. 5.28. Rotational strengths calculated for different conformations, adopted by N-acetyl-D-glycosaminides, by Schellman's procedure.

Rotational strengths are calculated for different values of the torsional angle around the C_2N_2 bond (τ_2). Units of the rotational strengths ($\frac{R}{C}$) are the same as those in Fig. 5.27.

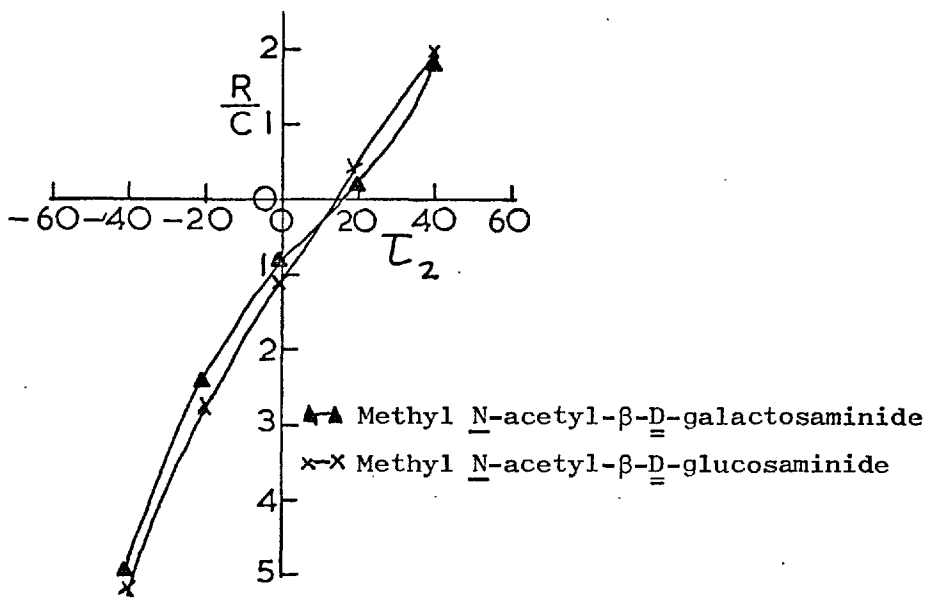
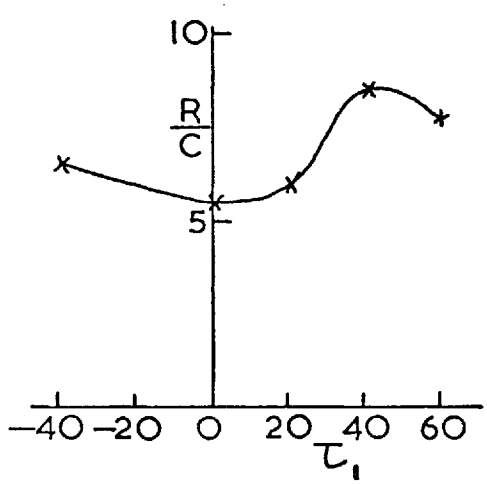


Fig. 5.29. Rotational strengths calculated for different conformations, adopted by GlcNAc-NHAc, by Schellman's procedure.

Rotational strengths are calculated for different values of the torsional angle around the C_1N_1 bond (τ_1), with a constant value for the torsional angle around the C_2N_2 bond ($\tau_2 = 0^o$). Units of the rotational strengths ($\frac{R}{C}$) are the same as those in Figs. 5.27 and 5.28.



Of more immediate interest is the need to calculate the rotational strengths of the $n \rightarrow \pi^*$ transitions of the chromophoric groups in GlcNAc-NHAc and GlcNAc-Asn. A complicating factor, however, is that the shift of the wavelength of the $\pi \rightarrow \pi^*$ transition in these compounds, relative to those of the N-acetylglycosylamines, partly obscures the $n \rightarrow \pi^*$ Cotton effects. Probable values for the $n \rightarrow \pi^*$ transitions (Table 5.1) were calculated from the results, but they are each likely to be composite values for the two $n \rightarrow \pi^*$ transitions discussed earlier. This type of complication was met in studies on di-peptides (Bayley, Nielsen & Schellman, 1969), but a solution to the problem was found by inclusion of factors which take into account the effects of dipole coupling between the electronic transitions of the two peptide groups. A similar answer was obtained when the rotational strengths were calculated on the basis of the one electron mechanism alone.

The quadrant rule has now been applied to calculate the rotational strength of the $n \rightarrow \pi^*$ transition of GlcNAc-NHAc.

Values for the partial charges lying on atoms of the amide groups of GlcNAc-NHAc were taken from the mean of the values listed by Ramachandran & Sasisekharan (1968). The partial charges of the carbon and hydrogens of the methyl groups were taken from the values calculated for the β-carbon and hydrogens of L-asparagine (Del Re, Pullman & Yonezawa, 1963). Considerations of the limiting distances for interatomic contacts limited the torsional angles of τ_1 and τ_2 to $+80^\circ$ to -40° and $+40^\circ$ to -30° respectively. The

rotational strengths in arbitrary units calculated for a model where $\tau_2 = 0^\circ$ with various values of τ_1 are shown in Fig. 5.29.

These methods do not lead to an unambiguous assignment of conformation to GlcNAc-NHAc and GlcNAc-Asn. The results would however agree with the acetamido groups at C_1 having a closely similar torsional angle (τ_1) to those found for the N-acetylglycosylamines, and for the acetamido group at C_2 having the same angle (τ_2) as the comparable group in methyl N-acetyl-β-D-glucosaminide.

FURTHER RESULTS AND DISCUSSION.

The chiroptical properties of GlcNAc-Asn, GlcNAc-NHAc, Glc-NHAc and methyl N-acetyl-β-D-glucosaminide in aqueous alkaline solutions.

The ORD and CD curves of these compounds in aqueous solution of alkali are shown in Figs. 5.30-5.33, and it may be seen that the shapes of the curves are changed but little compared with the corresponding curves obtained with substances in water. One of the interesting questions is whether the group in glycopeptides, which contain the GlcNAc-Asn linkage and which titrate at a pH in excess of 12 (see Chapter 2), is the imino hydrogen of the carbohydrate linkage. If ionisation of this group occurs, one might expect significant changes in the ORD and CD patterns. Thus, the zwitterionic forms of amino acids have $n \rightarrow \pi^*$ transitions at lower wavelengths than do the forms in which the carboxyl group is unionised (Fowden, Scopes & Thomas, 1971).

Fig. 5.30. The ORD of solutions of GlcNAc-NHAc in alkali.

Measured on Bendix II

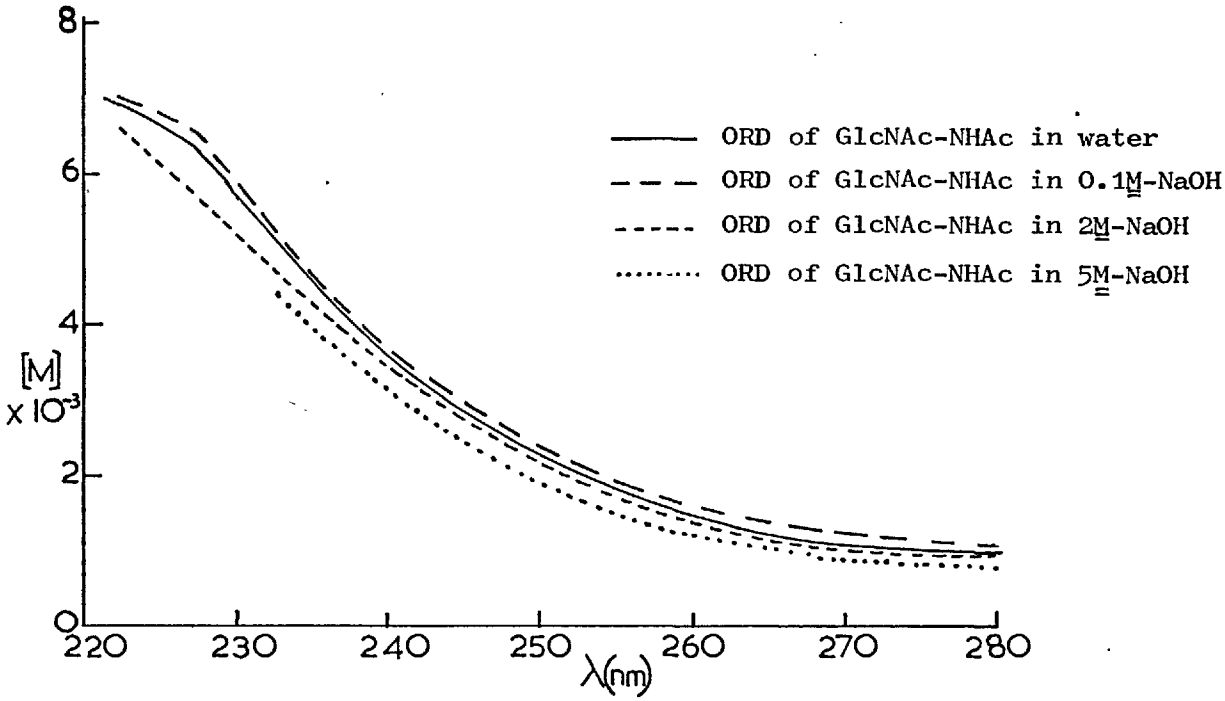


Fig. 5.31. The ORD of solutions of GlcNAc-Asn in alkali.

Measured on Bendix I

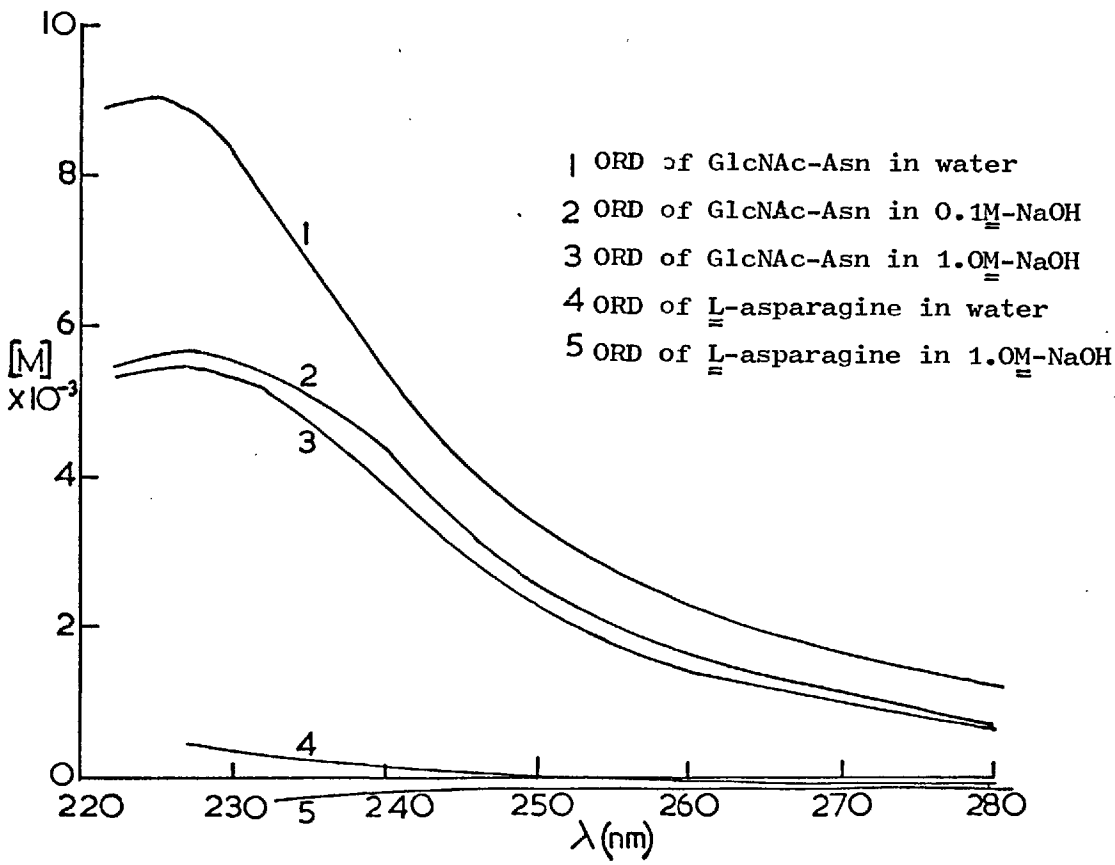
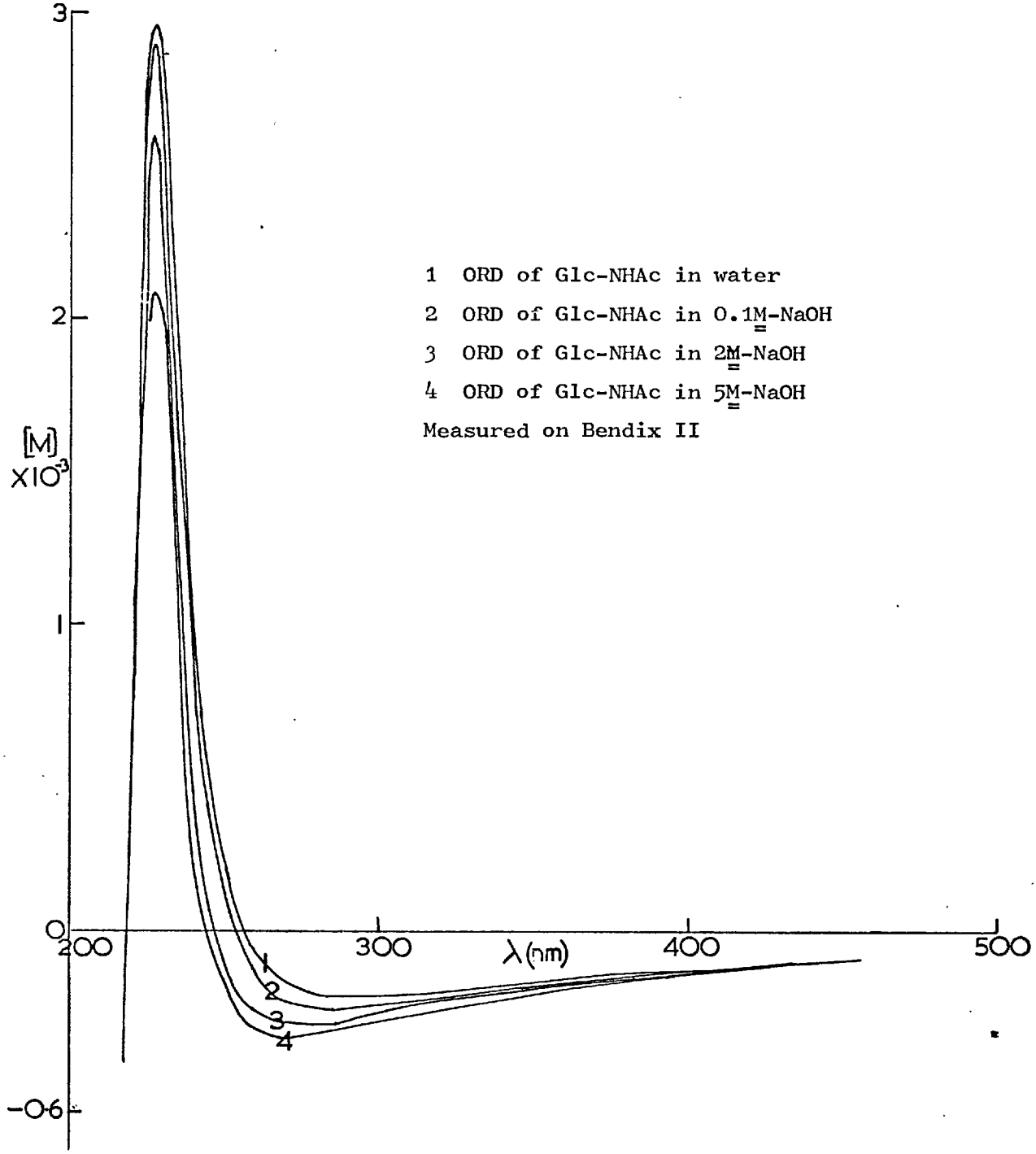
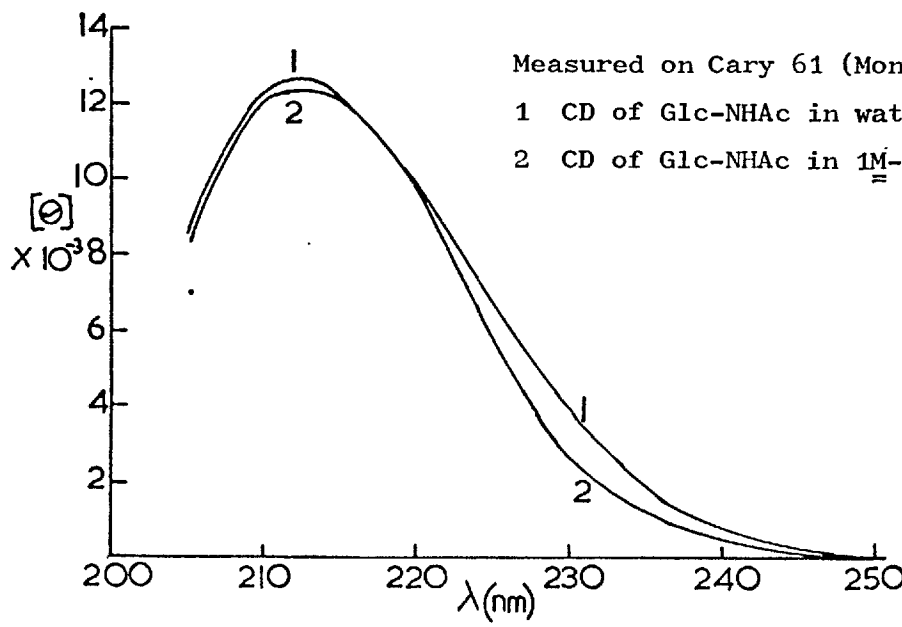


Fig. 5.32. The ORD and CD of solutions of Glc-NHAc in alkali.

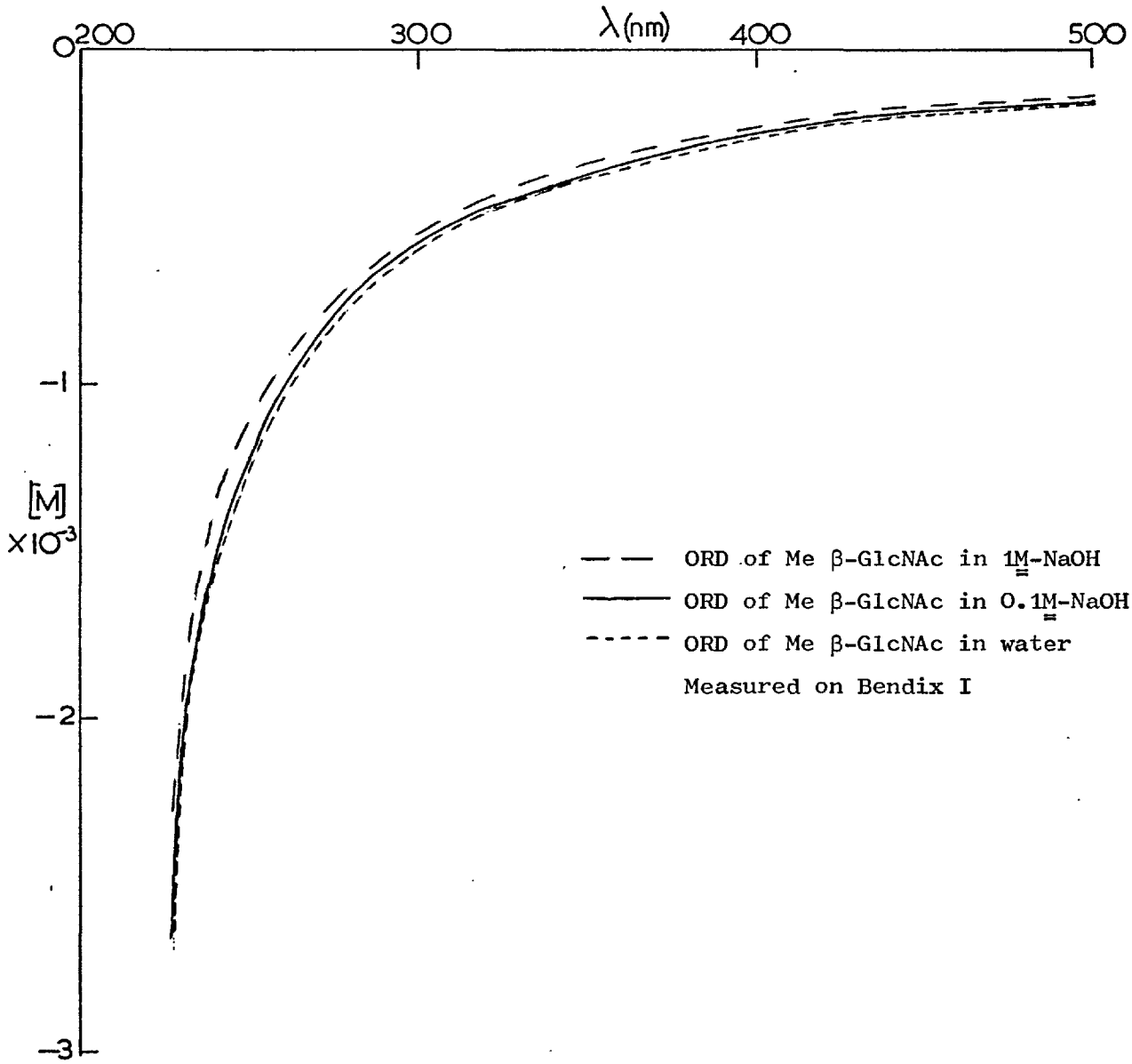


1 ORD of Glc-NHAc in water
2 ORD of Glc-NHAc in 0.1M-NaOH
3 ORD of Glc-NHAc in 2M-NaOH
4 ORD of Glc-NHAc in 5M-NaOH
Measured on Bendix II



Measured on Cary 61 (Monrovia)
1 CD of Glc-NHAc in water
2 CD of Glc-NHAc in 1M-NaOH.

