PROBLEMS IN THE ANALYSIS OF THE CARBOHYDRATE MOIETY OF GLYCOPROTEINS

PROBLEMS IN THE ANALYSIS OF THE CARBOHYDRATE MOIETY OF GLYCOPROTEINS

A thesis submitted by

EDWIN RUPERT BRUCE GRAHAM

in partial fulfilment of the requirements

for the Degree of

Doctor of Philosophy

in the

University of London.

May, 1966

St. Mary's Hospital Medical School,

London, W.2.

ABSTRACT

Available data on the general structure of glycoproteins was reviewed and a subdivision into serum-type and epithelial-type glycoproteins was suggested on the basis of the polypeptide content per carbohydrate prosthetic group, the predominant type of linkage between carbohydrate and protein and the type of sugar residue involved in the linkage.

The principle of isotope dilution, as used previously for the estimation of mannose in egg albumin, was applied on a greatly reduced scale to the estimation of fucose, mannose and galactose in glycoproteins. The method obviates the lack of specificity of colorimetric methods applied to the intact glycoprotein and allows the use of severe conditions of acid hydrolysis for the release of sugars without regard to destruction by acid of the released sugars. From model experiments it appeared that in some situations the result obtained may be higher than the true sugar content and means to avoid this difficulty were suggested. The released sugars were well separated by Celite chromatography which was also found to be useful for the resolution of anomeric glycosides. The release of mannose from egg albumin in 2 N HCl at 100° after various times was determined by the radioisotope dilution method and found to have a half-time of 7 minutes. The destruction of mannose in 2 N HCl at 100° in the absence of air was found to be enhanced to a much greater extent by free tryptophan than by the same amount of tryptophan in a protein. Addition of ribose reduced the destructive effect of tryptophan.

A radioisotope dilution method was applied to the estimation of glucosamine and galactosamine in some glycoproteins after hydrolysis under strongly acidic conditions which gave a quantitative yield of glucosamine from methyl N-acetyl-D-glucosaminide. The hexosamines were isolated and estimated as their naphthylisothiocyanate derivatives.

ACKNOWLEDGEMENTS

I am grateful to Professor A. Neuberger, C.B.E, F.R.S. for his encouragement and for the ideas upon which this work was based. I wish to thank other members of this department for their help and in particular Dr. R.D. Marshall for many useful discussions.

I am indebted to Dr. J.E. Scott of the Canadian Red Cross Memorial Hospital, Taplow and Dr. A. Barrett, Strangeways Research Laboratory, Cambridge for unpublished details of modifications to the arylisothiocyanate method for hexosamine estimation, to Professor W.T.J. Morgan, F.R.S., of the Lister Institute, London for samples of blood-group specific substances and to Miss E.M. Press of the Wright-Fleming Institute, London for a sample of rabbit γ-globulin.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

I INTRODUCTION

1. General Structure of Glycoproteins

(a) The occurrence of carbohydrate in proteins

It has long been known that carbohydrate is intimately associated with protein in many biological materials. A century ago Eichwald (1865) showed that on acid hydrolysis of mucins from various organs, a reducing substance, then assumed to be glucose, was released. Later some purified serum and milk protein fractions were found to give a positive furfural reaction after treatment with concentrated sulphuric acid (Udranszky 1888).

Crystalline derivatives of D-glucosamine were isolated and identified from the hydrolysis products of various purified proteins such as egg albumin (Seeman 1898, Langstein 1900), bronchial mucin (Müller 1901) and seromucoid (Zanetti 1903). Much later D-mannose was isolated and identified as the phenylhydrazone from hydrolysates of egg albumin (Fränkel and Jellinek 1927) and from blood protein fractions (Bierry 1929, Dische 1928, Rimington 1929). D-Galactose, isolated as the methylphenylhydrazone, was also found to be a component of the latter (Bierry 1929). Sialic acid was first isolated in crystalline state from submaxillary gland glycoproteins of various species (Blix 1936, Blix et al 1956), and found to be widely distributed in the mammalian body (see Gottschalk 1960). It is present in all vertebrates tested so far and in some invertebrates; the lowest member in the phylogenetic scale, excluding bacteria, to contain sialic acid is the primitive platyhelminthe, Polychoerus carmelensis (Warren 1963). L-Fucose was recognized as a constituent of blood group A substance by the isolation of 2,3,4 trimethyl-α-methyl-L-fucoside from the

methylated substance (Bray et al 1946).