Fig. 5.33 The ORD of solutions of methyl N-acetyl-β-D-glucosaminide in alkali



The 1-N-acetamido N-H proton of GlcNAc-NHAc would be expected to have a very similar pK_a to that of GlcNAc-Asn, but very little change is observed in the ORD and CD curves of GlcNAc-NHAc in 0.1M-NaOH (about pH 13.0) compared to the curves measured in water. The decrease in positive rotation observed in the ORD curves of GlcNAc-Asn in 0.1M-NaOH and 1.0M-NaOH when compared to ORD of GlcNAc-Asn in water is due, in part at least, to changes in the state of ionisation of the α-amino group, because a similar decrease is observed when L-asparagine is examined in 1M-NaOH (Fig. 5.31).

The first ORD peak of the positive Cotton effect exhibited by Glc-NHAc is visible both in water and in alkali up to concentrations of 2M (Fig. 5.32) and the first positive extremum in the CD at 212nm is also visible in 1M-NaOH. There appears to be no change in the wavelength in concentrations of alkali up to 2M, suggesting that there is no ionisation of the N-H proton of the 1-N-acetamide group up to about pH 14.3. However, the rotation becomes slightly less positive as the alkalinity of the solvent is increased. The difference in the value of $[M]$ at 235nm exhibited by Glc-NHAc in water and by Glc-NHAc in 5M-NaOH was about -1000 deg. cm^2/dmole, is similar to the change in $[M]$ observed at 235nm in the ORD curves of GlcNAc-NHAc under the same conditions.

If the 1-N-acetamido group of Glc-NHAc in alkali remains in the conformation described above for water, an increase of negative charge in the "right-lower quadrant" would be expected to lead to a less positive rotational strength. Such a change would be expected to accompany an ionisation

of either the C_2 or C_3 hydroxyl groups.

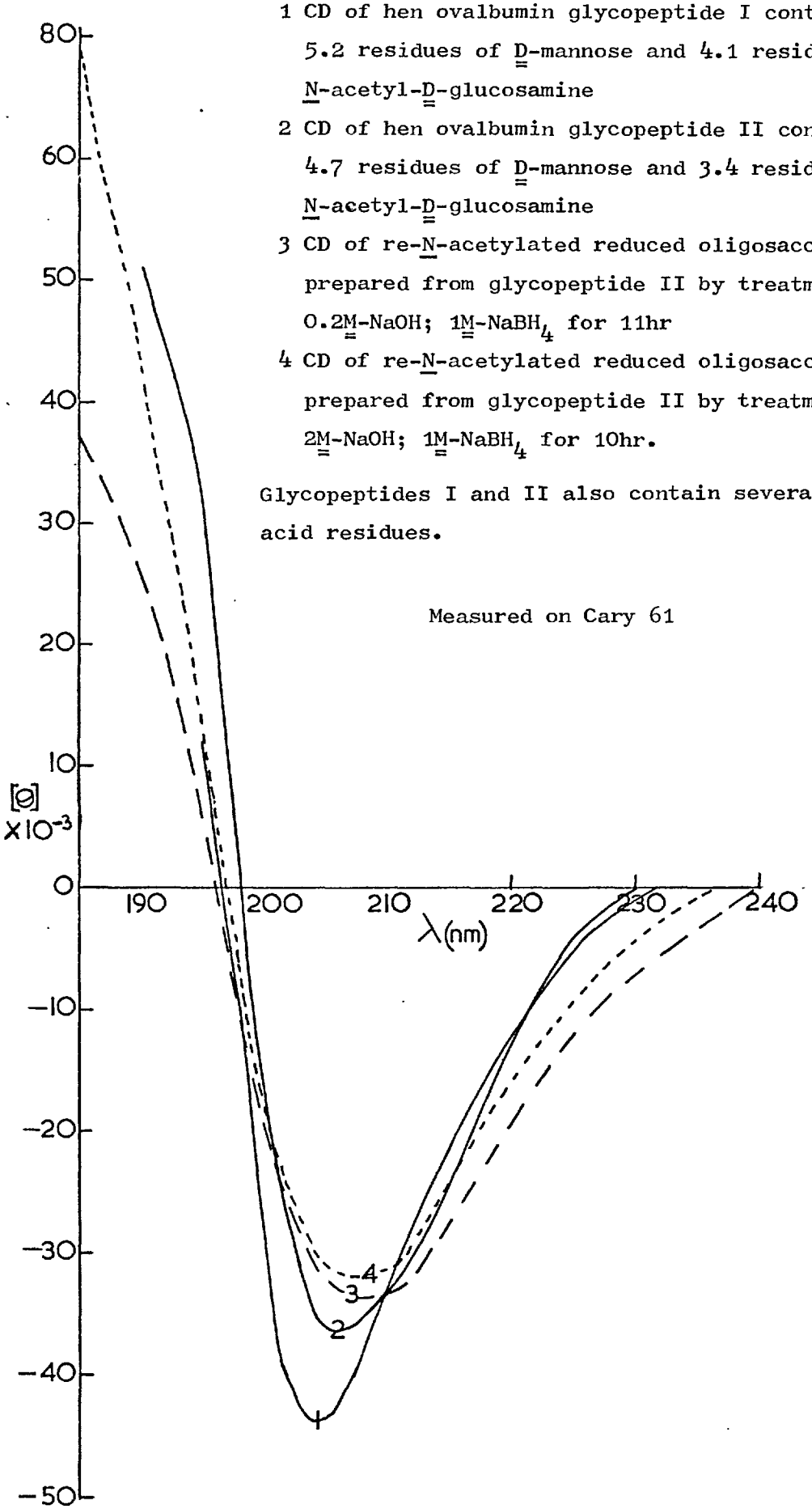
There were only very slight differences in the ORD curves of methyl N-acetyl-β-D-glucosaminide in water, 0.1M-NaOH or 1.0M-NaOH (Fig. 5.33); this suggests that the 2-acetamido N-H proton does not ionise at values of pH below about 14.0.

<u>The chiroptical properties of hen ovalbumin glycopeptide and of the products obtained by treating the glycopeptide with alkaline borohydride</u>.

The compositions of glycopeptides I and II are listed in Tables 3.5 and 3.6 (Chapter 3). Whereas the peptide portion of these two fractions of glycopeptide material contain roughly equivalent amounts of each amino acid, the carbohydrate portions differ. Glycopeptide I contains 5.2 residues of D-mannose and 4.1 residues of N-acetyl-D-glucosamine, whereas glycopeptide II contains 4.7 residues of D-mannose and 3.4 residues of N-acetyl-D-glucosamine. These differences are reflected in the CD spectra (Fig. 5.34). Glycopeptide I possesses a more negative ellipticity at its minimum ($[\theta]$ - 44,000 deg. cm^2/dmole) than glycopeptide II ($[\theta]$ - 36,500 deg. cm^2/dmole).

The chiroptical properties of the glycopeptides at wavelengths between 250nm and 185nm are mainly the result of contributions from optically active transitions in the 2-acetamido groups of the N-acetyl-D-glucosamine residues, the 1-acylamido group at the linkage region, and the peptide bonds. The additional negative rotational strength of glycopeptide I compared to that of glycopeptide II probably reflects the negative contributions of an additional

Fig. 5.34. The CD of aqueous solutions of glycopeptides and re-N-acetylated reduced oligosaccharides prepared from hen ovalbumin.



1 CD of hen ovalbumin glycopeptide I containing 5.2 residues of D-mannose and 4.1 residues of N-acetyl-D-glucosamine

2 CD of hen ovalbumin glycopeptide II containing 4.7 residues of D-mannose and 3.4 residues of N-acetyl-D-glucosamine

3 CD of re-N-acetylated reduced oligosaccharide prepared from glycopeptide II by treatment with 0.2M-NaOH; 1M-$NaBH_4$ for 11hr

4 CD of re-N-acetylated reduced oligosaccharide prepared from glycopeptide II by treatment with 2M-NaOH; 1M-$NaBH_4$ for 10hr.

Glycopeptides I and II also contain several amino acid residues.

Measured on Cary 61

0.7 mole of N-acetyl-D-glucosamine in non-reducing terminal positions.

The CD curves from 240nm to 195nm of oligosaccharides prepared from glycopeptide II by alkaline borohydride treatment followed by re-N-acetylation (Chapter 3) are very similar to the CD curve of the intact glycopeptide II (Fig. 5.34). Cleavage of the 1-acylamido group in a glycopeptide that contained aspartic acid as the only amino acid would be expected to yield a product that exhibited a more negative Cotton effect at the first extremum, because GlcNAc-Asn itself shows strong positive ellipticities at these wavelengths ($[\theta]$ = + 25,500 deg. cm^2/dmole at 205nm). It is reasonable to believe, therefore, that the peptide portion which is removed during alkaline borohydride reduction and subsequent purification of the oligosaccharide material of the intact glycopeptide contributes strong, negative ellipticities to the CD curves of glycopeptides I and II.

It is also instructive to compare the ORD curve of glycopeptide I with the ORD curves, of glycopeptides containing aspartic acid as the only amino acid, that have been reported by Montgomery (1972). The ORD curve reported by him of a glycopeptide that contains the same number of N-acetyl-D-glucosamine residues as glycopeptide I, and the ORD curve of glycopeptide I itself, are shown in Fig. 5.35. Glycopeptide I exhibits more negative rotations at wavelengths from 300nm to 203nm than the glycopeptide containing L-aspartic acid as the only amino acid, and it seems likely that the contribution to the rotation by the peptide portion

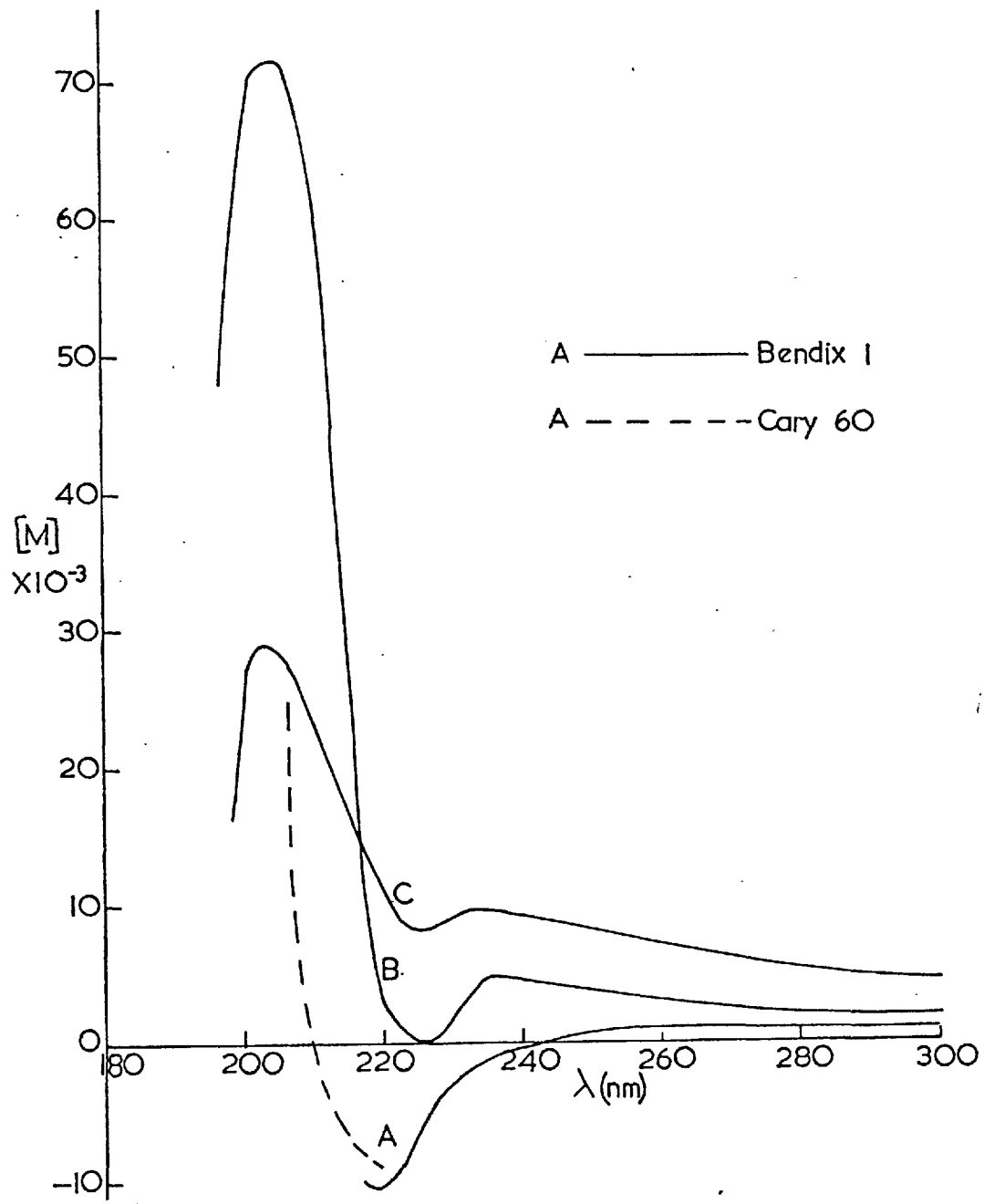
Fig. 5.35. The ORD of aqueous solutions of glycopeptides prepared from hen ovalbumin.

A ORD of glycopeptide I containing 5.2 residues of D-mannose and 4.1 residues of N-acetyl-D-glucosamine and several amino acid residues

B ORD of a glycopeptide containing 6 residues of D-mannose and 4 residues of N-acetyl-D-glucosamine, and L-aspartic acid as the only amino acid residue.

C ORD of a glycopeptide containing 5 residues of D-mannose and 2 residues of N-acetyl-D-glucosamine, and L-aspartic acid as the only amino acid.

B and C are from the data of Montgomery (1972)



is negative. The apparent rotational strength of the peptide portion of the glycopeptides studied might suggest that the peptide has a relatively fixed conformation, but it is not possible to establish the nature of this conformation at present.

EXPERIMENTAL.

Instruments.

Chiroptical properties were measured on a number of instruments. Most of the ORD measurements were made on Bendix-Ericcson Polarmatic 62 spectropolarimeters, which were based on the design of Gillham & King (1961). Samples were studied both on a first-generation instrument fitted with a 250 Watt arc Xenon lamp and Advance Electronics HR96T recorder at the Department of Pharmacy, Chelsea College of Technology, London, and also on a more recent instrument, fitted with a 450 Watt Xenon lamp, a Hewlett Packard 7035B recorder, and an improved optical system, at the Department of Chemistry, Imperial College, London. In the discussion, these instruments are referred to as Bendix I and Bendix II respectively. A small number of ORD measurements were also made on Cary 60 spectropolarimeters by Cary Instruments Ltd. at Monrovia, California and by Dr. P.M. Scopes and her staff at Westfield College London.

The majority of CD measurements were performed on the Cary model 61 CD system at Unilever Research Laboratories, Colworth House, Bedford. Some measurements were also made on a Jouan dicrograph at Westfield College and on Cary 61 and Cary 60 CD instruments at Monrovia, California. The

optical systems of all instruments were purged with dry nitrogen, and the temperatures of the cell compartments were about 25°C.

A cell of optical path-length of 0.5cm was used in the Bendix I and II instruments. Optically active substances were studied on the Bendix I in solution at concentrations sufficient to rotate light of wavelengths between 500 and 240nm by about 200 millidegrees (m^{o}) and between 240 and 220nm by about 50m^{o}. On the Bendix II, rotations of about 200m^{o} were obtained between 500 and 220nm, and about 50m^{o} between 220 and 197nm. The concentrations were therefore adjusted according to the rotational strengths of the compounds under study. For example, the N-acylglycosylamines were studied at concentrations of about 1% (w/v) from 500 to 300nm, 0.1% from 300nm to 220nm, and 0.01% from 220 to 197nm.

After the ORD of an optically active sample had been measured, the base line given by the solvent was recorded under the same conditions. Measurements were discontinued when the noise level became too high for accurate measurement. The lowest wavelengths at which reasonable measurements could be read were 220nm on the Bendix I instrument and 197nm on the Bendix II.

Compounds were studied at concentrations of about 0.1% (w/v) on the Cary 60 instrument. The lowest permissible wavelength of the Cary 60 at Westfield College was about 200nm when solutions were studied in a 1mm path-length cell, whereas the lower limit of the Cary 60 at Monrovia appeared to be 185nm with a 0.1mm path-length cell.

The ORD curves of GlcNAc-NHAc measured on four different instruments are compared in Fig. 5.14. Good agreement was obtained with the curves measured on the Bendix instruments, and the Cary 60 at Westfield College, at all wavelengths except those between 220 and 230nm, where the Bendix I repeatedly recorded larger rotations than those recorded on the other instruments. The wavelength at which the peak was recorded at Monrovia was lower than that recorded on the Bendix II, but it was noted that longer response times had been selected for the pen-recorder than those selected on the other instruments.

Reproducibility on the Bendix II of the rotations of separate samples of equivalent concentrations of the same material was $\pm$ 3m^{o} at 225nm for measured rotations of about 200m^{o}, and $\pm$ 3m^{o} at 208nm for rotations of about 30m^{o}, and $\pm$ 8m^{o} at 197nm for rotations of about 60m^{o}. Noise levels increased as the wavelengths decreased, which is almost invariably observed.

The ORD curves were constructed by plotting molecular rotation (deg. cm^{2}/dmole) as a function of wavelength.

The CD measurements made on the Cary 61 instrument at Colworth House were performed on solutions in a 0.5mm path-length cell. Concentrations (between 0.06 and 0.15% w/v) were chosen to give ellipticities of about 30m^{o} at 210nm. Penetration to a wavelength of 185nm was achieved in most cases. Baselines were constructed by scanning the solvent in the same cell under the same conditions; typical traces are shown (Fig. 5.36).

Three separate samples of N-acetyl-β-D-xylopyranosylamine at similar concentrations in water which gave

Fig. 5.36. A typical trace obtained on the chart of a Cary 61 CD instrument at Colworth House.

Measurements were made on a solution of Glc-Asn (0.077% w/v) in water in a cell of 0.5mm path-length. The centre trace is the base-line given by water in the same cell.

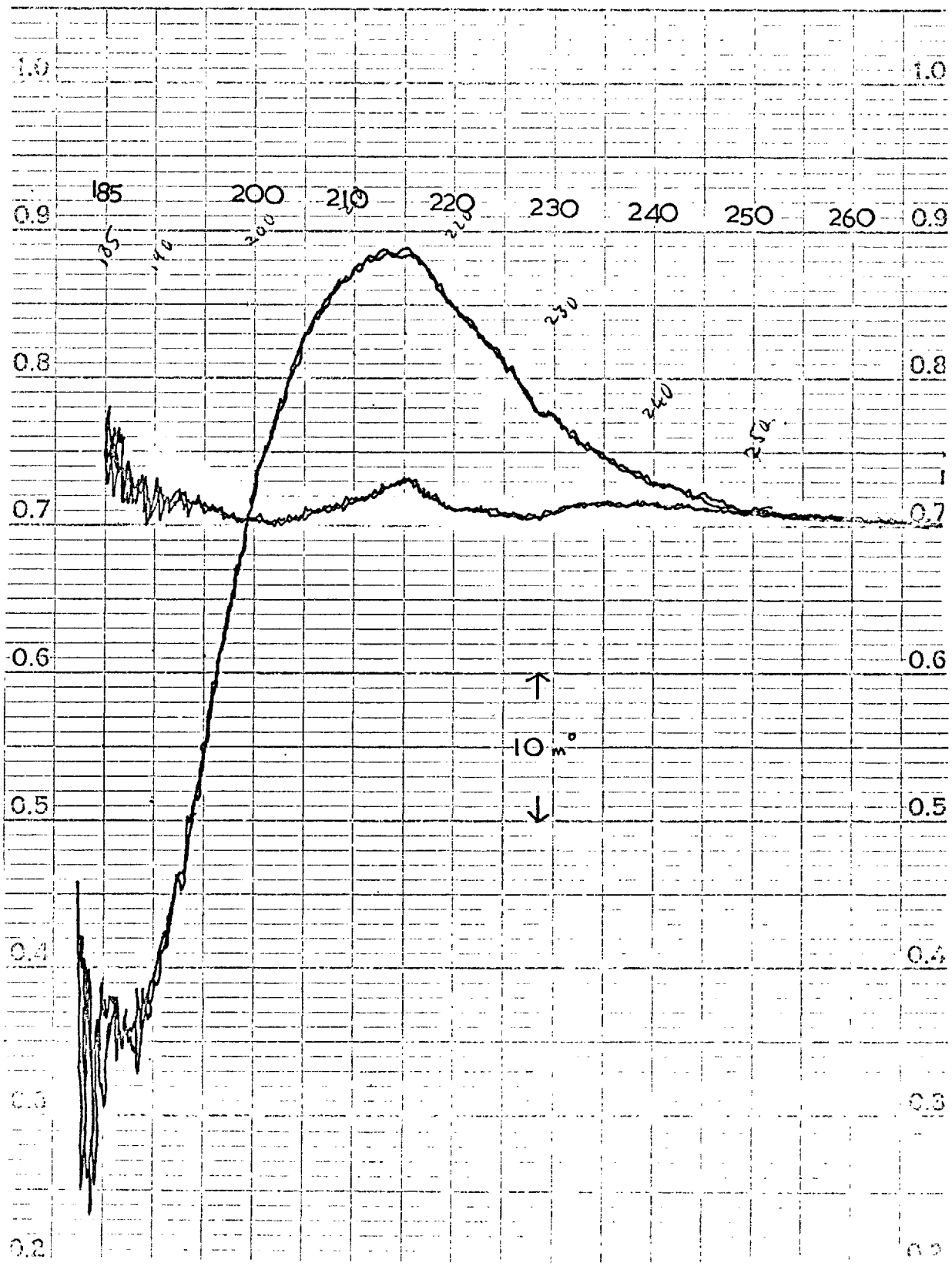


Table 5.4. Maximum molecular ellipticities, measured on the Cary 61 and Jouan CD instruments, of some N-acetylglycosylamines.

Values measured on

	Cary 61		Jouan	
	λ_o^I(nm)	$[\epsilon_o^I]$ (deg cm^2/dmole)	λ_o^I(nm)	$[\epsilon_o^I]$(deg cm^2/dmole)
βGal-NHAc	212	+13,600	212	+11,090
Ara-NHAc	212	+13,200	212	+11,550
Xyl-NHAc	212	+14,960	212	+12,210

Extreme molecular ellipticities measured on three different solutions of Xyl-NHAc on the Cary 61 CD instrument.

	λ_o^I(nm)	$[\epsilon_o^I]$ (deg cm^2/dmole)	λ_o^{II}(nm)(a)	$[\epsilon_o^{II}]$(deg cm^2/dmole)
Xyl-NHAc (1)	212	+15,006	185	-21,006
(2)	212	+14,700	185	-19,440
(3)	212	+15,180	185	-19,200

(a) Inflection points occur at this wavelength.

ellipticities of about 23m° at 212nm, and 30m° at 185nm, gave molecular ellipticities which were within ± 1.6% of each other at 212nm, and ± 5% at 185nm.

The Jouan dicrograph measured differential dichroic absorption and this was converted to units of molecular ellipticity. All the CD curves were recorded by plotting molecular ellipticity (deg. cm^2/dmole) as a function of wavelength.

The molecular ellipticities of three N-acylglycosylamines as measured on the Cary 61 and the Jouan dicrograph are compared in Table 5.4. Maximum molecular ellipticities measured on the Jouan dicrograph were about 84% of those measured on the Cary 61 at 212nm, but penetration was achieved only as far as 200nm on the Jouan instrument. All rotational strengths are based on measurements made on the Cary 61.

Materials.

Most of the compounds studied were synthesised as described in Chapter 4. The N-acetyl-D-glycosaminides had been prepared previously in this laboratory (Neuberger & Wilson, 1971). 2-Acetamido-2-deoxy-3-O-methyl-D-glucopyranose and methyl 2-acetamido-2-deoxy-3-O-methyl-β-D-glucopyranoside were kindly provided by Dr. A.K. Allen.

N-Acetyl-D-mannosamine was purchased from Koch-Light Laboratories and L-asparagine from British Drug Houses. The preparation of glycopeptide I and II from hen ovalbumin (Chapter 2) and the preparation of the reduced, N-acetylated oligosaccharides (Chapter 3) are described earlier.

SECTION 2. THE CRYSTAL STRUCTURE OF GlcNAc-Asn.$3H_2O$ AND Glc-Asn.H_2O.

As the methods of calculating rotational strengths by application of the ideas of Schellman and his colleagues have not previously been applied to carbohydrate derivatives, it was of interest to discover if the stereochemistry of GlcNAc-Asn and related compounds are the same in the solid state as they are in aqueous solution. It was of particular interest to elucidate the nature of the forces that controlled the conformation of the C_1-amide, which appears from chiroptical studies to have a relatively fixed orientation. The number of degrees of freedom of rotation about the C_1N_1 bond will determine the orientation that a carbohydrate moiety of a glycoprotein may adopt with respect to the protein chain.

The marked differences in the properties of the trihydrated and anhydrous forms of GlcNAc-Asn have been noted (see Chapter 4). Although it was not possible to grow crystals of the anhydrous form of GlcNAc-Asn large enough for X-ray diffraction studies, it was of interest to determine the nature of the intramolecular bonding to water molecules in the trihydrated form. As the chiroptical properties of the N-acylglycosylamines are easier to interpret than those of the di-N-acyl sugar derivatives, the crystalline structure of Glc-Asn was also studied, and the conformation adopted in the solid state compared to that of GlcNAc-Asn.

Crystals of GlcNAc-Asn.$3H_2O$ and Glc-Asn.H_2O were grown from solutions in aqueous ethanol at $+4^{\circ}C$. Photographs

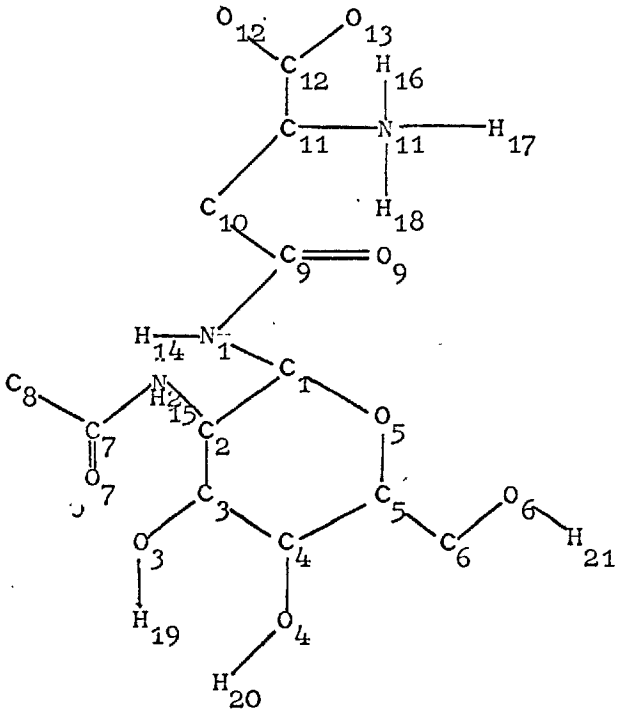
of the crystals viewed under a microscope set at 25 magnification are shown in Plate 4.2.

The crystals of both compounds belong to the monoclinic system and a $\underline{P}2_1$ space group. The dimensions of the unit cells are recorded in Chapter 4.

The structures were solved by direct methods, and all carbon nitrogen and oxygen atoms were located to a satisfactory resolution ($\underline{R} < 0.07$). Two water molecules were located in unique positions in the unit cell, but the third water molecule was variable in its position from one unit cell to another. This resulted in the appearance of two less intense peaks of electron density. The single water molecule in the crystal of Glc-Asn was located. Hydrogen atoms were located from a difference electron density map. The numbering of atoms and the bond lengths are shown in Figs. 5.37 and 5.38. The bond lengths lie within the ranges previously established for other compounds (Pauling, 1948). The parameters of the acylamide groups at C_1 and C_2 are in agreement with the parameters of other acylamide bonds (Corey & Pauling, 1953).

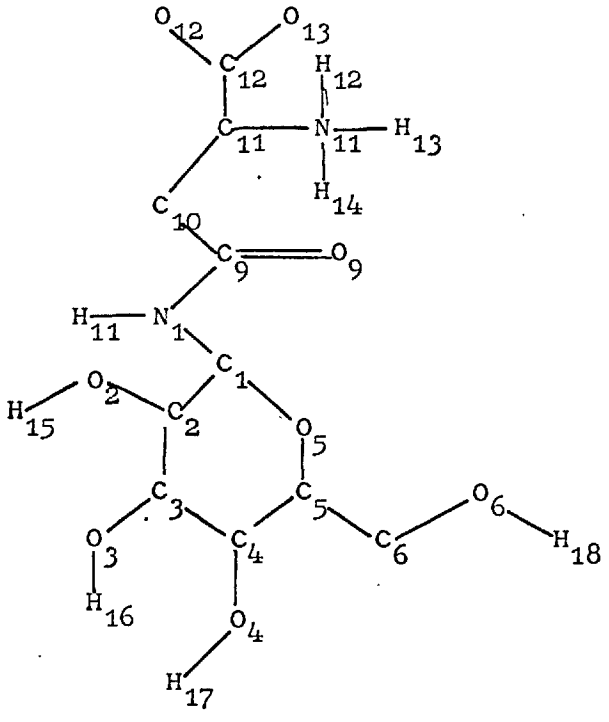
Representations of the conformations which are adopted by the two molecules in the crystalline state are given in Figs. 5.39 and 5.40. The conformation around $C_{10}C_{11}$ results in the projections of bonds $C_{11}N_{11}$ and $C_{10}C_9$ having synclinal relationships in both GlcNAc-Asn. $3H_2O$ and Glc-Asn. H_2O. A similar relationship is found between the γ-carbon atom and α-amino group of $\underline{L}$-asparagine monohydrate (Kartha & De Vries, 1961) and in the $\underline{L}$-asparaginyl moiety of glycyl-$\underline{L}$-asparagine (Pasternak, Katz & Corey, 1954) although these

Fig. 5.37. Bond lengths of GlcNAc-Asn. $3H_2O$



$O_{12}C_{12}$	1.246	C_3C_4	1.519
$O_{13}C_{12}$	1.231	C_4C_5	1.532
$C_{12}C_{11}$	1.554	C_5C_6	1.516
$C_{11}N_{11}$	1.468	C_2N_2	1.455
$C_{11}C_{10}$	1.517	N_2C_7	1.334
$C_{10}C_9$	1.536	C_7O_7	1.226
C_9O_9	1.201	C_7C_8	1.497
C_9N_1	1.360	C_3O_3	1.420
C_1N_1	1.441	C_4O_4	1.435
C_1O_5	1.424	C_6O_6	1.416
C_1C_2	1.524		
C_2C_3	1.543		

Fig. 5.38. Bond lengths of Glc-Asn.H_2O



$O_{12}C_{12}$	1.262	C_2C_3	1.543
$O_{13}C_{12}$	1.283	C_3C_4	1.552
$C_{12}C_{11}$	1.488	C_4C_5	1.553
$C_{11}N_{11}$	1.532	C_5C_6	1.534
$C_{11}C_{10}$	1.521	C_2O_2	1.437
$C_{10}C_9$	1.526	C_3O_3	1.425
C_9O_9	1.214	C_4O_4	1.422
C_9N_1	1.342	C_6O_6	1.431
C_1N_1	1.462		
C_1O_5	1.454		
C_1C_2	1.521		

Fig. 5.39. A representation of the conformation adopted by GlcNAc-Asn.$3H_2O$ in the crystalline state.

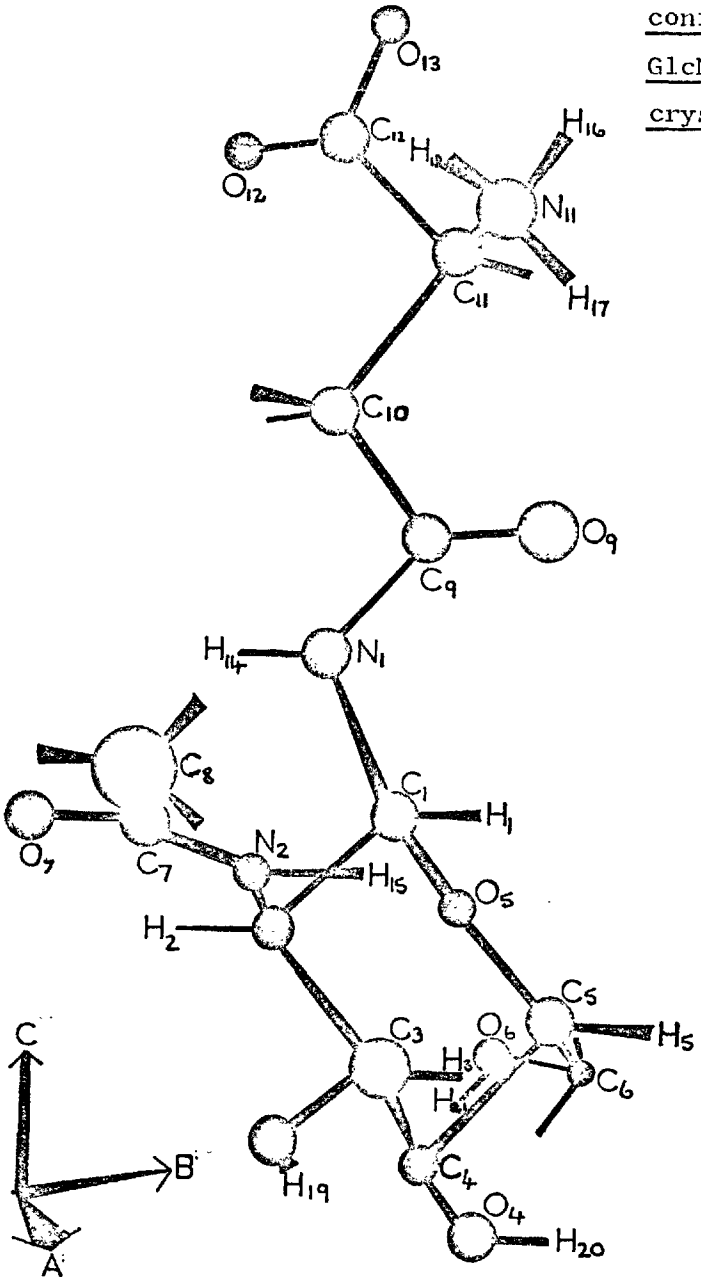
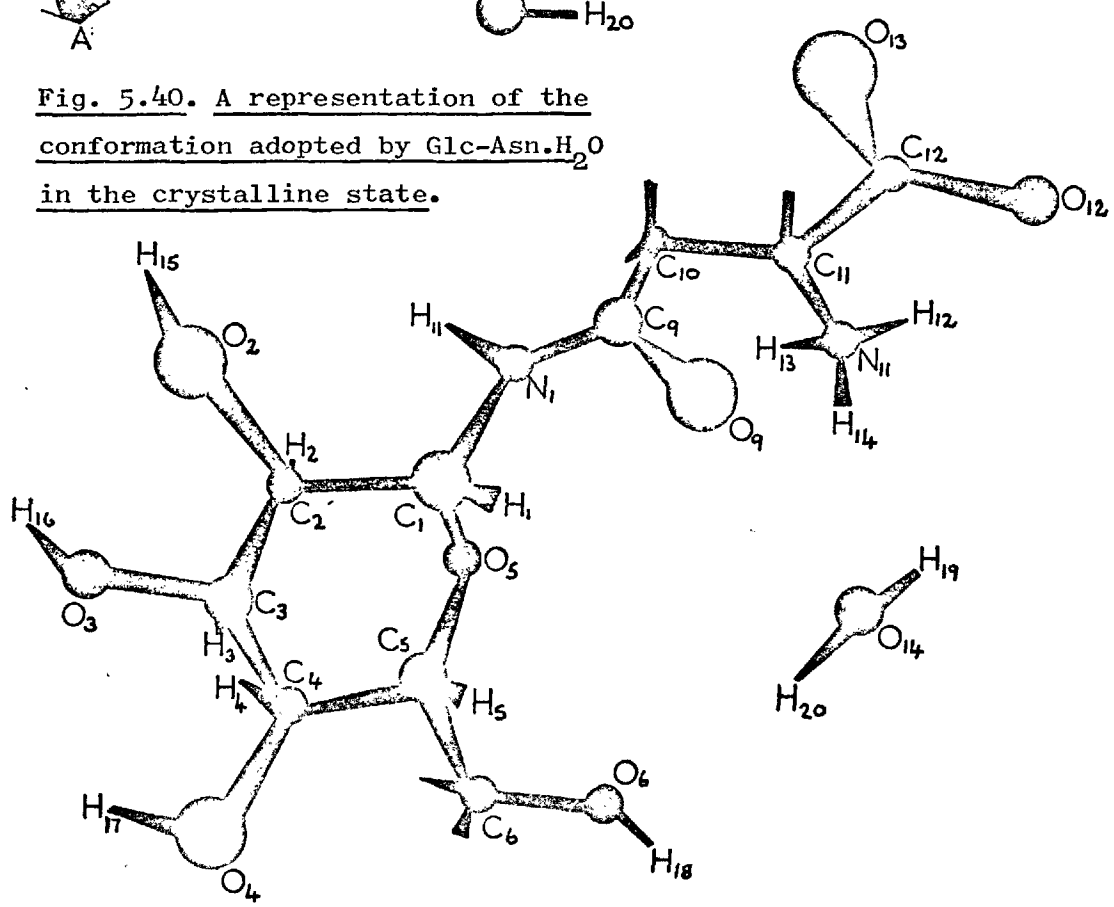


Fig. 5.40. A representation of the conformation adopted by Glc-Asn.H_2O in the crystalline state.



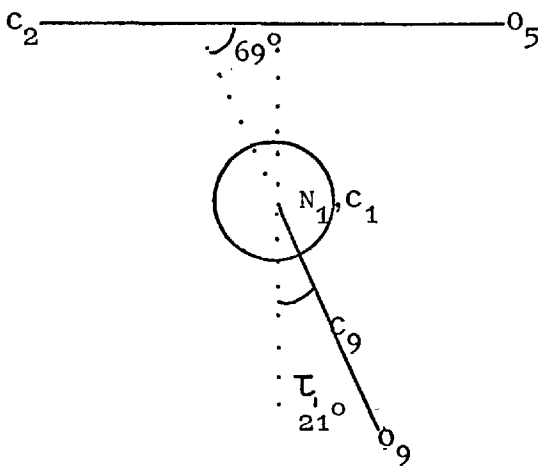
authors presented a structure containing the D-isomer of asparagine. In both GlcNAc-Asn. $3H_2O$ and Glc-Asn. H_2O, if we look down bond $C_{10}C_9$, the projection of bonds $C_{10}C_{11}$ and C_9O_9 are superimposed. This type of structural feature occurs in a number of amino acids including L-asparagine (Ramachandran & Sasisekharan, 1968). The intramolecular non-bonded distances between the α-amino atom (N_{11}) and the amide oxygen (O_9) are fairly short (2.95Å in GlcNAc-Asn; 2.88Å in Glc-Asn). A similar distance is found in the structure of L-asparagine monohydrate (Kartha & De Vries, 1961). There is no hydrogen bonding between these two atoms, because the hydrogen atoms of the α-amino group are directed away from the carbonyl oxygen. Electrostatic interaction between the two groups may occur.

It is immediately obvious from the structures that the sugar rings are in the C1 conformation, that the configurations at the anomeric carbon atoms are β, and that the amide groups are trans.

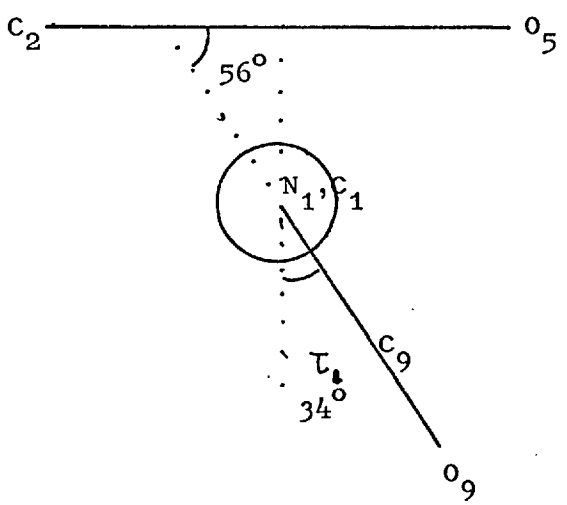
The angles between the planes defined by the ring atoms $C_2C_3C_5O_5$ and the atoms of the C_1 amide group $C_{10}C_9O_9N_1H_{14-11}C_1$ were calculated to be 69° in the structure of GlcNAc-Asn. $3H_2O$ and 56° in that of Glc-Asn. H_2O, with the carbonyl oxygens closer to the ring oxygen than in an assumed conformation in which the oxygen atom of the amide group is syn-periplanar with the C_1 ring proton (H_1). The O_9-O_5 non-bonded intramolecular distances are 3.5Å in GlcNac-Asn. $3H_2O$ and 3.22Å in Glc-Asn. H_2O. The conformations of these groups are shown schematically in Fig. 5.41. Approximate angles of 21° and 34° for these two compounds

Fig. 5.41. The conformations adopted by the acylamido groups at C_1 in the crystalline forms of GlcNAc-Asn.$3H_2O$ and Glc-Asn.H_2O.

GlcNAc-Asn.$3H_2O$



Glc-Asn.H_2O



respectively may be calculated for the angle τ_1 defined in Section 1 of this Chapter. Chiroptical methods yielded a limited range of values including the ones now found by X-ray diffraction methods.

The angle between the plane containing the ring atoms $C_2C_3C_5$ and O_5, and the atoms of the C_2 acetamide group of GlcNAc-Asn. $3H_2O$ ($C_2N_2H_{15}C_7O_7O_8$) is 65.2° in the solid state. In this conformation, the carbonyl oxygen is very nearly syn-periplanar with the ring proton at C_2 (H_2), and the plane of the amide group also includes C_5 C_6 and O_6. The orientation of the acetamide group is similar to that found previously in the crystal structure of N-acetyl-α-D-glucosamine (Johnson, 1966).

The atoms of the amide groups of GlcNAc-Asn. $3H_2O$ are planar to within 0.05Å. The C_1-amide group of Glc-Asn H_2O is less planar; N_1 is 0.09Å out of the mean plane of the amide group.

There are complete systems of intermolecular hydrogen bonding throughout both crystals. Most atoms able to take part in hydrogen bonding do so. The hydrogen bonds involving N_1 are fairly long (GlcNAc-Asn: $N_1 - H_{14}\cdots O_9 = 3.13$Å, and in Glc-Asn: $N_1 - H_{11}\cdots O_2 = 3.27$Å) (Pimental & McClellan, 1960), and may therefore be fairly weak and not important factors in determining the conformation adopted by the C_1 acylamido group.

SECTION 3. THE STEREOCHEMISTRY OF 4-N-(2-ACETAMIDO-2-DEOXY- -D-GLUCOPYRANOSYL)-L-ASPARAGINE IN ITS COMPLEX WITH LYSOZYME.

As GlcNAc-Asn is a derivative of N-acetyl-D-glucosamine, an inhibitor of hen egg lysozyme, it seemed possible that a complex might be formed between GlcNAc-Asn and the enzyme. The stereochemistry of this complex could then be investigated by an extension of the X-ray diffraction methods applied to complexes of lysozyme and of various inhibitors by Professor D. Phillips and his colleagues.

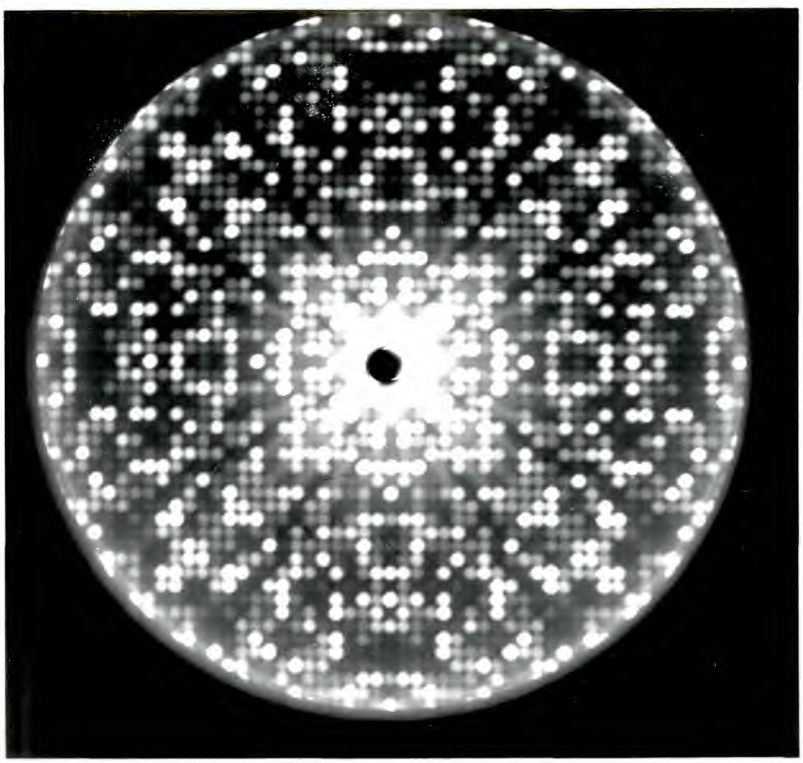
Experimental details.