That carbohydrate is attached to protein by chemical bonds has been shown for many glycoproteins. In purified submaxillary mucin Hammarsten (1888) found the carbohydrate to be firmly bound to the protein. In serum globulins the carbohydrate was considered to be integral with the protein since it could only be released by strong acid hydrolysis (Hewitt 1934). Crystalline egg albumin, containing only about 3% carbohydrate, did not lose any sugars despite repeated recrystallizations (Sorensen 1934, Neuberger 1938, Hewitt 1938) or ultrafiltration of native and denatured material (Neuberger 1938).

Investigation of the carbohydrate unit (or units) of glycoproteins, by preliminary digestion with proteolytic enzymes was begun with seromucoid (Rimington 1929) and egg albumin (Neuberger 1938), both of which gave rise to polysaccharide material. In egg albumin the carbohydrate (i.e. 8 sugar residues) was found to have a molecular weight of about 1500 and thus was accounted for in one unit. A rough estimate of the size of a saccharide isolated from alkali-treated urinary glycoprotein suggested that the carbohydrate occurred as many groups attached to the protein, rather than as one large piece (Gottschalk 1952). In the submaxillary gland glycoproteins of the ox and sheep the carbohydrate was found to be present as the disaccharide, sialyl (2 $\longrightarrow$ 6) N-acetylgalactosamine, many hundreds of which are attached along the protein chain (Gottschalk and Graham 1959, Graham and Gottschalk 1960). By analysis and molecular weight estimation of glycopeptides isolated from enzymic digests the carbohydrate, in many glycoproteins has been shown to exist in several units, of molecular weight up to about 4,000 (see Table 1).

Since many more methods have recently become available for the isolation and purification of carbohydrate-protein substances (such as low temperature ethanol fractionation, ion-exchange chromatography, gel filtration and column electrophoresis) and for testing the homogeneity of preparations of macromolecules (for instance gel electrophoresis, immunoelectrophoresis, amino acid terminal analysis, etc.,) the presence of carbohydrate-protein complexes, in almost all mammalian tissues and fluids investigated, has been established.

(b) Classification and terminology of carbohydrate-protein complexes in animals

Until recently general agreement on terminology and classification of carbohydrate-protein complexes has been lacking: in 1958 it was stated "there is no generally accepted definition of a mucopolysaccharide" (Morgan 1958). Many classifications and definitions have been proposed, for example by Levene (1925), Meyer (1945, 1953), Stacey (1946), Blix (1951), Masamune (1955), Kent and Whitehouse (1955), Montreuil (1957), Winzler (1958), Bettelheim-Jevons (1958), Schultze (1958), Jeanloz (1960), Gottschalk (1962) and Schultze (1963), and the same names have been used to describe different substances. The feature common to most classifications is the distinction made between the "mucopolysaccharides" and the "glycoproteins".

The mucopolysaccharides are polysaccharides which contain hexosamine and have a repeating unit: examples are chitin, hyaluronic acid and keratan sulphate. These substances have recently been given the generic name of "glycosaminoglycans" (Balazs and Jeanloz 1965). Of these the polysaccharides which contain uronic acid are called "glycosaminoglyc-

uronans" e.g. heparin hyaluronic acid, dermatan sulphate and chondroitin 4- and 6- sulphates. Most, if not all, of the glycosaminoglycans seem to be bound by covalent bonds to polypeptide in their natural state, since extraction by mild procedures at low temperatures, avoiding the use of agents such as alkali and proteolytic enzymes, produces material which contains some protein (see Muir 1964).