A tetragonal crystal (Alderton & Fevold, 1946), measuring about 1mm in diameter, of hen egg lysozyme was placed in a quartz capillary. The crystal was surrounded by its mother liquor (pH 4.7) containing GlcNAc-Asn at a concentration of 0.2M. The ends of the capillary were sealed, and the crystal was left to soak in this solution for 22 hr at about 22°C. The solution was removed from the crystal, and the tube was mounted on a goniometer head.

The crystal was aligned on a Supper precession camera in such a way that X-rays (CuKα 1.522Å) passed straight down the unique c axis (Holmes & Blow, 1966). A precession photograph was taken with a precession angle of 4°. The symmetry of the diffraction pattern obtained (see Plate 5.1) showed that the crystal was correctly aligned. The goniometer head carrying the crystal was mounted on a Hilger & Watts linear diffractometer, and intensities were collected to a resolution of 2.5Å.

Plate 5.1. The diffraction pattern exhibited by the lysozyme - GlcNAc-Asn complex.

A precession photograph was taken on a Supper precession camera with X-rays (CuKα = 1.522Å) at an angle of 4° to the c axis of the lysozyme crystal.



The change in electron density due to the incorporation of GlcNAc-Asn was calculated by subtracting the known structure amplitudes of native lysozyme, previously determined by Professor Phillips and his colleagues, from the observed structure amplitudes of the complex of GlcNAc-Asn with lysozyme. A Fourier synthesis was performed on the observed changes by applying the phase information observed for native lysozyme.

The data were obtained in the form of electron densities recorded in planes perpendicular to the c-axis (c = 37.9Å) at intervals along this axis of 0.63Å. These electron densities were recorded on each plane at distances apart of 0.66Å. Contours were drawn at intervals of an estimated $0.1eA^{-3}$ above a level of about $0.3eA^{-3}$. The sections were then stacked together (Blake et al, 1967a) and the three-dimensional position of the electron density related to a model of lysozyme, built with Kendrew models at a scale of 2cm = 1Å.

The conformation of GlcNAc-Asn in the lysozyme complex.

It was immediately apparent that GlcNAc-Asn was bound in the cleft of lysozyme (Blake et al., 1967b), with the sugar ring in site C and the L-aspartyl residue extending down into site D.

Various stereoisomers representing GlcNAc-Asn in a variety of configurations were constructed with the Kendrew models. The relationships of the atoms in these models were then directly related to the three-dimensional contours

of electron density constructed as described above.

In other methods used to assess the configuration, the electron density was displayed at a contour level of $0.36eA^{-3}$ on a Ferranti display system on-line to the Argos 500 computer. It was possible to display simultaneously a line representation of the molecule. The programme facilities allowed the conformation of the molecule to be adjusted by performing rotations about a specified axis through the whole molecule, or by rotating about certain specified bonds.

As the crystal structure of GlcNAc-Asn trihydrate had not been ascertained at that time, the structural parameters chosen for line representation was built up from those of L-asparagine as determined in the crystalline form of L-asparagine monohydrate (Kartha & De Vries, 1961) and, for a model in β-anomeric configuration, the structure of β-D-glucose (Chu & Jeffrey, 1968). The amide geometry established by Corey & Pauling (1953) was employed for the 2-acetamido group. The possibility was also considered that GlcNAc-Asn was of α-anomeric form, although this was extremely unlikely (see, for example, Marshall & Neuberger, 1964). For the construction of models representing the latter type, the parameters of N-acetyl-α-D-glucosamine (Johnson, 1966) were chosen.

Considerable freedom was allowed in all the models for the geometry of the linkage region, the bond lengths and angles of which were adjusted by programming until the best fit to the electron density was obtained.

It was found from building Kendrew models that the

N-acetyl-D-glucosaminyl moiety of the molecule could be accommodated in the electron density in a position such that the 2-acetamido group made specific hydrogen bonds with the main chain of the protein in the same manner found previously in inhibitor-lysozyme complexes (Blake et al., 1967b). The N_2-atom (see Fig 5.37) was finally located at a distance of 2.8Å from the main chain carbonyl oxygen of residue 107 (alanine), and the O_7 atom was at a distance of 3.5Å from the main chain NH group of residue 59 (asparagine). With the 2-acetamido group in this position, most of the ring atoms were accommodated in the electron density. The atom O_6 was not generally located in electron density, but Dr. L. Johnson said that this situation also occurs in other lysozyme-inhibitor complexes.

The photographs which are shown in Plates 5.2 to 5.6 depict the electron density displayed on a television screen, together with line representations of the bonds in GlcNAc-Asn. Points corresponding to density levels of $0.36eA^{-3}$ are joined up with thin lines to form a three-dimensional contour of electron density. GlcNAc-Asn is represented by thicker lines. Two models side by side represent stereoscopic views of the same model, and are best viewed through a stereomagniscope. A suitable viewer is provided in the back of Advances in Protein Chemistry (1967) Vol. 22, and Plates 3.6 and 3.7 are best viewed through the coloured glasses provided at the back of this thesis.

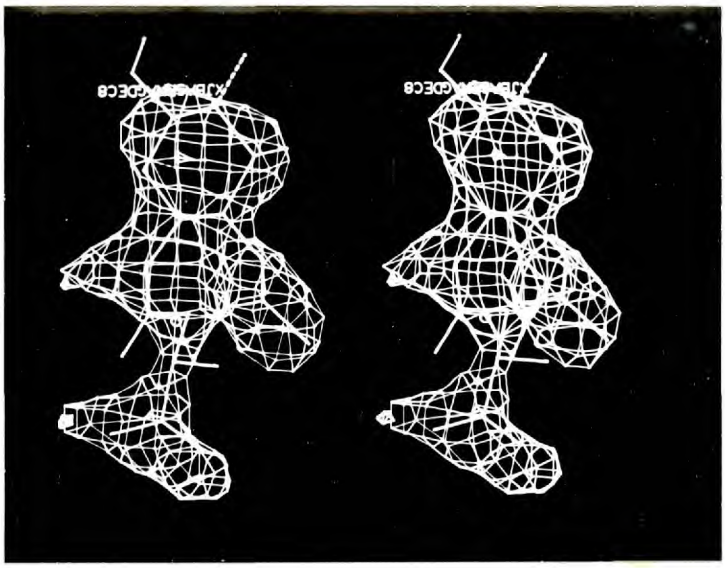
It is evident that the α-amino acid moiety of GlcNAc-Asn should fit into the lower lobes of electron density (Plates 5.2-5.6) and that the narrow part of the electron density that joins the upper and lower lobes of electron density limits the configuration of C_1.

Models of the α-anomeric form with a $\underline{Cl}$ sugar conformer leave the L-aspartyl residue well outside the limits of the electron density when the sugar residue is placed in the correct position. The fit obtained when attempts were made to accommodate a model of the β-anomeric configuration, but with a $\underline{cis}$-amide at C_1, is shown in Plate 5.2. This configuration leaves O_7 and N_{11} outside the electron density, and much of the electron denisty is left unaccounted for.

Plates 5.3 and 5.4 show the best fits that could be obtained with the C_1-amide group in a normal β-$\underline{trans}$ configuration. In plate 5.3, the carbonyl oxygen is approximately syn-periplanar ($\underline{cis}$) with the C_1 ring portion (H_1). In this conformation, O_7 and the carboxyl group lie outside the electron density. In Plate 5.4 a conformation is adopted in which the carbonyl oxygen is closer to the ring oxygen O_5. The carboxyl group and O_7 now lie inside the regions of electron density, but the rest of the aspartyl residue lies well outside. In both cases, much electron density is left unaccounted for.

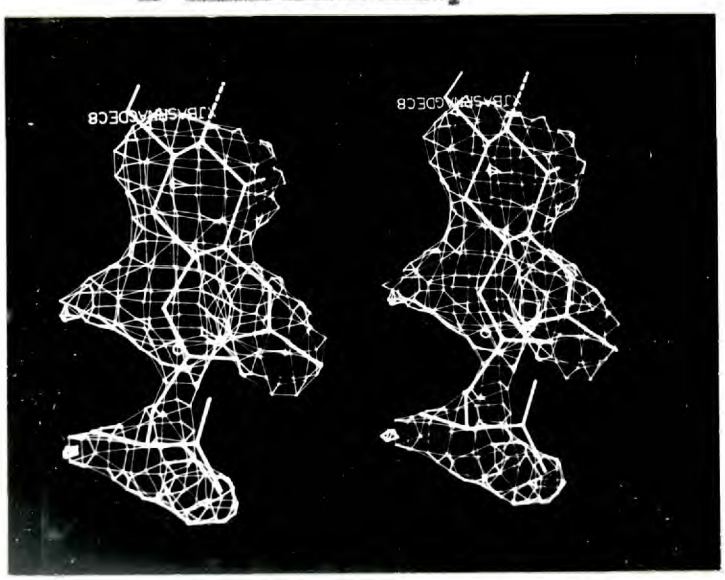
Models were considered in which distortions were applied to the C_1-amide group in such a way that the atoms $C_1N_1C_9O_9C_{10}$ no longer lay in a plane. Two good fits that

Plate. 5.2. A model, built by computer, of GlcNAc-Asn containing a cis-amide at C_1



The upper half of the three-dimensional representation of the electron density contains the sugar ring, and the aspartyl residue extends down into the lower half.

Plate. 5.3. A model, built by computer, of GlcNAc-Asn containing a trans-amide at C_1



The photographs show a television screen, displaying a line model of the compound and the electron density of the compound when bound to lysozyme. For details, see text.

Plate 5.4. A model, built by computer, of GlcNAc-Asn containing a trans-amide at C_1

The groups around C_1N_1 bond are oriented differently in this model compared to the model shown in Plate 5.3.

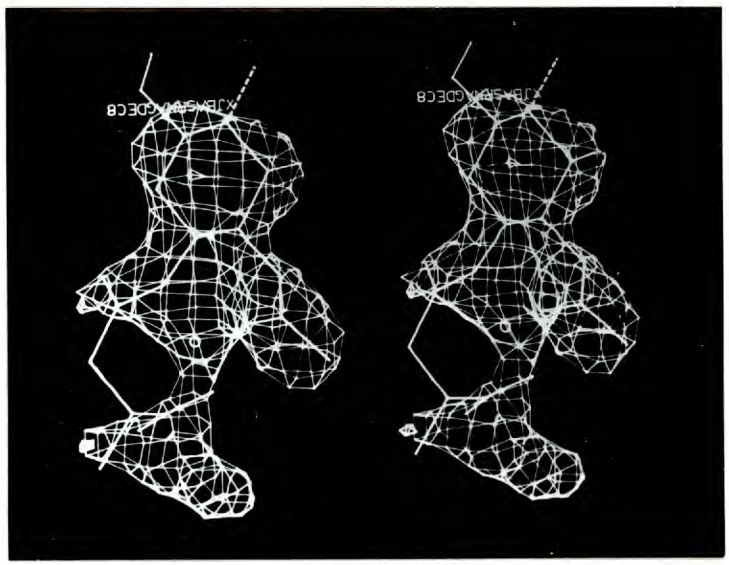
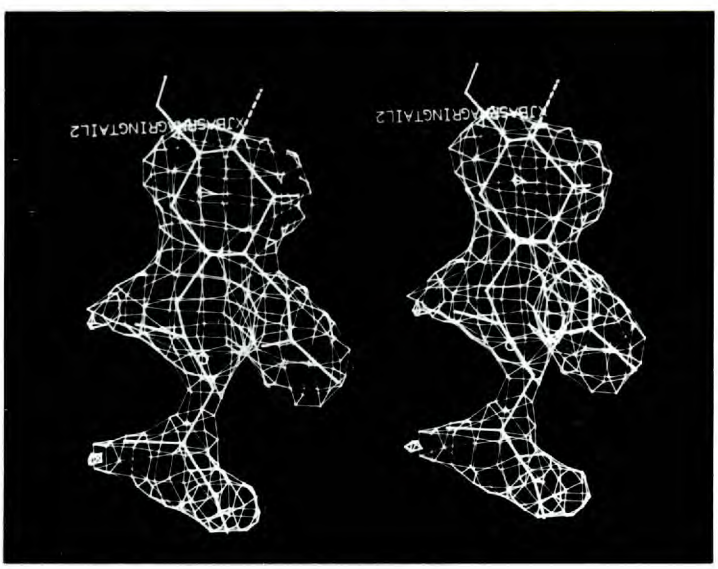


Plate 5.5. A model, built by computer, of GlcNAc-Asn containing a distorted amide at C_1



Interpretation of these photographs is described in the text.

were obtained by programming changes in the C_1-amide geometry are shown in Plates 5.5 and 5.6. In these models, most of the atoms are accommodated inside the electron density contour, although one of the carboxyl oxygens lies on the edge of the electron density. Moreover, most of the electron density is now accounted for.

It seems that the structure of GlcNAc-Asn in its complex with lysozyme does not have a planar acylamido group at C_1 so that the structure is different from that which it is in the crystalline state. The asparaginyl moiety has a structure similar to that of L-asparagine monohydrate with an O_9N_{11} distance equal to 3.1Å.

The final distance adopted for the C_1N_1 bond (1.40Å) was slightly shorter than that found for GlcNAc-Asn trihydrate in the crystalline state (1.44Å), but the bond angles around the C_1 atom ($\angle C_2C_1N_1 = 108.1^\circ$; $\angle O_5C_1N_1 = 106.8^\circ$ and $\angle C_2C_1O_5 = 108.9^\circ$) were about the same.

Interactions of GlcNAc-Asn with lysozyme.

The position that GlcNAc-Asn adopts in the cleft of lysozyme is shown in Plate 5.7. As well as the interactions involving the 2-acetamido group discussed above, the NH side chain groups of residues 62 and 63 (tryptophan) form hydrogen bonds with O_4 (3.6Å) and O_3 (2.9Å). There is a strong electrostatic interaction (2.8Å) between the α-amino group of GlcNAc-Asn and the carboxyl group of residue 52 (aspartic acid). The distance of O_{12} to the carboxyl oxygen of residue 35 (glutamic acid) is

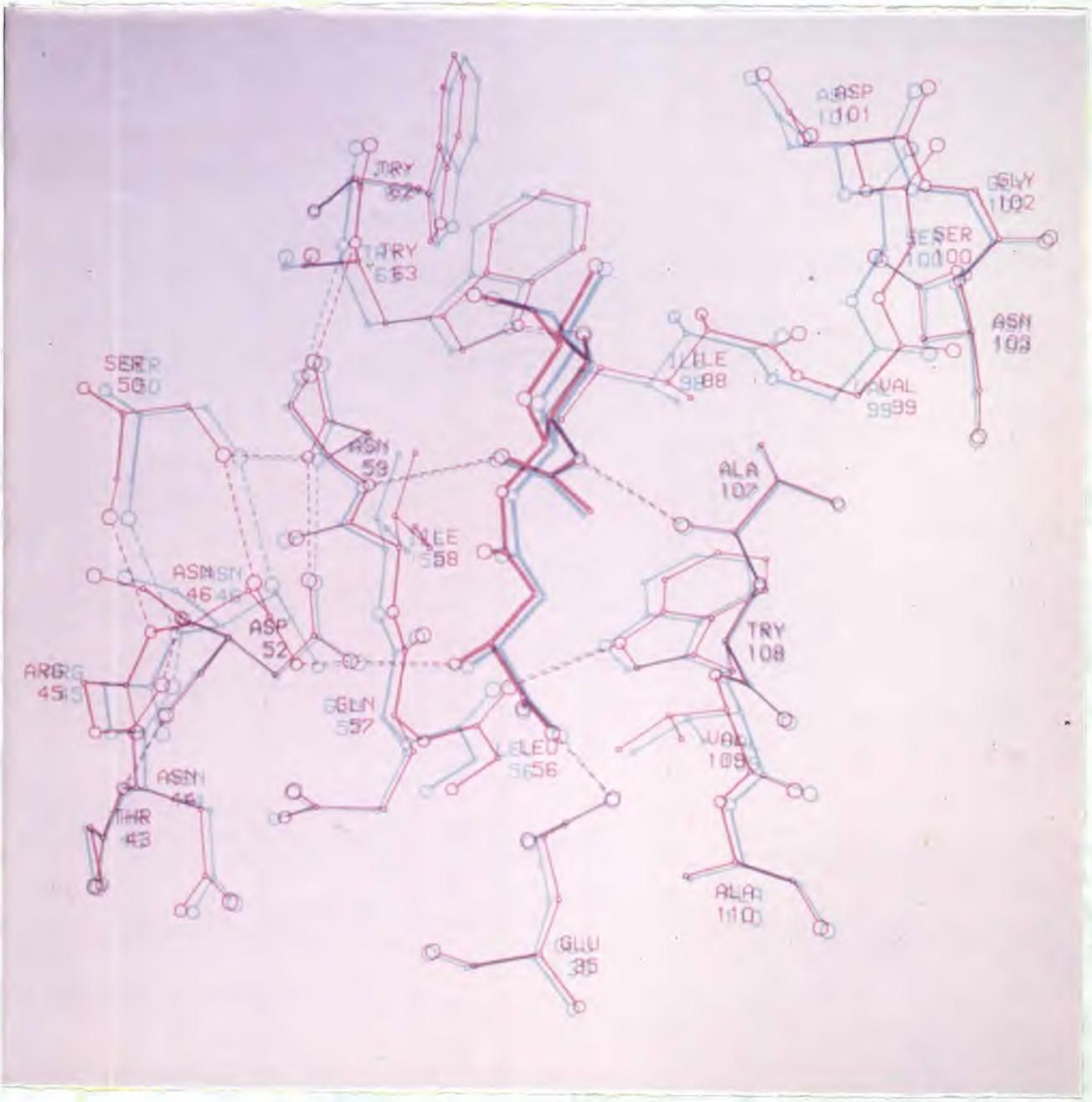
Plate. 5.6. A model, built by computer, of GlcNAc-Asn, containing a distorted amide at C_1

This plate is in the form of a slide placed at the back of this thesis. The slide should be projected onto a screen placed at a distance of about six feet from the projector. The image should be viewed through the glasses also provided at the back of the thesis.

Plate. 5.7. The mode of binding of GlcNAc-Asn to lysozyme.

This plate is in the form of a slide placed at the back of this thesis. The slide should be projected as described for Plate 5.6.

The model of GlcNAc-Asn that provided the best fit to the electron density, as shown in the two proceeding Figures, is drawn with thicker lines. The sugar residue is at the top and is bound at site 'C'. The aspartyl residue extends down into site 'D'. Possible hydrogen bonds are marked with dotted lines.



2.9Å, and there is a possibility of hydrogen bonding here as the residue is presumed to be unionised (Blake et al., 1967b).

The occupancy of GlcNAc-Asn in the lysozyme molecules in the crystal was calculated to be 24%.

SECTION 4. DISCUSSION.

Important features of the chiroptical properties of certain types of glycopeptides and of glycoproteins are the Cotton effects, in the wavelength regions below 250nm exhibited by the acylamido groups at C_1 and C_2 of the linking N-acetyl-D-glucosaminyl residue and by the C_2-acetamido groups of other N-acetyl hexosamine residues. These studies have shown that these effects are more easily isolated by CD techniques from those arising from other centres of optical activities in the molecule than by ORD. Moreover, Cotton effects arising from $n \rightarrow \pi^*$ transitions have been resolved from those arising from $\pi \rightarrow \pi^*$ transitions in CD spectra (Figs. 5.10-5.13), but not in ORD spectra (Fig. 5.9).

Valuable information concerning the preferred orientations adopted by the compounds studied here has been obtained by application of symmetry rules. The present studies have employed symmetry rules which take into account the electrostatic nature of the perturbing atoms. Other workers have applied the octant rule as formulated by Moscowitz et al., (1962) to interpret the chiroptical properties of carbohydrate derivations (Beychok & Kabat,

1965; Beychok, Ashwell & Kabat, 1971; Paulsen, 1968; Okuda, Harigaya & Kiyomoto, 1964), but there are dangers in doing so as this rule was derived for use on certain ketones.

From the chiroptical properties of the compounds studied, it has been possible to define a limited range for the torsional angles about C-N bonds of the C_1 and C_2 acetamido groups. The preferred conformations are likely to lie within these ranges. It is interesting to find that the torsional angles around the C-N bonds of the acylamido groups of GlcNAc-Asn and Glc-Asn in the crystalline states lie within the ranges of values evaluated from chiroptical properties. Similar correlations have been established previously for nucleoside derivatives (Miles et al., 1970).

Examination of the ORD and CD of GlcNAc-Asn provides a sensitive and rapid method for the identification of this compound as the Cotton effects are very distinctive. Some information about structure and conformation has been obtained by examination of the chiroptical properties of glycopeptides and their breakdown products, but further studies on model disaccharides and trisaccharides would be desirable (see also Lloyd, Beychok & Kabat, 1967; Etzler, et al., 1970). In this respect, ORD may prove to be a more useful tool than CD as configuration-dependent effects are more marked in ORD.

Chiroptical studies may also prove to be of use in elucidating the nature of conformational changes that occur when glycoproteins and glycopeptides interact with other macromolecules. For example, it should be possible

to observe the changes induced in GlcNAc-Asn by lysozyme with difference spectropolarimetry (Ikeda & Hamaguchi, 1969; Adkins & Yang, 1968).

Rotations about the $C_{11}C_{10}$, $C_{10}C_9$ and N_1C_1 bonds of the linking moiety, GlcNAc-Asn, in glycoproteins are theoretically possible, and have been commented upon (Rees & Scott, 1971). The present studies have shown that the groups around the N_1C_1 bond are orientated differently in the substance when it is in the crystalline state and when it is in aqueous solution, compared to when it is bound to lysozyme. The forces required to bring about this change result from interactions with the polypeptide chain. It is reasonable to ask whether linkages of this type in glycoproteins are stereochemically identical with that occurring in the model compound itself and, furthermore, whether the linkage differs in its special orientations from one glycoprotein to another. These questions can be answered only by further studies.

CHAPTER 6.

SOME PROPERTIES OF THE OVALBUMIN OF EMDEN GOOSE.

At the time these studies were commenced, in the autumn of 1968, there were relatively few sequences known in the neighbourhood of glycosylated L-asparaginyl residues of glycoproteins (see Marshall & Neuberger, 1968). The frequency with which a β-hydroxy - amino acid residue occurs in a position next-but-one on the C-terminal side of the glycosylated residue had been discussed (Marshall, 1967), and it was later suggested that the necessary sequence for glycosylation of an L-asparaginyl residue to occur, was -ASN-X-Thr(Ser)- (Neuberger & Marshall, 1969). It was pointed out at that time, that the occurrence of this sequence does not guarantee that glycosylation of the L-asparaginyl residue will occur, and that other factors, including the whole stereochemistry of the protein, are important.

At the time, reports were known of several examples of sequences which did not fit into this pattern, but these have since been shown to be in error (see Marshall, 1972), with the exception of hen egg yolk phosvitin (Shainkin & Perlmann, 1971).

Because of these considerations, it was decided, while the experiments described in Chapter 2 were in progress, to examine other glycoproteins for the sequences of amino acids in the neighbourhood of glycosylated L-asparagine residues. However, during the time that the experiments were in progress, many examples of the triplet amino acid

sequence became known (see for example, Catley, Moore & Stein, 1969; Jackson & Hirs, 1970; and also Table 1.4). Partly for this reason, and partly because other aspects of the problem became of greater interest, the project was not pursued extensively. Nevertheless, some interesting results were obtained and these are now reported.

It has been firmly established that hen ovalbumin contains a carbohydrate moiety at a unique site in the polypeptide chain, although the sequence -Asn-Leu-Ser- occurs elsewhere in the polypeptide chain of one genetic variant of ovalbumin (Wiseman, Fothergill & Fothergill, 1972).

It has been found (Fothergill & Fothergill, 1970) that there are greater differences in the structures of ovalbumin belonging to the different orders, galliformes and anseriformes, than between species of the same order. It had earlier been decided, because of the not unexpected finding just mentioned, that a comparison of the ovalbumins of the Embden goose (anseriformes) and the hen (galliformes) was desirable.

RESULTS.

Preparation and properties of goose ovalbumin.

Goose ovalbumin was prepared by the procedure which was basically that of Sørenson & Høyrup (1915-1917), after adsorption of the lysozyme of the egg-white on carboxymethyl cellulose. A photograph of the preparation of goose ovalbumin under 400 magnification is shown in Plate 6.1. The ovalbumin does not form crystals on separation

Plate 6.1. The preparation of goose ovalbumin viewed under 400 magnification.

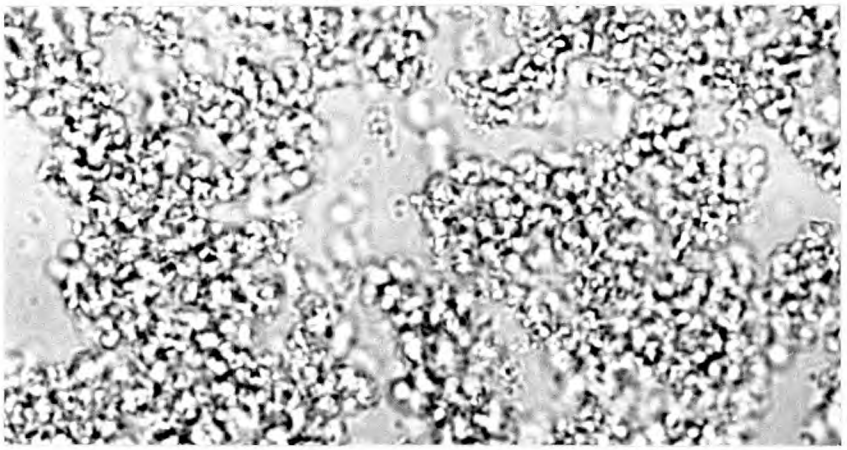
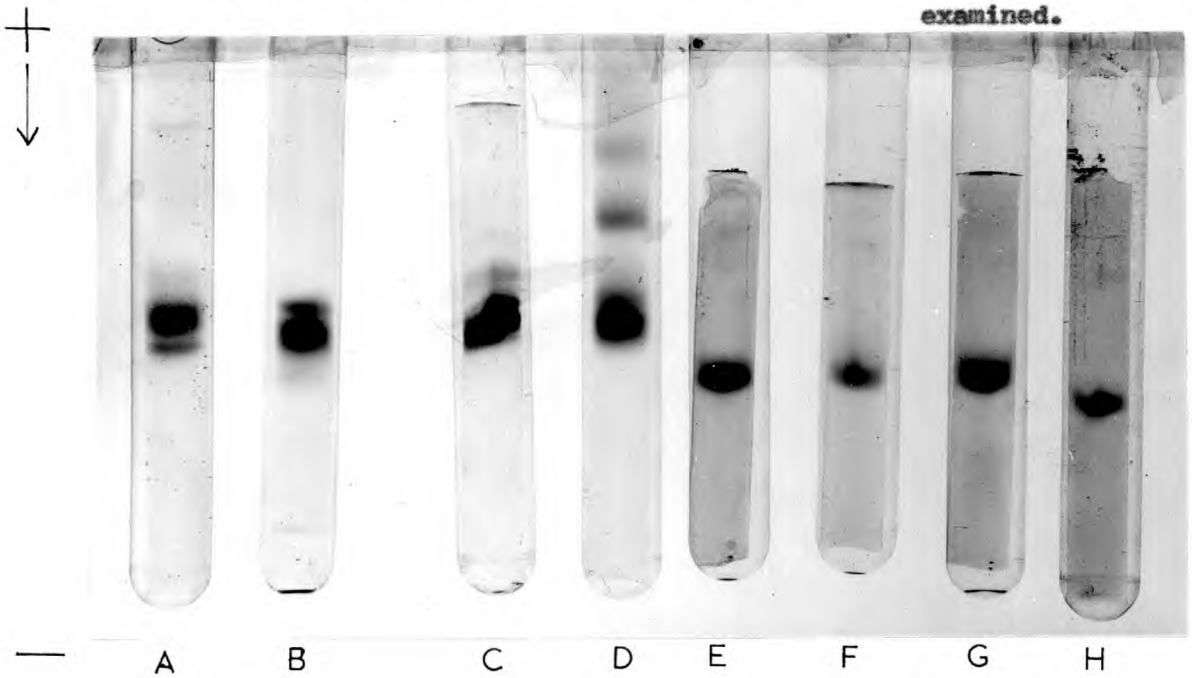


Plate 6.2. Polyacrylamide disc gel electrophoresis of goose ovalbumin.

Electrophoresis was performed at pH 8.5 either in the presence or absence of 0.1% sodium dodecyl sulphate. Conditions are described by Davis (1964) or Marshall & Zamecnik (1969). 50 µg amounts of protein were examined.



A Hen ovalbumin

B Goose ovalbumin

C Hen ovalbumin (on the left)
Goose ovalbumin (on the right)

D An aged sample of goose ovalbumin

E Goose ovalbumin in 0.1% sodium dodecyl sulphate

F Goose ovalbumin, reduced in mercaptoethanol, in 0.1% sodium dodecyl sulphate

G Goose ovalbumin, reduced and carboxymethylated, in 0.1% sodium dodecyl sulphate.

H Hen ovalbumin in 0.1% sodium dodecyl sulphate.

from ammonium sulphate solution, whereas hen ovalbumin crystallises as small, cigar-shaped needles (compare Plates 6.1 and 2.1). Nevertheless, an opalescent sheen is observed when suspensions of goose ovalbumin are swirled in daylight.

The preparation contained a protein which possessed a very low mobility when subjected to disc electrophoresis, by the method of Davis (1964), at pH 8.5. This protein was removed by gel filtration on Sephadex G-100 (Fig. 6.1). The protein in the major peak was isolated by dialysis, dried from the frozen state and used in all further studies. Multiple bands are exhibited by goose ovalbumin on polyacylamide discs at pH 8.5 (Plate 6.2; gel B) and also by hen ovalbumin (Plate 6.2; gel A). The electrophoretic heterogeneity of hen ovalbumin has been discussed previously (see Chapter 2). The intensities of staining and the mobilities of the bands exhibited by the two proteins differ slightly, suggesting, perhaps, differences in the degrees of phosphorylation. The hen ovalbumin variants run faster during electrophoresis than those of goose ovalbumin, when the two proteins are placed on either side of a plastic partition placed at the top of a single gel, and then subjected to electrophoresis (Plate 6.2; gel C). Other bands, observed when samples of goose ovalbumin which have been stored for some time in the frozen state are subjected to electrophoresis are probably due to aggregation of the protein (Plate 6.2; gel D).

When electrophoresis is performed in 0.1% (w/v) sodium dodecyl sulphate, goose ovalbumin gives one band

Fig. 6.1. Gel chromatography of the preparation of goose ovalbumin.

Column: Sephadex G-100, suspended in 0.1M-NaCl at 25°C, (85 x 2.5cm).

Eluting agent: 0.1M-NaCl at 8.3ml/hr.

Protein (0.2g), obtained by ammonium sulphate fractionation of the egg-white, was placed on the column.

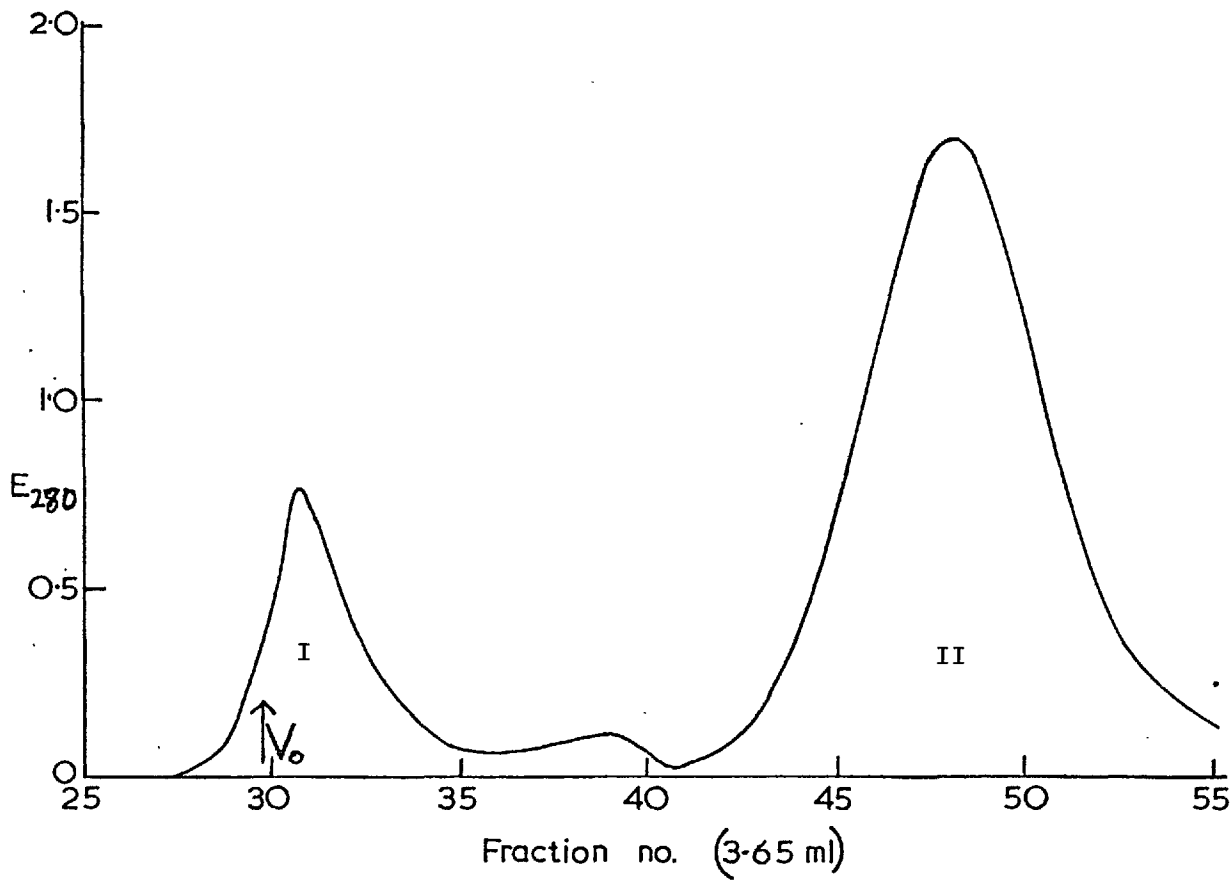


Fig. 6.2. Calibration of Sephadex G-100 column with proteins of known molecular weight.

Protein (<10mg) was dissolved in 0.1M-NaCl and placed on the column described in Fig. 6.1. Eluting agent was 0.1M-NaCl, and chromatography was performed at 25°C.

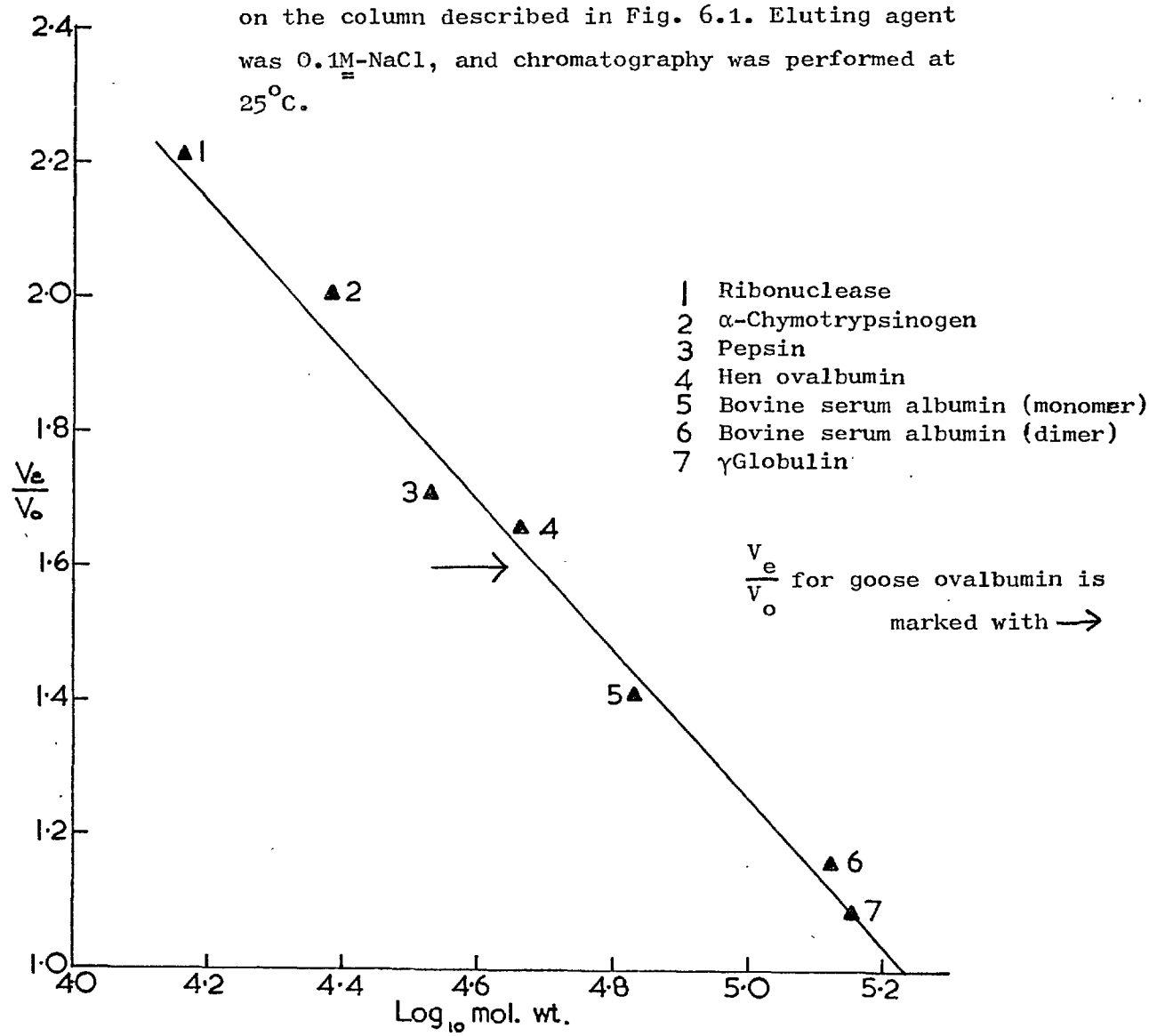


Fig. 6.3. Calibration of polyacrylamide gels, run in 0.1%(w/v) sodium dodecyl sulphate, with proteins of known molecular weight.

Gels were run at pH 8.5 under the conditions described by Marshall & Zamecnik (1969). R_f values are the distances run by the proteins relative to that run by bromophenol blue.

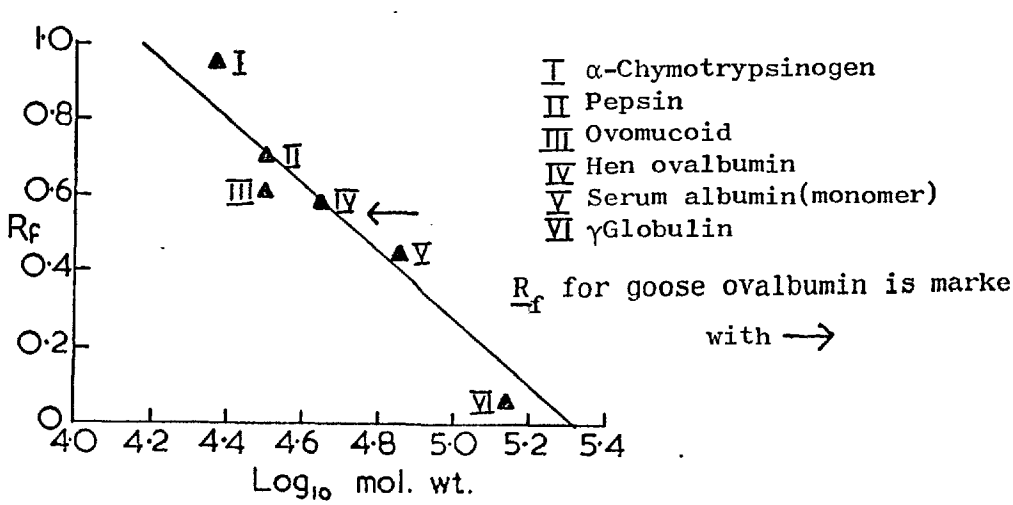


Table 6.1. The composition of Embden goose ovalbumin.

Residues (to nearest integer) per mole of ovalbumin.

Substance	Goose	Hen[a]
Alanine	24[b]	35
Glycine	21	19
Valine	31	32
Leucine	30[b]	31
Isoleucine	21[c]	24
Proline	15[b]	15
Phenylalanine	23[b]	19
Tyrosine	12[b]	10
Tryptophan	3[d]	3
Serine	42[e]	38
Threonine	28[e]	15
Cystine/2	8[f]	6
Methionine	19[b]	15
Arginine	12[b]	15
Histidine	6[b]	7
Lysine	23[b]	20
Aspartic acid	36[b]	32
Glutamic acid	51[b]	52
Glucosamine	8[g]	3[i]
Mannose	12[h]	5[i]
Moisture content	(10.1%)	
Ash content	(9.6%)	

(a) Amino acid analyses are from Smith & Back (1970)

(b) Mean value of seven estimations made after different times of hydrolysis

(c) Measured after 78 hr of hydrolysis

(d) Measured spectrophotometrically

(e) Values obtained by extrapolation to zero time of hydrolysis

(f) Measured as carboxymethyl-L-cysteine formed by reduction and alkylation of protein

(g) Measured by modification of the Rondle & Morgan (1955) procedure

(h) Measured by the orcinol-H_2SO_4 procedure

(i) Johansen, Marshall & Neuberger (1960)

(gel E). Gels F and G show that the preparation of goose ovalbumin is not dissociable into sub-units by cleavage of disulphide bonds.

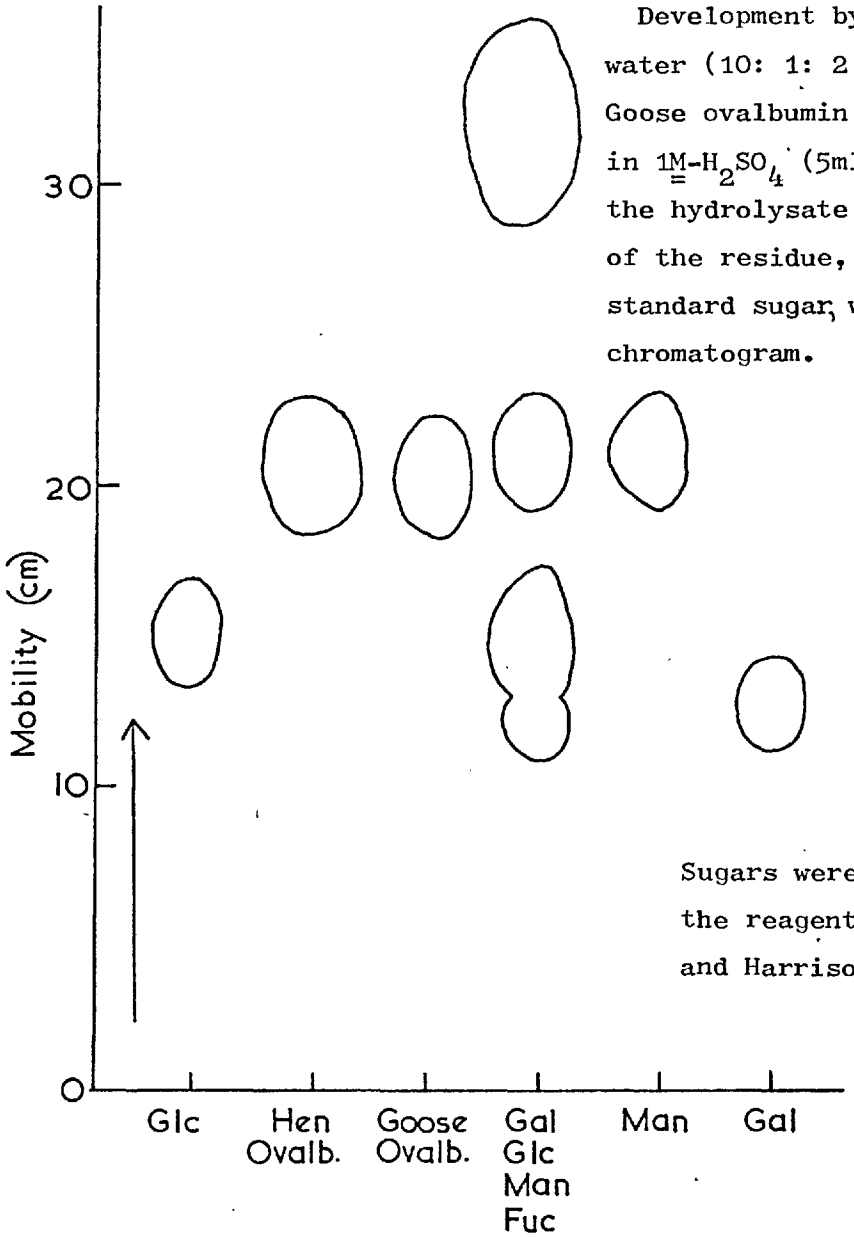
Calibration of the Sephadex G-100 column (Fig. 6.2) with standard proteins, and calibration of the polyacrylamide gels (Fig. 6.3) with standard proteins that had been left overnight at +4°C in 0.1% (w/v) sodium dodecyl sulphate in electrophoresing buffer, suggested that goose ovalbumin has a slightly higher molecular weight than does hen ovalbumin; a value of 47,500 for the goose protein was estimated.