Glycoprotein was a term used originally to denote "compounds of the protein molecule with a substance or substances containing a carbohydrate group other than nucleic acid" (Committee on Protein Nomenclature 1908), a definition which covers all carbohydrate-protein complexes. Current usage has excluded the glycosaminoglycans and greater knowledge about the general structure of carbohydrate-protein complexes has led to a better description of glycoproteins "as conjugated proteins containing as prosthetic group one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain" (Gottschalk 1962, 1966). Implicit in this definition is that removal of all peptide material from a glycoprotein would result in saccharides of low molecular weight (up to 4000). Conversely, removal of all carbohydrate from a glycoprotein would give a protein.

(c) Description of glycoproteins

The glycoproteins, as defined above, cover a great range of natural polymers whch can be widely different in physico-chemical properties, molecular weight, carbohydrate content, composition, distribution, biological activity and even in the type of linkage between carbohydrate and protein. For example, egg albumin behaves physically

as a protein, it has a molecular weight of 45,000 and its carbohydrate content of 3.2% consists of mannose and N-acetylglucosamine only. The carbohydrate occurs as a single unit attached to the protein by a relatively alkali-stable N-glycosyl-amide linkage which involves C_1 of an N-acetylglucosamine residue and the nitrogen of an asparagine residue (see Neuberger and Marshall 1966). By contrast, the glycoprotein from sheep submaxillary gland gives viscous solutions, is precipitated by acetic acid and it has a particle weight of about 10^6. It contains 40% carbohydrate made up of N-acetylgalactosamine and sialic acid in the form of a disaccharide, many hundreds of which are joined to the protein in a very alkali-labile glycosidic linkage between C_1 of N-acetylgalactosamine and the hydroxyl groups of the hydroxyamino acid (see Pigman and Gottschalk 1966, and Gottschalk and Graham 1966).

These two glycoproteins can be taken as extreme forms of the general structure of many glycoproteins which is now apparent. The heterosaccharides are each joined to the peptide chain by a single covalent bond which involves the reducing terminal of the carbohydrate and the functional group of an amino acid side chain. It appears that more than one type of heterosaccharide prosthetic group may be present on the same peptide chain, for example thyroglobulin (Spiro 1963) and bovine submaxillary gland glycoprotein (Tsuiki et al 1961). The heterosaccharides, which have been studied up till now and which contain more than two sugar residues, appear to be branched e.g. egg albumin (Clamp and Hough, 1963), α_1-acid glycoprotein (Eylar and Jeanloz 1962), fetuin (Spiro 1964), γ-globulin (Clamp and Putman 1964), ovomucoid (Bragg and Hough, 1961), and the blood-group substances

(Painter et al 1965) in contrast to the linear chain of the glycosaminoglycans. Although there are some sugar sequences which occur more than once within a heterosaccharide, such as sialyl-galactosyl-N-acetylglucosaminyl- in for example α_1-acid glycoprotein (Eylar and Jeanloz 1962) and fetuin (Spiro 1962), no simple repeating unit is evident as in the glycosaminoglycans. When present both sialic acid and fucose occupy non-reducing terminal positions in the molecule.

The position of the carbohydrate prosthetic groups in the protein chain is unknown for all glycoproteins except for ribonuclease B where the asparagine residue at position 34 is involved in the attachment of the carbohydrate (Plummer and Hirs 1964). The blood-group substance polypeptide appears to be fairly resistant to the relatively specific proteolytic enzymes such as trypsin and chymotrypsin (Morgan 1963) suggesting that the proximity of carbohydrate groups sterically inhibits the approach of proteolytic enzymes to the polypeptide (Watkins 1966). It has been shown for α_1-acid glycoprotein (Yamashina 1956) and for ovine submaxillary gland glycoprotein (Gottschalk and Fazekas de St. Groth 1960) that removal of sialic acid increases the susceptibility to proteolytic enzymes. In the latter glycoprotein the numerous disaccharides appear to be fairly uniformly distributed. In γ-globulin most of the carbohydrate is associated with two of the four polypeptide chains - the H chains (Press and Porter, 1966).