The amino acid and sugar analyses of goose ovalbumin.

The neutral sugar in hen and goose ovalbumin is shown to be D-mannose only (Fig. 6.4).

The amino acid and sugar analyses of goose ovalbumin are reported in Table 6.1. The values are expressed as residues per mole (47,500 mol. wt.) of goose ovalbumin. The numbers of residues of amino acids released by acid hydrolyses for 15 hr, 30 hr, 48 hr and 78 hr were calculated relative to the number of glycine residues (21) released by hydrolysis for 20 hr. The analyses of all amino acids measured by autoanalysis, except serine, threonine, valine, isoleucine and cysteine, were independent of the times of acid hydrolysis, and mean values of seven determinations are given in Table 6.1. The analyses of serine and threonine extrapolated to zero time, and the analyses of valine and isoleucine obtained by 78 hr hydrolysis, (see Fig. 6.5) are reported. The changes suggested that serine

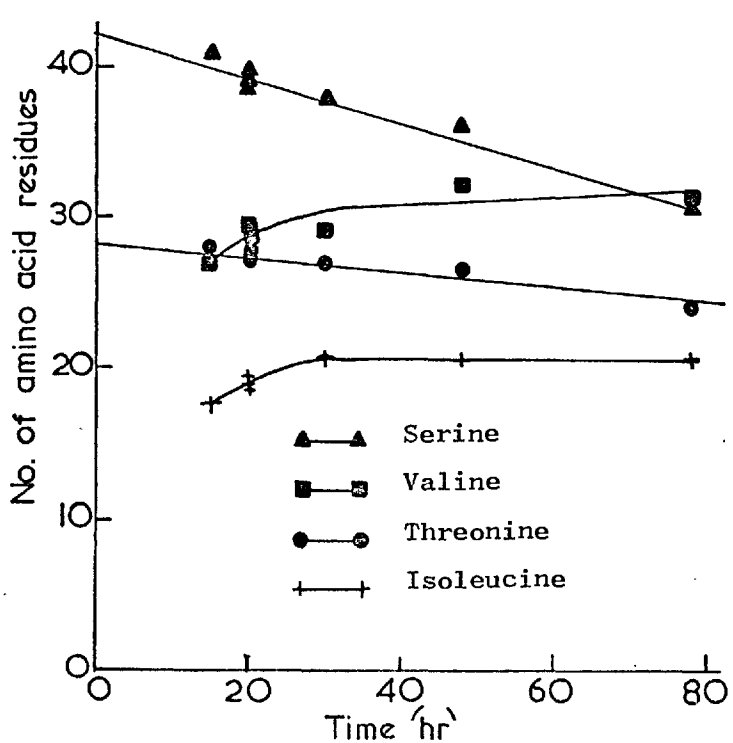
Fig. 6.4. Paper chromatography of neutral sugars.



Development by n-butanol-ethanol-water (10: 1: 2 by vol) for 2 days. Goose ovalbumin (11mg) was hydrolysed in 1M-H_2SO_4 (5ml) at 100°C for 8hr, and the hydrolysate was deionised. One fifth of the residue, and 100µg of each standard sugar, were placed on the chromatogram.

Sugars were located with the reagent of Trevelyan, Proctor and Harrison (1950)

Fig. 6.5. Recovery of amino acids following hydrolysis of goose ovalbumin in 6M-HCl at 110°C for increasing periods of time.



and threonine were destroyed to extents of 7.7% and 3.6% respectively by 20 hr acid hydrolysis (Fig. 6.5). Rees (1946) found that 10.5% and 5.3% of serine and threonine contents of various proteins were destroyed under the same conditions of hydrolysis during 24 hr.

The mean value of the numbers of hexosamine residues released from the protein by hydrolysis in 4M-HCl at 100°C for periods of 2 hr, 4 hr and 6 hr was calculated; there was less than 4% difference in the values measured. D-glucosamine was the only hexosamine detected on the autoanalyser.

Tyrosine and tryptophan were measured spectrophotometrically (Table 6.1). The results obtained were interpreted through the equations of Goodwin & Morton (1946) which are described by Beaven & Holiday (1952). The ultra-violet spectra of a centrifuged solution of goose ovalbumin in water and in alkali are recorded in Fig. 6.6. There is no change in the spectrum measured in 0.3M-NaOH after storage of the solution for 6 hr at room temperature. The tyrosine value agrees with that obtained by autoanalysis.

The analyses are compared to those of hen ovalbumin in Table 6.1. The main differences are higher threonine (13 additional residues), lower alanine (11 residues less) and greater carbohydrate content of goose ovalbumin compared with hen ovalbumin.

Proteolytic digestion of goose ovalbumin.

This part of the work was carried out with the help of Dr. E. Romanowska of the Polish Academy of Sciences, Wroclaw, who spent six weeks as a British Council Visitor

Fig. 6.6. Ultra-violet spectra of aqueous and alkaline solutions of goose ovalbumin.

I Ultra-violet spectrum of a solution of goose ovalbumin (0.064% w/v) in water

II Ultra-violet spectrum of a solution of goose ovalbumin (0.026% w/v) in 0.3M-NaOH.

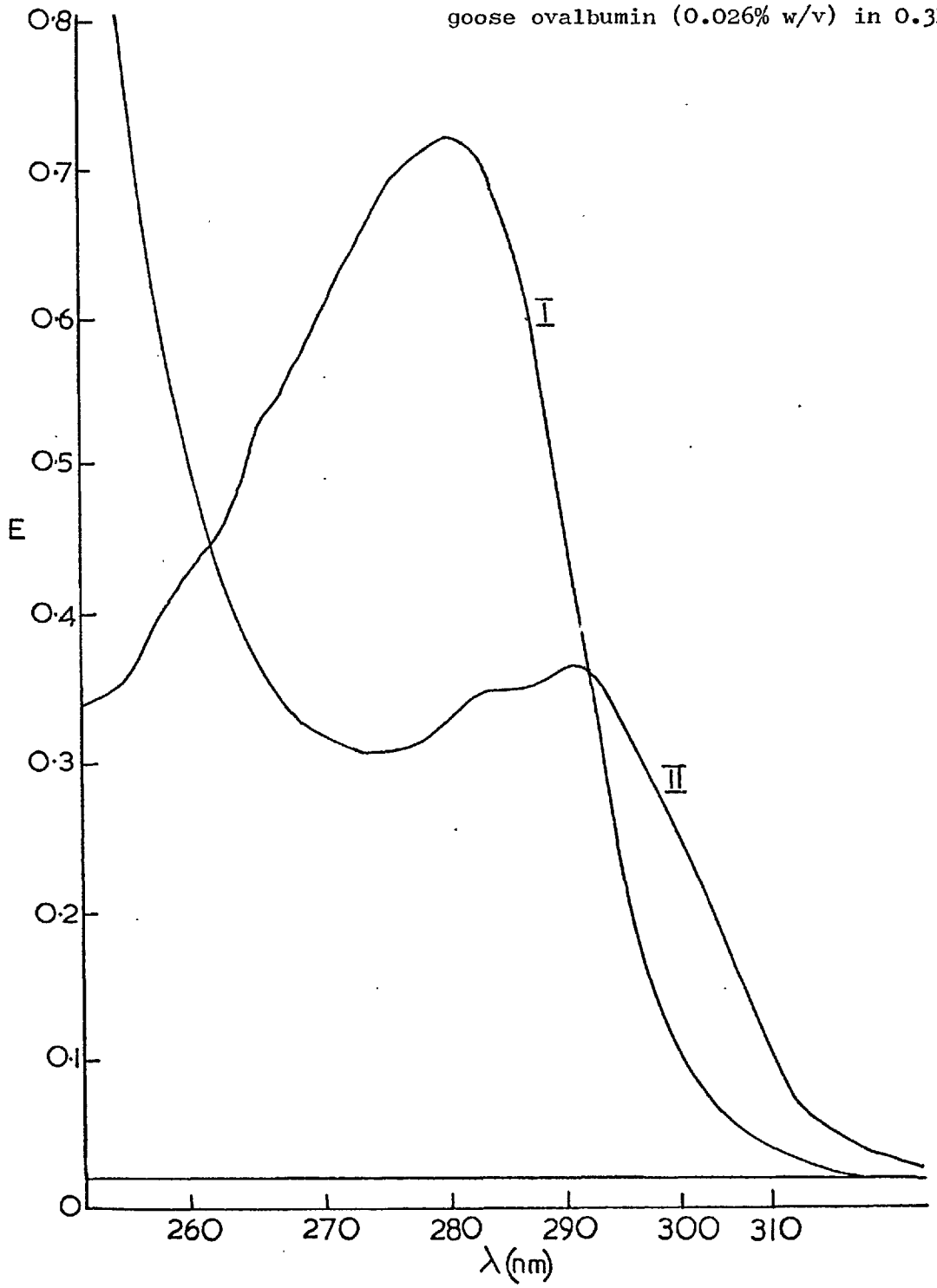


Table 6.2. Analysis of pooled fractions, containing glycopeptide material, obtained by gel chromatography of a proteolytic digest of goose ovalbumin.

	Fraction 1[a]	Fraction 2[a]	Fraction 3[a]
Asp	1.00	1.00	1.00
Thr[b]	0.57	0.55	0.62
Ser[c]	0.55	0.41	0.59
Glu	0.68	0.55	0.31
Pro	0.31	0.32	0.24
Gly	0.67	0.41	0.35
Ala	0.36	0.23	0.27
Val	0.58	0.53	0.38
½ Cys	-	0.47	0.26
Ile	0.11	0.13	0.18
Leu	0.38	0.26	0.34
Tyr	-	0.24	0.11
Phe	-	-	0.17
Lys	0.57	0.41	0.33
GlcN	0.69	0.87	1.75
Man	26.1	2.32	1.66
% Man[d]	22	20	50

(a) These are fractions pooled as shown in Fig. 6.7

(b) Corrected for 4% loss during acid hydrolysis

(c) Corrected for 7% loss during acid hydrolysis

(d) Percentage mannose of that contained in the original glycoprotein before digestion.

in this Department.

In order to discover if the carbohydrate was attached at more than one locus in the polypeptide chain, goose ovalbumin was digested with pepsin, followed by chymotrypsin and trypsin. Ninhydrin estimations suggested that about 45% of the peptide bonds were split after 72 hr, a similar number to those split in hen ovalbumin by the same enzymes(Chapter 2).

The elution profile obtained by chromatography on Sephadex G-50, of that portion of the digest which was insoluble in ethanol, is shown in Fig. 6.7. Fractions were assayed with ninhydrin, and with phenol-H_2SO_4 reagents. The material containing hexose was pooled into three fractions (Fig. 6.7). The column was calibrated with some oligosaccharides of known molecular weight, and a glycopeptide (mol. wt. 2140) from hen ovalbumin containing several amino acids in the peptide portion. Although V_e and molecular weight (Fig. 6.8) would not be expected to exhibit a rectilinear relationship under these circumstances (see Chapter 3), an approximate value of 1900 for the molecular weight of the glycopeptide material obtained from goose ovalbumin was estimated. As shown in Table 6.2, there are about six sugar residues in this glycopeptide material, so that there must be more than one carbohydrate moiety attached to the polypeptide chain of the original glycoprotein. The molecular weight of a glycopeptide containing the original numbers of sugar residues present in the glycoprotein would be at least 3700. The analyses of Fractions 1, 2 and 3 are shown in Table 6.2.

Fig. 6.7. Gel chromatography of material obtained by proteolytic digestion of goose ovalbumin.

Column: Sephadex G-50, 56 x 2.6cm, in 0.1M-acetic acid.

Goose ovalbumin (13μmoles) was digested with proteolytic enzymes, and that portion of the digest that was insoluble in ethanol was eluted from the column with 0.1M-acetic acid at 4°C. Fractions containing hexose were pooled into three lots.

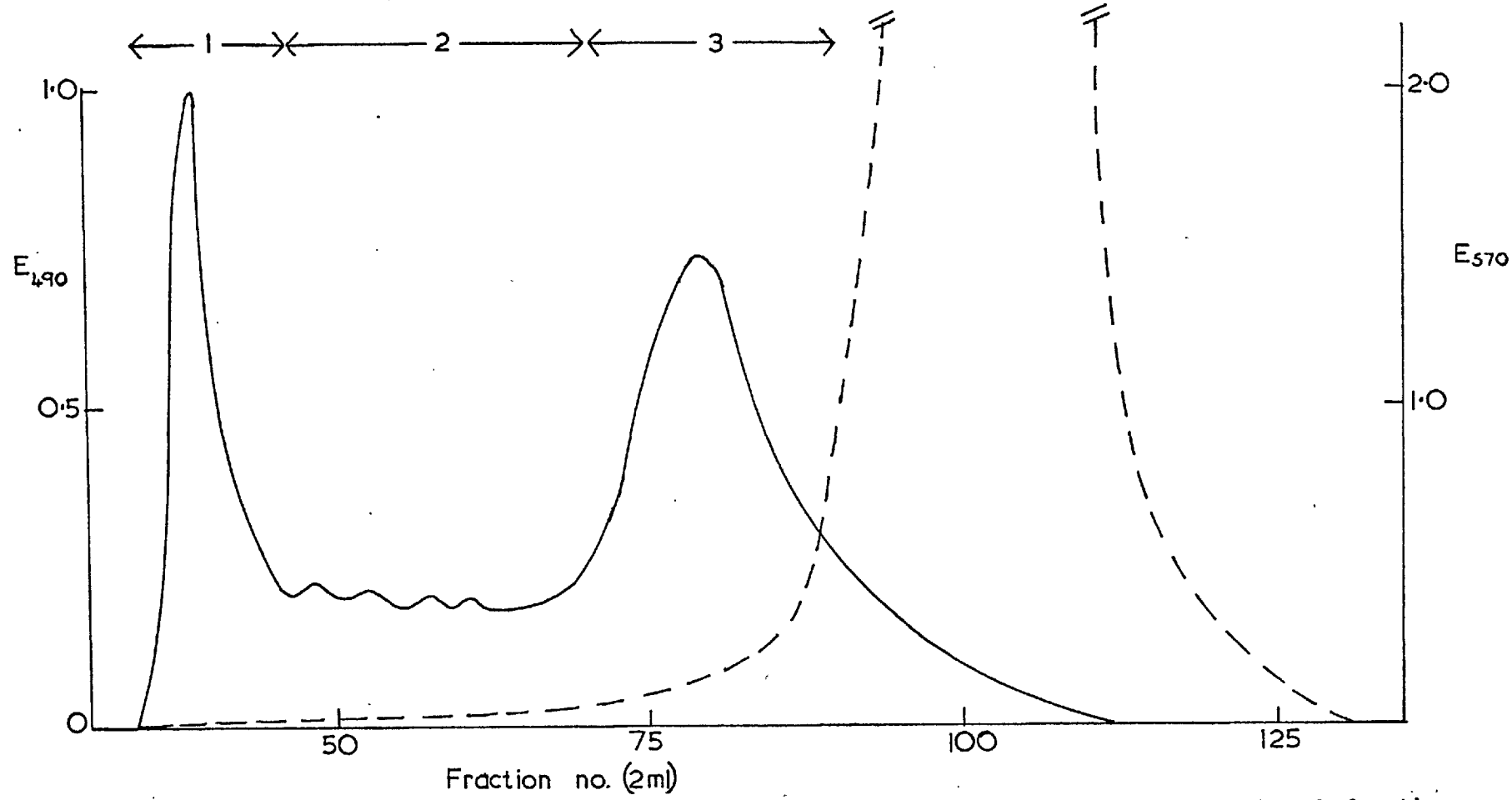
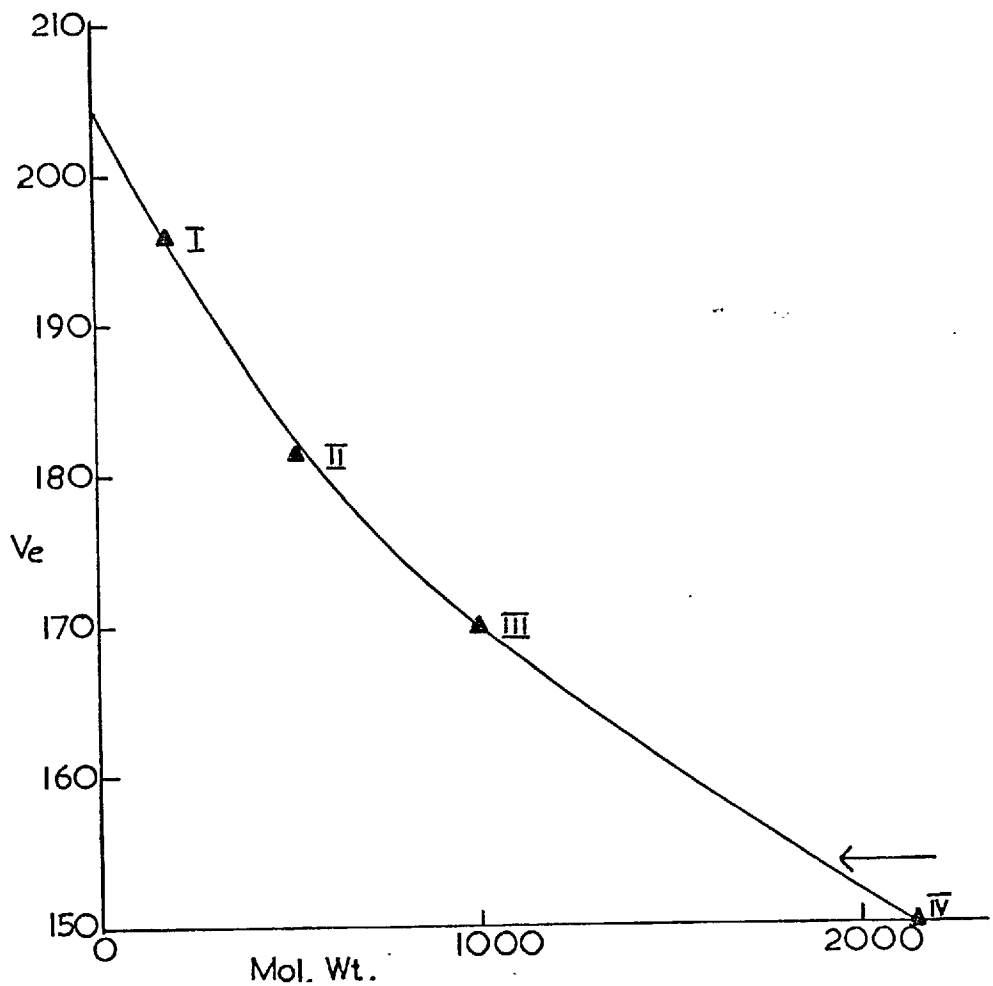


Fig. 6.8. Calibration of Sephadex G-50 column with substances of known molecular weight.

Column: Sephadex G-50 (56 x 2.6cm), in 0.1M-acetic acid.
Materials were dissolved in 0.5ml 0.1M-acetic acid, and eluted by 0.1M-acetic acid at 4°C.

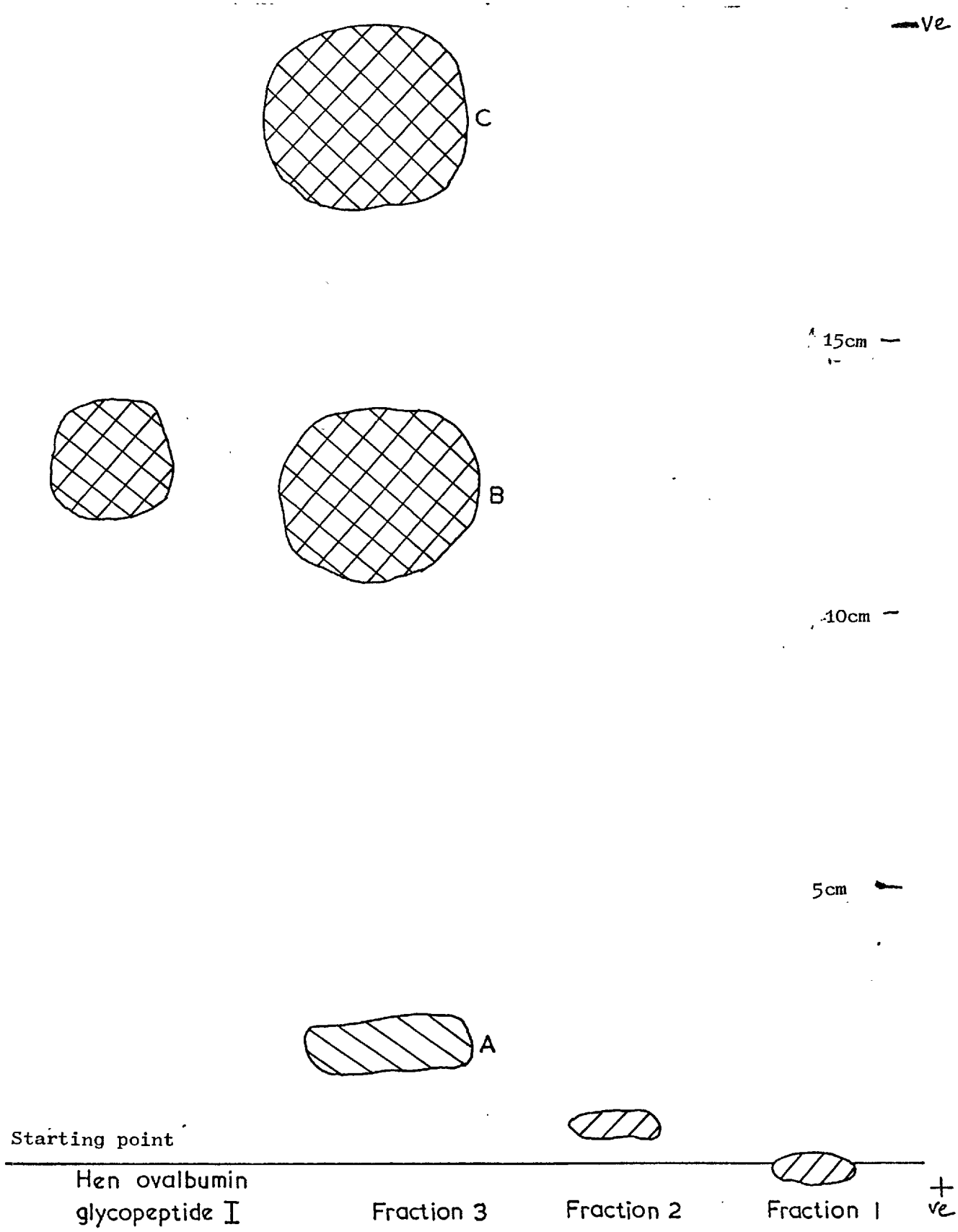


I Glucose
II Raffinose
III Lacto-N-difucohexaose I
IV Hen ovalbumin glycopeptide I (for composition, see Table 3.5)

V_e of goose ovalbumin glycopeptide (Fraction 3; Fig.6.7) is marked with ←

Fig. 6.9. Paper electrophoresis of material obtained by gel chromatography of digested goose ovalbumin.

Material (pooled into three fractions as described in Fig. 6.7) was subjected to electrophoresis at pH 2.0 and 32V/cm for 2hr.



Areas shaded XXX stained with both the periodate/permanganate and ninhydrin reagents. Areas shaded \\\ stained only with the ninhydrin reagent, and those shaded /// stained only with the periodate/permanganate reagent.

Table 6.3. Analysis of three ninhydrin-positive components that were separated from glycopeptide material obtained by gel chromatography of the proteolytic digest of goose ovalbumin.

Components were obtained by paper electrophoresis of Fraction 3.(13mg) (see Fig. 6.9).

	Moles per mole of Aspartic acid[a]		
	Component A	Component B	Component C
Asp	1.00	1.00	1.00
Thr[b]	0.50	0.85	0.54
Ser[c]	3.2	0.71	0.69
Glu	3.9	0.23	0.26
Pro	-	0.11	0.48
Gly	6.7	0.19	0.42
Ala	6.9	0.15	0.13
Val	4.8	0.25	0.16
Ile	-	-	0.38
Leu	0.5	0.81	0.08
Phe	-	-	0.41
Lys	-	-	0.36
GlcN	1.3	3.80	1.91
Man[d]	1.6	4.60	1.61
	0.075 moles Asp[e]	1.0 mole Asp[e]	1.4 mole Asp[e]

(a) Amino acids were released by hydrolysis in 6$\underline{M}$-HCl at 110°C for 20 hr, and GlcN by hydrolysis in 4$\underline{M}$-HCl at 100°C for 3 hr.

(b) Corrected for 4% loss on acid hydrolysis.

(c) Corrected for 7% loss on acid hydrolysis.

(d) Corrected for orcinol-positive colour eluted from paper. This amounted to E_{505} of about 0.3 while the samples gave an E_{505} of 0.7. The values for mannose are therefore not particularly reliable.

(e) Total amounts of aspartic acid contained in the material that was eluted from the electrophoretogram.

The materials obtained from Fractions 1, 2 and 3 (Fig. 6.7) were subjected to paper electrophoresis at pH 2 for 2 hr. Duplicate electrophoretograms were stained with periodate/permanganate and the ninhydrin reagent (see end of this Chapter). Fractions 1 and 2 contain material that stains only for carbohydrate, and does not move from the origin (Fig. 6.9). Fraction 3 produces three ninhydrin-positive bands, two of which stain also for carbohydrate (Fig. 6.9). Band B stains a greenish brown with ninhydrin and band C stains blue.

The analyses of the three components isolated by paper electrophoresis of Fraction 3 (Fig. 6.7) are shown in Table 6.3. Component A possesses an identical mobility with that of the extraneous peptide obtained from some fractions of hen ovalbumin glycopeptide. It also has a similar composition to that of the peptide derived from hen ovalbumin (compare Table 6.3 and Table 2.2). Component B has a similar amino acid composition to that of hen ovalbumin glycopeptide (compare Tables 6.3 and 2.2), and also possesses a similar electrophoretic mobility (Fig. 6.9). Component C has an amino acid composition which is markedly different from that of component B, in that it contains little leucine, but increased amounts of proline, glycine, isoleucine, phenylalanine and lysine.

DISCUSSION.

The reluctance of goose ovalbumin to form clearly recognisable crystals may be due to the additional heterogeneity provided by the additional carbohydrate

content of this glycoprotein compared to that possessed by the hen. The ovalbumin of duck and turkey appear not to have been obtained in crystalline form from ammonium sulphate solutions (Smith & Back, 1970) and also contain increased amounts of carbohydrate. It is known, however, that aged hen ovalbumin crystallises less readily from ammonium sulphate solutions than fresh preparations (Smith & Back, 1965 and 1968). Possibly goose ovalbumin undergoes similar changes during storage.

The only sugars contained in goose ovalbumin are D-mannose and N-acetyl-D-glucosamine. In this respect, the ovalbumins of goose and hen are similar, although there are isolated reports that small amounts of D-galactose occurs in hen ovalbumin (Levvy, Conchie & Hay, 1966; Kim et al., 1967). The ovalbumins of duck and turkey contain additional sugars (Weintraub & Schlamowitz, 1970).

The main differences between the amino acid analyses of the two proteins are a higher number of threonine and lower number of alanine residues in goose ovalbumin. One might expect, on statistical grounds, the possibility of a larger number of the triplet amino acid sequences necessary for glycosylation of L-asparagine residues in the goose protein.

The analysis and molecular weight of glycopeptide material, isolated from goose ovalbumin by proteolytic digestion and gel chromatography, show that more than one carbohydrate moiety is attached to the polypeptide chain. Partial purification of this material by paper electrophoresis was achieved. There were sufficient differences

in the amino acid and carbohydrate contents of the glycopeptide separated to suggest that goose ovalbumin contains at least two carbohydrate moieties attached at different parts of the polypeptide chain. Evidence has been presented that there is also more than one carbohydrate moiety in the ovalbumins of duck and turkey respectively (Huang & Montgomery, 1972). Sequence studies on the glycopeptides of goose ovalbumin will be of interest and are now in progress.

EXPERIMENTAL.

Materials.

Fourteen non-fertile goose eggs were obtained from a number of Embden geese at Cherry Valley Farm, Bucks. Proteolytic enzymes, bovine serum albumin, ribonuclease A and chymotrypsinogen were obtained from Armour Laboratories. Ovomucoid was purchased from Worthington Biochemicals and rabbit γ globulin was prepared by Dr. R.D. Marshall.

Iodoacetic acid was obtained from British Drug Houses, recrystallised from ether by the addition of light petroleum (b.p. 60-80°C), and dried in a desiccator over paraffin wax *in vacuo*. Urea (240g) (May & Baker Ltd.) was dissolved in water (500 mls) and the solution was deionised by passage through a column of washed Amberlite MB-1 resin (20 x 1cm). The first 200ml of eluate were discarded and the next 200ml were retained and stored in the frozen state. Carboxymethyl-L-cysteine was obtained from British Drug Houses.

Preparation and properties of goose ovalbumin.

The egg-whites (about 1200g) of fourteen goose eggs were separated, pooled and diluted with 0.05M-NaH_2PO_4 (3600ml). The solution was stirred with CM-cellulose (50g) under toluene at +4°C for 24 hr to remove lysozyme, and the CM-cellulose was removed by centrifugation. The supernatant was stored at +4°C for several days, and was dialysed against several changes of water at +4°C. An equal volume (3200ml) of saturated ammonium sulphate solution was stirred into the egg-white solution, and the mixture was covered with toluene and left at room temperature overnight. The precipitate was centrifuged off, and the pH of the supernatant adjusted to 4.6 by addition of 0.2M-H_2SO_4. The preparation was continued as described by Sørenson & Høyrup (1915-1917).

The protein was obtained by dialysis against water at 4°C and drying in the frozen state to yield 68g.

Gel chromatography of goose ovalbumin.

Sephadex G-100 (fine grade, Pharmacia Ltd.) (18g) was suspended in 0.1M-NaCl at room temperature, and packed into a column (85 x 2.5cm). A small amount of the protein (0.2g) was dissolved in 0.1M-NaCl (0.5ml) and placed on the column. Elution was performed by 0.1M-NaCl at flow rate 8.3ml/hr kept constant by a pump. Fractions (3.7ml) were collected, and the extinctions at 280nm recorded. The material in the more retarded fractions (peak II; Fig. 6.1) was pooled, dialysed against water and dried in the frozen state.

The column was calibrated by chromatography of small amounts (10mg) of proteins of known molecular weight initially dissolved in 1ml of 0.1M-NaCl. The following proteins were used: Ribonuclease, 13,700; chymotrypsinogen, 24,000 (mol. wt. calculated from the amino acid sequences reported by Dayhoff, 1969); pepsin, 33,500 (Fruton, 1971); bovine serum albumin, 68,000 (Tanford, Kawahara & Lapanje, 1967); hen ovalbumin, 45,000 (Warner, 1954) and γ globulin, 137,000 (Cammack, 1962). Goose ovalbumin (10mg) in 0.1M-NaCl (1ml) was rechromatographed.

Disc-gel electrophoresis.

The protein obtained in peak II (Fig. 6.1) was examined by polyacrylamide disc-gel electrophoresis (Davis, 1964) as described in Chapter 2. Small amounts (50μl) of a solution of the protein (1mg/ml) in electrophoresing buffer containing sucrose (10% w/v) were placed on the top of gels.

A small plastic wedge was placed in the sample space at the top of one gel to divide the space into two, in such a way that samples of goose ovalbumin and hen ovalbumin could be placed separately on either side of the gel, without mixing the proteins in each sample. One part of the outside of the glass tube was marked, and the gel was subjected to electrophoresis as described above. Before removal of the gel, a small piece of it was cut away on one side of the partition in order to identify the side of the gel on which goose ovalbumin had been placed.

Samples of goose ovalbumin (1mg/ml) were left overnight at 4°C in the presence of 0.1% (w/v) sodium dodecyl sulphate in running buffer, both in the absence and presence of 0.1% (v/v) mercaptoethanol, and subjected to electrophoresis on gels containing 0.1% (w/v) sodium dodecyl sulphate as described by Marshall & Zamecnik (1969). A sample of reduced and carboxylmethylated goose ovalbumin and samples of chymotrypsinogen, hen ovalbumin, pepsin, ovomucoid (mol. wt. 32,000; Deutsch & Morton, 1961), bovine serum albumin and γ globulin were subjected to the same procedure, in the absence of mercaptoethanol.

Analysis of goose ovalbumin.

Protein that had been dried in the frozen state was left to equilibrate with the atmosphere at room temperature for 24 hr. Portions of this material were weighed out and analysed separately for amino acids, hexosamines, moisture and ash content.

Amino acid analysis.

Small, weighed amounts of protein (2mg) were hydrolysed in 6M-HCl (3ml) at 110°C, and the hydrolysates were dried and analysed as described later in the chapter. Two samples were each hydrolysed for 20 hr, and single samples each for periods 15 hr, 30 hr, 48 hr or 78 hr.

Hexosamine analysis.

Duplicate samples of protein (50mg) were each hydrolysed in 4M-HCl (3ml) at 100°C for 2 hr, 4 hr or 6 hr periods,

and the hydrolysates dried as described later. The residues were taken up in water (3ml) and amounts of 1ml were subjected to a modified Rondle & Morgan (1955) procedure.

Analyses of moisture and ash content.

Weighed portions (20mg) of goose ovalbumin were dried in an Abderhalden pistol in vacuo at 100°C for 48 hr, cooled in vacuo, and reweighed.

Further portions (20mg) were heated in an electric furnace at 460°C for 12 hr, cooled, and the amount of ash remaining was weighed.

The results of amino acid and hexosamine analyses were corrected for moisture and ash content, and calculated as numbers of residues per mole of glycoprotein (47,500 mol. wt.).

Spectrophotometric estimations and hexose analyses were performed on the supernatant of a centrifuged solution of goose ovalbumin (50mg) in water (5ml). A small portion of the supernatant was analysed for amino acids after hydrolysis in 6M-HCl at 110°C for 20 hr, and the tyrosine, tryptophan and hexose contents analysed, as described below, were related to the amount of glycine measured.

Spectroscopic determination of tyrosine and tryptophan.

The ultra-violet spectrum of a small portion of the supernatant, (0.2ml) diluted to 5ml by the addition of 1M-NaOH (1.5ml) and water, was measured in a 1cm path-

length cell with 0.3M-NaOH in the reference cell. The spectrum of supernatant (1ml) diluted to 10ml with water was also measured.

Hexose analysis.

The supernatant (1ml) was diluted to 10ml with water, and portions (1ml) were assayed by the orcinol-sulphuric acid procedure to be described later.

Alkylation of reduced goose ovalbumin.

A portion of the glycoprotein (90mg) in 8M-urea and 0.2% (w/v) EDTA (3ml) was reduced and carboxymethylated as described by Hirs (1967). The carboxymethylated protein was isolated by dialysis against water for 48 hr at +4°C, and dried in the frozen state. Carboxymethyl-L-cysteine was released by hydrolysis of the material (2mg) with 6M-HCl (3ml) at 110°C for 20 hr in the presence of 4'-fluoro-β-DL-phenylalanine and measured on the Locarte autoanalyser.

Paper chromatography of neutral sugars.

Goose ovalbumin (11mg) was hydrolysed in 1M-H_2SO_4 (5ml) at 100°C for 8 hr in vacuo, and the cooled hydrolysate was eluted from Dowex 50-X4 (200-400 mesh; H^+ form; 3g) with water (20ml). The eluate was itself eluted from Dowex 1-X8 (200-400 mesh; acetate form; 3g) with water (40ml). This eluate was dried in the frozen state, and the residue taken up in water (50μl). Small amounts (10μl) were developed on Whatman No. 1 paper (46 x 57cm) with n-butanol — ethanol — water (10 : 1 : 2, by vol)

(Spiro, 1960) for 2 days. Small amounts (100μg) of standard sugars were also chromatographed. Sugars were located by the silver nitrate method of Trevelyan, Proctor & Harrison (1950), and the background destained by an aqueous solution of sodium thiosulphate (15% w/v).

Preparation of glycopeptides from goose ovalbumin.

Proteolytic digestion of goose ovalbumin.

The glycoprotein (13μ moles) was digested with pepsin (15mg) in 0.04M-potassium hydrogen phthalate buffer (30ml; pH 2.8) at 37°C under a layer of toluene for 48 hr. The pH was increased to 7.8 with 2M-NaOH, and 0.125M-tris buffer (15ml; pH 7.8) was added, together with trypsin (7mg) and chymotrypsin (7mg). At the end of another 24 hr, the digest was brought to pH 5, concentrated on the rotary evaporator at 45°C, and extracted three times with 50 volumes of ethanol. The precipitate was taken up in water (3ml).

Gel chromatography of the digested material.

That portion of the digest that was insoluble in ethanol was placed on a column of Sephadex G-50 (fine grade; Pharmacia Ltd.) (56 x 2.6cm) equilibrated with 0.1M-acetic acid. Fractions (2ml) were collected, portions (50μl) of which were assayed with the phenol-sulphuric acid reagent and with ninhydrin. The fractions containing hexose were pooled as shown in Fig. 6.7, and dried in the frozen state to yield 15mg of dried material in Fraction 1

(8.5mg hexose), 26mg in Fraction 2 (5.9mg hexose) and 65mg in Fraction 3 (12mg hexose).

The column was calibrated with small amounts (0.5mg) of raffinose and lacto-N-difucohexose which were initially dissolved together in 0.1M-acetic acid (1ml). Small portions (0.5ml) of the fractions obtained were assayed for ketose by the carbazole-sulphuric acid reaction (Dische & Borenfreund, 1951) and for fucose by Dische & Shettles (1948) procedure (E measured at 430nm and 400nm). The column was also calibrated with hen ovalbumin glycopeptide (5μmoles) and glucose (1mg) dissolved initially in 0.1M-acetic acid (1ml), and fractions were assayed by the phenol-sulphuric procedure. Fraction 3 (Fig. 6.7) was re-chromatographed.

Analysis of isolated glycopeptide material.

Portions (1mg) of the materials obtained from the three fractions (Fig. 6.7) were each assayed by the phenol-H_2SO_4 reagent. Further amounts (1mg) were each hydrolysed in 6M-HCl for 20 hr at 110°C or in 4M-HCl at 100°C for 4 hr, and the hydrolysates were analysed for amino acids and hexosamines.

Paper electrophoresis of glycopeptides.

Small amounts of Fractions 1 (5mg), 2 (3mg) and 3 (3mg) (see Fig. 6.7) were subjected to electrophoresis on Whatman 3MM paper at pH 2.0 for 2 hr.

Preparative paper electrophoresis was performed on Fraction 3 (13mg). The components were eluted from the

paper, and concentrated to 2 ml (Components B & C) or 1ml (Component A) (see Fig. 6.7). Aliquots (100µl) were assayed for hexose, and for amino acids and hexosamine after acid hydrolysis.

Some methods of quantitative and qualitative analysis.

The following methods have been used in various parts of the studies described in this thesis.

High-voltage paper electrophoresis.

Paper electrophoresis was carried out on a cooled-plate apparatus (Atfield & Morris, 1961) on Whatman 3MM paper at a potential gradient of 32V/cm (50mA). One buffer used contained 98% formic acid (12.5ml), glacial acetic acid (43.5ml) made up to 1 l with water (pH 2.0), and the other contained 98% formic acid (28ml) and glacial acetic acid (20ml) made up to 1 l with water (pH 1.85) (Marshall & Neuberger, 1964).

Paper and thin-layer chromatography.

Chromatography was performed at 25°C in tanks into the base of which the developing solvent was generally placed several hours before use.

Descending paper chromatography was carried out on Whatman 3MM or Whatman No. 1 paper in glass tanks (60 x 60 x 23), and ascending chromatography on Whatman No. 1 paper in a tank measuring 20 x 20 x 37cm.

Thin-layer chromatography was carried out on layers

of silica gel G, H, GF_{256} or HF_{256} or microcrystalline cellulose (Merck, Darmstadt). The layers were 0.25mm thick and measured 20 x 10cm or 20 x 20cm. Development was performed in glass tanks (22 x 25 x 8cm).

Several indicators were used to assess the positions of the materials chromatographed, and these included the following:-

1. Ninhydrin/pyridine reagent.

Chromatograms were treated with ninhydrin (1% w/v) in acetone containing pyridine (2% v/v) and were heated at 80°C (Marks, Marshall & Neuberger, 1963).

2. Periodate/permanganate reagent.

Glycopeptides stained brown with this reagent, which is described by Lemieux & Bauer (1954). The chromatograms were washed under the tap once the brown spots had appeared. Approximately 0.4μ moles of glycopeptide could be located, by this reagent, after chromatography.

3. Periodate/2,4-pentane dione reagent.

The reagent is described by Weiss & Smith (1967). About 0.1μ moles of reduced oligosaccharide were detected.

Automated amino-acid analysis.

Samples were hydrolysed at 110°C *in vacuo* for various periods of time, and were dried at about 40°C in a rotary evaporator which contained sodium hydroxide pellets in a separate container in the condenser. The residues were taken up in pH 2.2 citrate (0.2M-Na^+) buffer, and auto-analysed.

Analyses were carried out either on a Technicon or

a Locarte autoanalyser. Ninhydrin-positive components were separated and detected on the Technicon by the buffer gradient and ninhydrin reagent which are described in "Techniques in Amino Acid Analysis" (Technicon Instruments Ltd., 1966), and which are based on the techniques described by Moore & Stein (1954a and b). D-Glucosamine and D-galactosamine were resolved by lowering the pH of the starting buffer from pH 2.875 to pH 2.80. DL-Norleucine was incorporated as an internal standard.

The Locarte autoanalyser is based on the design of Spackman, Moore & Stein (1958). Ninhydrin-positive components were eluted from the resin by stepwise elution of buffers of increasing pH and ionic strength, and were detected by a ninhydrin reagent containing stannous chloride.

The conditions employed for amino acid analysis were those described in the manufacturer's handbook. D-Glucosamine eluted in the same position as DL-norleucine, and 4'-fluoro-β-DL-phenylalanine was generally incorporated as an internal standard. D-Glucosamine and D-galactosamine were resolved from amino acids by the buffer programme described in Table 3.2.

Samples in pH 2.2 (0.2M-Na^+) citrate buffer were loaded onto glass columns (6 x 1cm) of an automatic loading accessory incorporated into the Locarte autoanalyser, and were forced into coils of Teflon tubing. Further quantities of pH 2.2 buffer were packed into these coils behind the samples, and the columns were then filled

with methanolic starting buffer [pH 3.25 or pH 3.15 (0.2M-Na^+) citrate buffer containing 20% (or 30%; see Chapter 3) methanol].

The autoanalysers were calibrated by application of 0.25 μmoles (for the Technicon) or 0.025 μmoles (for the Locarte) of standard amino acids (Beckman Instruments Inc., Palo Alta, California) and D-glucosamine, together with the internal standard.

Quantitative ninhydrin reagent for determination of free amine groups.

The method is described by Rosen (1957). Recrystallised glycine and D-glucosamine were used as standards.

Determination of total nitrogen.