The blood-group substances having a high carbohydrate content have been referred to as "mucopolysaccharides" (Stacey and Barker, 1962, Brimacombe and Webber 1964) and also as "glycoproteins" (Gottschalk 1966). If, as Morgan (1963) has suggested that in these materials "relatively

short carbohydrate chains are attached at intervals as branches to a common peptide backbone", the blood-group substances would be included in the above definition of glycoproteins. Additional support for their inclusion is as follows:

1. All the sugars apart from mannose which commonly occur in glycoproteins are found in the blood-group substances.

2. Some of the blood-group substances show inhibitory activity against the influenza virus haemagglutinin (Pusztai and Morgan 1961a), a property shown by many glycoproteins.

3. Other substances in epithelial secretions have similarities in carbohydrate and amino acid composition to the blood-group substances e.g. isolated components of cervical and bronchial mucus (Gibbons and Roberts 1963) and some of the animal submaxillary and sublingual gland glycoproteins (Hashimoto and Pigman 1962).

4. The bovine submaxillary gland glycoprotein has been shown to possess blood-group activity (Horowitz et al 1963).

(d) Some features of glycoproteins

Since the term "glycoprotein" embraces so many natural substances from different sources and since there are such large differences between some glycoproteins it may be useful to generalise and sub-classify, at least roughly, these substances, even though rigid subdivisions may not be possible. Various criteria have been used by different authors. (i) The acid nature of the substance has been used in classifications such as those of Meyer (1953) and Blix (1954). This does not appear to be a useful basis for subdivision of glycoproteins since many acid groups may be present eg sialic acid as in many glycoproteins (Gottschalk 1960),

phosphate as in egg albumin (Perlman 1955), sulphate as in sheep colonic mucin (Kent and Marsden 1963), and a high dicarboxylic amino acid content without much amide present as in ovomucoid (see Melamed 1966).

(ii) The relative amounts of carbohydrate and protein, as a single criterion, is unlikely to be satisfactory, as the carbohydrate content of glycoproteins ranges from 3.2% for egg albumin (Johansen et al 1961), 10% for human thyroglobulin (Spiro 1964), 20% for fetuin (Spiro 1960), 40% for α_1-acid glycoprotein (Eylar and Jeanloz 1962), 60% for porcine submaxillary glycoprotein (Hashimoto et al 1964) and up to 85% for blood-group substances (Pusztai and Morgan 1961b). Although the α_1-acid glycoprotein of serum and the submaxillary gland glycoprotein of sheep have a similar carbohydrate content about 40%, the two glycoproteins have quite different physical properties, sugar components and molecular weight.

(iii) The types of sugar present. Amino sugars appear to be present in almost all carbohydrate-protein complexes found in animals. There are possible exceptions, for example, collagen contains only galactose and glucose (Blumenfield et al 1963). Uronic acid and sulphate have been used as distinguishing features of the mucopolysaccharides (Stacey 1946, Masamune 1955). However sulphate has been found in glycoproteins from sheep colonic mucin (Kent and Marsden 1963), from corneal stroma (Robert and Dische 1963) and from gastropod liver (Inoue and Egami 1963), all of which contain sialic acid. Uronic acid and sialic acid are not usually found together in the one molecule but they are both present in small amounts in a chondromucoprotein from human cartilage (Anderson

1962). Sialic acid-containing mucins (sialo mucins) and fucose-containing mucins (fucomucins) were suggested as divisions (Blix 1951, Odin 1958) but although these sugars do not seem to occur in great amount together, they are commonly present in the same molecule, e.g. blood group Le^a substance (Pusztai and Morgan 1961a) and α_1-acid glycoprotein (Eylar and Jeanloz 1962). Dische (1963) has pointed out that in a group of glycoproteins from the same source, such as serum or urinary glycoproteins, the sialic acid and fucose contents, when related to either the hexose or hexosamine contents, bear a reciprocal relation to each other, in that when the fucose ratio is rising in a series the sialic acid ratio is falling.

However the carbohydrate compositions of some groups of glycoproteins are sufficiently different to be characteristic of the group. Glycoproteins from serum contain the sugars L-fucose, N-acetylneuraminic acid, D-galactose, D-mannose and D-glucosamine, e.g. α_1-acid glycoprotein (Eylar and Jeanloz 1962), and γ-globulin (Rothfus and Smith 1961). D-Galactosamine is present to a small extent in fetuin (Spiro 1960, Graham 1961) but is usually absent from serum proteins.