Total nitrogen was measured by a modification, described by Melamed (1965), of the method of Jacobs (1962). The ninhydrin procedure of Rosen (1957) was used to determine the ammonia released. Glycine was also subjected to the digestion procedure, the ammonia released was measured, and a standard curve was constructed from the results.

Determination of total hexose.

The orcinol-sulphuric acid procedure (François, Marshall & Neuberger, 1962), and the phenol-sulphuric acid procedure (Dubois et al., 1956) were employed. The two procedures gave values, which were within 5% of each other, for the hexose content of hen ovalbumin glycopeptide. Orcinol was

purified by heating the monohydrate on a rotary evaporator at reduced pressure at 65°C for 30 min in order to remove the water of crystallisation followed by recrystallisation of the residue from benzene. Analar phenol and conc. sulphuric acid (British Drug Houses) were used. D-Mannose was recrystallised from aqueous ethanol, dried *in vacuo* at 64°C, and employed as a standard for each determination.

Determination of hexosamine.

Samples were hydrolysed in 4M-HCl, which was prepared from constant-boiling HCl by dilution with deionised water, at 100°C *in vacuo* for various periods of time. The hydrolysates were dried at room temperature over NaOH pellets and conc. H_2SO_4 *in vacuo*.

The hexosamine contents of the residues were measured by the procedure of Rondle & Morgan (1955) as modified by Kraan & Muir (1957). 2,4-Pentane dione was redistilled (b.p. 138-140°C) and stored in the deep freeze, and p-dimethylaminobenzaldehyde was purified as described by Adams & Coleman (1948).

Spectrophotometry and polarimetry.

Spectra were recorded on a Unicam SP 700 and monochromatic measurements were made on a Unicam SP 600. Infrared spectra were recorded on a Unicam SP 200 and monochromatic optical rotations were measured on a Perkin-Elmer model 141.

CHAPTER 7.

CONCLUDING REMARKS.

The study of the structure of proteins has proceeded in a number of distinct stages. First came the realisation that these substances are composed of amino acids and it was postulated that the chemical union between these amino acids was mediated through peptide linkages (Hofmeister, 1902). It was found that the number of types of amino acids are relatively restricted. By 1935, when L-threonine was discovered by Rose and his colleagues (McCoy, Meyer & Rose, 1935), the total number of these amino acids was known to be about twenty. Earlier methods for the analysis of the amino acid composition of proteinswere somewhat cumbersome, although considerable data were accumulated by methods involving crystallisation (see, for example, Greenstein & Winitz, 1961). The publication of analytical data for a number of proteins led to the realisation that this information was unlikely to provide any real insight into the way in which proteins function. Analysis of the primary structure of proteins presented insuperable difficulties until the development of chromatographic techniques (Martin & Synge, 1945). These techniques provided the basis for Sanger's attack on the sequence of insulin (Ryle, Sanger, Smith & Kitai 1955)and, with the development of methods for sequencing protein chains, it was realised that each type of protein molecule has an amino acid sequence which is largely unique. Although

Sanger's pioneering work was followed by intensive activity, which resulted in our present knowledge of the amino acid sequences of a large number of proteins (Dayhoff, 1971), there was still no appreciation of the manner in which a protein exerts its particular function.

During this period, Perutz, and later Kendrew*, carried out X-ray crystallographic studies in Sir Laurence Bragg's department at Cambridge on the three-dimensional structure of carboxyhaemoglobin and on myoglobin. The solution to this problem led to an understanding of the nature of the cleft into which the haem prosthetic group fitted. Later studies by Phillips and his colleagues (Blake et al., 1967a) on lysozyme led to the first elucidation of the structure of a protein with proven enzymatic activity. Since that time, the stereochemistry of a number of proteins has been established (Blow & Steitz, 1970), and it is clear that the folding of the protein chain leads, in each example, to a cleft which is believed to be required for the particular activity of the protein. There has been no report of the overall stereochemistry of a glycoprotein up to the present time.

Study of the carbohydrate prosthetic groups of glycoproteins has also demonstrated that relatively few types of monosaccharides are found in this situation. The general structural features of glycoproteins as we

* Perutz (1962)

know them at the present time have been discussed in Chapter 1. A few studies of the primary structures of carbohydrate moieties had been published before the demonstration by Cunningham and his colleagues (Cunningham, 1968) that the carbohydrate entity of hen ovalbumin is heterogeneous. Heterogeneity is a structural feature common to most, if not all, glycoproteins. At the moment it is difficult to ascertain whether the carbohydrate moiety, attached at a given position in a protein chain, varies from one molecule to another solely because there are differences in the numbers of specific sugar molecules attached at non-reducing terminal positions, or whether there is a much more extensive variation in structure involving the more "internal" parts of the prosthetic group. However, the fact that a specific biological activity of a glycoprotein may reside largely in a carbohydrate moiety, implies that there are likely to be highly specific structural features of these prosthetic groups. Until the problem of heterogeneity has been solved, there is little hope of discovering the nature of the total stereochemistry of carbohydrate moieties.

The work in this thesis was aimed mainly at three interrelated problems. It seemed possible that acid-base titration of glycopeptides might yield information about the way in which the code contained in the amino acid sequence -Asn-X-Thr,Ser- (see Chapter 1) is trans-

lated to result in the formation of carbohydrate-protein linkages. It had been suggested earlier that hydrogen-bonding occurs between the oxygen atom of the amide group of the asparaginyl residue and the hydroxyl group of the 3-hydroxyamino acid that lies in the position next-but-one towards the C-terminus of the protein. The results of titration showed that any hydrogen-bonding of this type cannot be observed by this procedure. Further studies on the acid-base titration properties and chiroptical properties of a pure peptide, such as -Asn-Leu-Thr-Ser-, will be of interest, although it is possible that the nature of the specificity of the L-asparaginyl residue that becomes glycosylated will not be solved until investigations on the enzyme responsible for synthesizing the linkage have been completed.

There is a need to obtain partial degradation products from the carbohydrate moieties of glycoproteins by procedures which do not involve a general breakdown of the prosthetic group. Some years ago, Morgan (1958) suggested the use of alkaline conditions to obtain, from blood group substances, degradation products in which L-fucose residues were still present. Application of this method enabled Marr, Donald & Morgan (1968) to elucidate the structures of fragments in which the sugar, which is bound by a glycosidic linkage that is very labile in acid, was present. Conditions of alkali and alkaline borohydride have been widely used in studies on glycoproteins

which contain sugars linked to the 3-hydroxy groups of L-seryl and L-threonyl residues and which are readily degraded by alkali. Other types of carbohydrate-protein linkages with which N-acetyl-D-glucosamine residues are attached to the amide groups of L-asparaginyl residues have been described as "alkali stable" linkages (Marshall & Neuberger, 1972). The term "alkali stable" is, of course, a relative one; this was clear from the earlier studies of Neuberger (1938) and Johansen, Marshall & Neuberger (1961).

Treatment of GlcNAc-Asn with alkali has been shown to result in a complex reaction. Furthermore, treatment of this model compound with sodium borohydride in alkali leads to reactions additional to those of alkaline hydrolysis and reduction of the released sugar. Treatment of hen ovalbumin glycopeptide with solutions containing high concentrations of sodium borohydride (1M) in high concentrations of alkali (0.2M - 2.0M) at 100°C gives the maximum yield of reduced oligosaccharides. These conditions are likely to be widely applicable to studies of the primary structures of carbohydrate moieties and should yield information which is not readily obtainable by the use of other techniques. It has been shown in the experiments described that an N-acetyl-D-glucosaminyl residue, attached to an L-asparaginyl residue in hen ovalbumin, does not form a branch point. This finding is in contrast to earlier reports (Montgomery, Lee & Wu, 1965). Furthermore, the N-deacetylation of

N-acetylhexosaminyl residues that occurs under the conditions of alkaline borohydride described above enables disaccharides containing glycosidic linkages, which are stable to acid, to be isolated. This has been demonstrated by the isolation of β-D-glucosaminyl-(1 → 4)-D-glucosaminitol from hen ovalbumin.

The chiroptical and X-ray diffraction studies that have been made on GlcNAc-Asn and related compounds might be extended to other model substances. The change in conformation which GlcNAc-Asn undergoes when it forms a complex with lysozyme suggests that the conformation of this "internal" part of the prosthetic group may vary from one glycoprotein to another. At present we do not know if oligosaccharide moieties attached to L-asparaginyl residues in intact glycoproteins can interact with lysozyme. This aspect requires further study and it may lead to an understanding of, for example, the nature of binding of β-ovomucin to lysozyme (Robinson, 1972; Dam, 1971). This interaction may be implicated in the thinning reaction of egg white.

There are relatively few reports of studies devoted to the stereochemistry of carbohydrate moieties of glycoproteins. This stage of development of the subject is likely to give considerable insight into the way in which these moieties influence the biological activities of some glycoproteins.

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obtained. However, the adsorbed antisera to fraction I agglutinated 'tanned' sheep erythrocytes coated with fraction I or its fractions giving titres [$\log_2$(maximum dilution for observable agglutination)] of 7–10. Antisera to sheep articular cartilage extract gave a very low titre against cells coated with sheep serum; the serum-adsorbed antisera had little reactivity with cells coated with cortical-bone fractions although, with articular-cartilage extract, they gave haemagglutination titres up to 14 and precipitin lines were obtained by double diffusion.

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Some Properties of Embden-Goose Egg-White Albumin

By B. M. Austen, Elzbieta J. Romanowska and R. D. Marshall. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London W.2, U.K.*)

Goose egg-white albumin was prepared by a procedure closely related to that (Warner, 1954) used in the isolation of the similar protein from the hen's egg. Final purification of the monomeric form was achieved by chromatography in 0.1M-sodium chloride on a Sephadex G-100 column. The protein exhibits three closely running bands on polyacrylamide-gel disc electrophoresis (Davis, 1964). A single band was observed on electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate, and the molecular weight was found to be 47500 by this technique (Marshall & Zamecnik, 1969) as well as by chromatography on Sephadex G-100. The glycoprotein contains 24 alanine, 21 glycine, 30 valine, 30 leucine, 21 isoleucine, 13 proline, 23 phenylalanine, 12 tyrosine, 7 tryptophan, 39 serine, 27 threonine, 8 half-cystine, 19 methionine, 12 arginine, 6 histidine, 23 lysine, 36 aspartic acid and 51 glutamic acid residues as well as 8 glucosamine and 12 mannose moieties/molecule. The main differences from hen egg-white albumin are higher threonine (11 additional residues/molecule), lower alanine (10 residues less/molecule) and greater carbohydrate contents.

An enzymic digest (pepsin at pH 2.8 followed by trypsin and chymotrypsin at pH 7.8) of 660mg of heat-denatured albumin was neutralized (to pH 5.4) and evaporated under reduced pressure to a volume of about 3ml. The portion of the residue that was insoluble in ethanol was chromatographed in 0.1M-acetic acid on a Sephadex G-50 column. Three fractions were obtained: the first (fraction I) (at the void volume of the column) accounted for 22% of the original mannose of the protein, the second (fraction II) accounted for 20% of the mannose and the third (fraction III) accounted for about 50% of the mannose. The mannose/glucosamine molar ratios decreased in the order fraction I > fraction II > fraction III. Fraction III had a molecular weight, by gel-filtration methods, of about 1900 and contained 1.8mol of glucosamine, 1.7mol of mannose, 0.69mol of threonine, 0.55mol of serine and 0.35mol of glycine/mol of aspartic acid. Smaller amounts of other amino acids were also present.

Goose egg-white albumin therefore contains more than one carbohydrate prosthetic group/molecule. It is reasonable to deduce from this observation, and from the amino acid composition of the whole protein, that the necessary amino acid sequence for the formation of 4-*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparagine (see Marshall & Neuberger, 1970) occurs more frequently in this glycoprotein than it does in hen egg-white albumin.

E.J.R. is a British Council Visitor from the Polish Academy of Sciences, Wroclaw, Poland.

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Cross-links in Protein from Cataractous Lenses

By R. H. Buckingham. (*Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, U.K.*)

Human cataractous lenses removed at operation vary widely both in opacity and depth of colour when examined after extraction. Cataractous lenses with deep-brown or black nuclei contain about 15% of their total protein in a form that is insoluble in 6M-guanidinium chloride–50mM-dithiothreitol, a medium that dissolves the normal lens completely (Pirie, 1968). Cataractous lenses that are lighter in colour, but still with a golden-yellow nuclear core, yield little or no insoluble protein under such conditions. The proteins from these lenses, classed by Pirie (1968) as group III cataracts, have been studied in an attempt to account for the lens pathology and for the insolubility of proteins from darker lenses.

Group III cataractous lenses were decapsulated

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Alkaline Reductive Cleavage of 4-*N*-(2-Acetamido - 2 - deoxy - β - D - glucopyranosyl) - L - asparagine

By B. M. AUSTEN and R. D. MARSHALL. (*Department of Chemical Pathology, St Mary's Hospital Medical School, London W.2, U.K.*)

Treatment of glycoproteins, which contain *O*-glycosidic linkages to serine and threonine residues, with alkaline sodium borohydride was introduced to obtain reduced oligosaccharides (Schiffman, Kabat & Thompson, 1964) and to examine the amino acid residues forming the point of attachment of the carbohydrate prosthetic groups (Tanaka, Bertolini & Pigman, 1964). Present results suggest that similar procedures might be applied to the preparation of reduced oligosaccharides from glycoproteins or glycopeptides that contain 4-*N*-(2-acetamido - 2 - deoxy - β - D - glucopyranosyl) - L - asparagine (I) as a carbohydrate–peptide linking moiety. Studies of the products formed when compound (I) and 4-*N*-(β-D-glucopyranosyl)-L-asparagine (II) are treated with hot aqueous alkali, both in the presence and in the absence of sodium borohydride, have been carried out.

First-order rate constants for the destruction of compounds (I) and (II) in 0.2M-sodium hydroxide at 100°C were 6.44×10^{-3} and $5.97 \times 10^{-3}\,min^{-1}$ respectively. The rate of release of ammonia under these alkaline conditions from compound (I) followed complex kinetics, with an initial lag phase. Approx. 1.5mol of ammonia was produced after a time equivalent to five half-lives for destruction of compound (I). U.v.-absorbing material was formed ($\lambda_{max.}$ 264nm) with a minimum molar extinction coefficient of 1300, this value being based on the concentration of compound (I). L-Aspartic acid was formed from compounds (I) and (II) with first-order rate constants of about 2.8×10^{-3} and $2.6 \times 10^{-3}\,min^{-1}$ respectively. Thus after five half-lives for the reactions in which compounds (I) and (II) are destroyed only about 45% of the theoretical amount of aspartic acid had been released in each case. Free L-aspartic acid was found to be stable under the conditions employed, both in the presence and the absence of free *N*-acetyl-D-glucosamine.

In 0.05M-sodium hydroxide at 100°C the first-order rate constants for the destruction of compound (I) and for the formation of aspartic acid from it were 3.1×10^{-3} and $0.87 \times 10^{-3}\,min^{-1}$ respectively. These data and those obtained in 0.2M-sodium hydroxide may suggest that the reaction whereby compound (I) is destroyed without formation of aspartic acid is intramolecular. *N*-Deacetylated compound (I) is unlikely to be the major product in which the aspartic acid that is not released is incorporated, since acid hydrolysis of the alkali-treated material does not release aspartic acid except from undegraded compound (I).

Compound (I) was destroyed at rates of $21 \times 10^{-3}\,min^{-1}$ and $11 \times 10^{-3}\,min^{-1}$ at 100°C in 0.2M- and 0.05M-sodium hydroxide respectively in the presence of 1M-sodium borohydride. Approx. 60 and 50% of compound (I) respectively were degraded to give aspartic acid with, in each case, similar amounts of reduced sugars. Substances with the chromatographic mobilities of asparagine and of homoserine are formed in the alkaline reductive cleavage of compound (I). The identities of these amino acids were confirmed from thin layer chromatographic studies of their dansyl derivatives.

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PRELIMINARY NOTE

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Optical rotatory dispersion of 1-N-β-L-aspartyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine

Optical rotatory dispersions (ORD) of a number of glycoproteins, which contain 1-*N*-β-L-aspartyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn) as carbohydrate–peptide linking moieties, have been measured. Some of these studies involved glycoproteins with a relatively large number of such linkages, and as examples we may consider orosomucoid[1], the haptoglobins[2] and fetuin[3]. Although oligosaccharides which contain β-linked *N*-acetyl amino sugar residues are known to exhibit Cotton effects with negative extrema at 220 nm[4,5], probably caused by a weak n–π^* transition in the acetamido group, we do not know the extent to which the β-L-aspartyl group at *N*1 in GlcNAc–Asn may contribute to the optical rotatory dispersion of a glycoprotein or glycopeptide. We have therefore measured the ORD curves of GlcNAc–Asn and of 1-*N*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc–NHAc), and compared them with those obtained with structurally related compounds. Measurements were made with both Bendix–Ericcsen Polarmatic 62 and Cary 60 spectropolarimeters, and we are grateful to Dr. A. R. Rosen and to Dr. P. M. Scopes for the use of these respective instruments.

The ORD curves for *N*-acetyl methyl α- and β-D-glucosaminides (Fig. 1) are

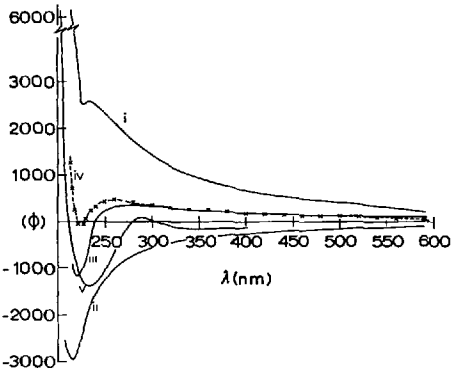

Fig. 1. ORD curves of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (indicated as i), methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (ii), α- and β-anomers of methyl 2-acetamido-2-deoxy-D-glucopyranosides in a molar ratio of 0.49:0.51 (iii), mutarotated *N*-acetyl-D-glucosamine (iv) 2-acetamido-2-deoxy-β-D-glucopyranosylazide (v).

in agreement with those described earlier[4,5]. The resultant curve for a solution containing these α- and β-glycosides in a molar ratio of 0.49:0.51 (Curve iii, Fig. 1) almost coincides with that obtained with a solution of mutarotated *N*-acetyl-D-

Abbreviation: ORD, optical rotatory dispersion; GlcNAc–Asn, 1-*N*-β-L-aspartyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine; GlcNAc–NHAc, 1-*N*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosylamine.

glucosamine (iv) except in the region of the Cotton effects. This might suggest that the anomers of *N*-acetyl-D-glucosamine at equilibrium in water are present in similar proportions to the above values. The calculated $[\Phi]_D$ values for pure *N*-acetyl α- and β-D-glucosamines may be calculated to be $+182°$ and $+10°$, respectively, with the use of HUDSON's[6] rules and accepting $A = \pm 86$ (ref. 6) and $B = 96$ (ref. 7). The measured $[\Phi]_D$ value for *N*-acetyl-D-glucosamine at equilibrium in water is $+87.5°$, and these values therefore support the suggestion made above, assuming a simple equilibrium between pyranose anomers. It might be noted that while the measured value of $[\Phi]_D$ for *N*-acetyl-α-D-glucosamine is $+181°$ (refs. 8, 9), that reported for the β-anomer is $-49°$, a value which was calculated from measurements made on a 0.054% solution in water[8]. In deuterium oxide, the anomers of *N*-acetyl-D-glucosamine are estimated from NMR measurements to be present in the ratios of 0.68 part of α- to 0.32 part of β-form[10].

N-Acetyl-D-glucosamine in the crystalline state[9] and in its complex with lysozyme[11] exhibits a structure such that carbon atoms 6, 5 and 2 as well as all the atoms of the acetamido groups are almost co-planar, with the carbon oxygen *cis* to the proton at ring carbon atom 2. Our ORD data may suggest that the anomers of *N*-acetyl-D-glucosamine and the *N*-acetyl-methyl-D-glucosaminides in solution are all structurally very similar with respect to the acetamido substituent. Application of the octant rule to these glycosides[4] leads to the deduction that the acetamido group is orientated in a direction similar to that described above for *N*-acetyl-D-glucosamine in the solid state.

2-Acetamido-2-deoxy-β-D-glucopyranosylazide has a molar ultraviolet absorption maximum of 36.2 at 272 nm. This is attributable to an n–π^* transition in the organic azido group for which closely similar values were found from studies with other compounds[12–14]. The absorption in the ultraviolet of this chromophore in a β-anomeric configuration is associated with a positive Cotton effect, with a first extremum at 288 nm (Curve v, Fig. 1). This might be expected in general terms from an application of the octant rule[15], although interpretation of the curve is complicated by the presence of the chromophoric acetamido group at carbon atom 2. β-D-Glucopyranosylazide also exhibits a positive Cotton effect at about the same wavelength[16].

Both GlcNAc–Asn and GlcNAc–NHAc also exhibit positive Cotton effects with

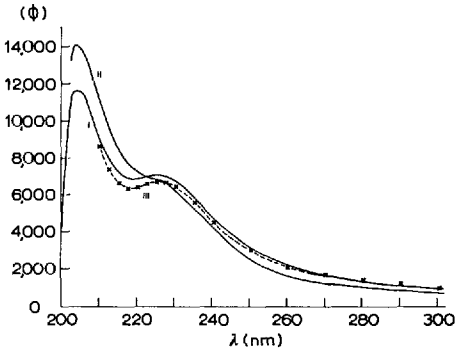

Fig. 2. ORD curves of GlcNAc–Asn (i), GlcNAc–NHAc (ii). The difference curve of $[\Phi]$ values for GlcNAc–Asn *minus* that of L-asparagine is indicated as Curve iii.

the first extremum in each case occurring at a wavelength of 227 nm (Fig. 2) and a second occurring at 204 nm. The contribution by L-asparagine to the ORD curve of GlcNAc–Asn is small as is indicated by the difference curve (iii, Fig. 2). The magnitude of the Cotton effect of the two *N*-acyl glycosides suggests that the 1-acylamido moiety, like the 2-acetamido group, adopts a preferred orientation with respect to its neighbouring atoms. The azido, acetamido, and β-L-aspartamido chromophores that we have examined appear to give rise to positive Cotton effects when they are present in the β-anomeric position. These observations are of value in comparisons of the configurations of compounds of this type, and are likely to to be useful in the identification of carbohydrate–peptide bonds occurring in glycopeptides.

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