In the blood-group substances L-fucose, N-acetylneuraminic acid, D-galactose, D-glucosamine and D-galactosamine occur, D-mannose being absent (Morgan 1963). A similar composition exists in the glycoproteins of the submaxillary and sublingual glands of some animals e.g. bovine, canine and porcine submaxillary glands, and bovine sublingual gland (Pigman 1966) and in the bovine cervical glycoproteins (Gibbons and Roberts 1963). The submaxillary gland glycoprotein of sheep has practically only N-acetylneuraminic acid and D-galactosamine as its constituents.

Egg albumin (Johanson et al 1960) and ribonuclease B (Plummer and Hirs 1964a) contain only D-mannose and D-glucosamine; thyrotropin contains these two sugars and much smaller amounts of fucose and galactosamine (Carsten and Pierce 1963). In these three glycoproteins the carbohydrate is present as one unit.

(iv) The type of linkage between carbohydrate and protein. Two types of linkage between carbohydrate and protein have been demonstrated: (1) An N-glycosyl-amide linkage, which involves a bond between C_1 of an N-acetylglucosamine residue and the nitrogen atom of the amide group of an asparagine residue, was unequivocally demonstrated in egg albumin by identification of synthetic 1-(L-β-aspartamido-)-2-acetamido-1,2-dideoxy-D-glucose (see Fig 1) with a compound isolated by acid hydrolysis from egg albumin glycopeptide (Marks et al 1963, Yamashina et al 1963, Bogdanov et al 1964, Tsukamoto et al 1964, Marshall and Neuberger 1964). Various pieces of evidence suggest that the same linkage probably occurs in ovomucoid (Montgomery and Wu 1963), in α_1-acid glycoprotein (Eylar 1962, Satake et al 1965), transferrin (Jamieson 1965a), γ-globulin (Rothfus and Smith 1963), fetuin (Spiro 1963), thyroglobulin (Spiro 1963), and interstitial cell-stimulating hormone (Papkoff 1963). The N-glycosyl amide linkage is more stable to alkaline treatment than acetamide (Marks et al 1963). N-Acetylglucosamine is the only sugar so far found in this type of linkage.

(2) An O-glycosidic linkage, involving C_1 of a sugar and the hydroxyl group of a serine or threonine residue, (see Fig. 2) has been found in bovine and ovine submaxillary gland glycoprotein (Hashimoto et al 1963, Anderson et al 1964, Bhavanandan et al 1964, Tanaka et al 1964, Harbon

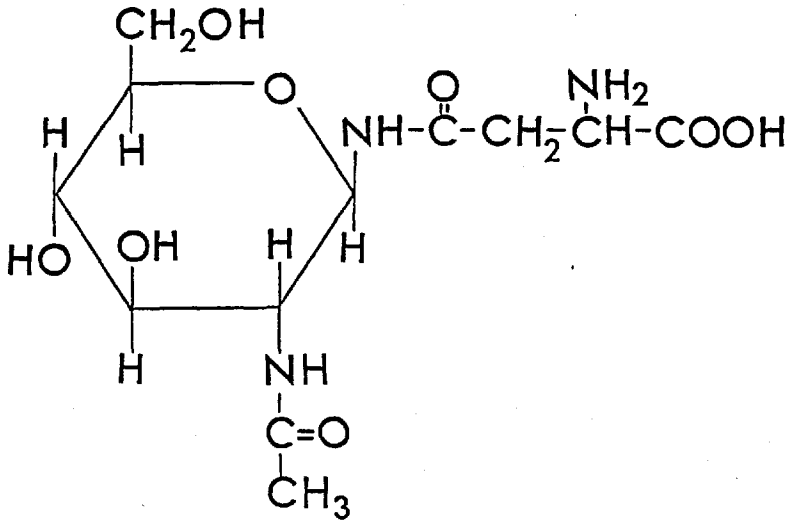


Fig. 1. 2-Acetamido-1-β-(L-β-aspartamido-)-1,2-dideoxy-D-glucopyranose, the compound which incorporates the linkage between carbohydrate and protein in egg albumin and probably many other serum-type glycoproteins.

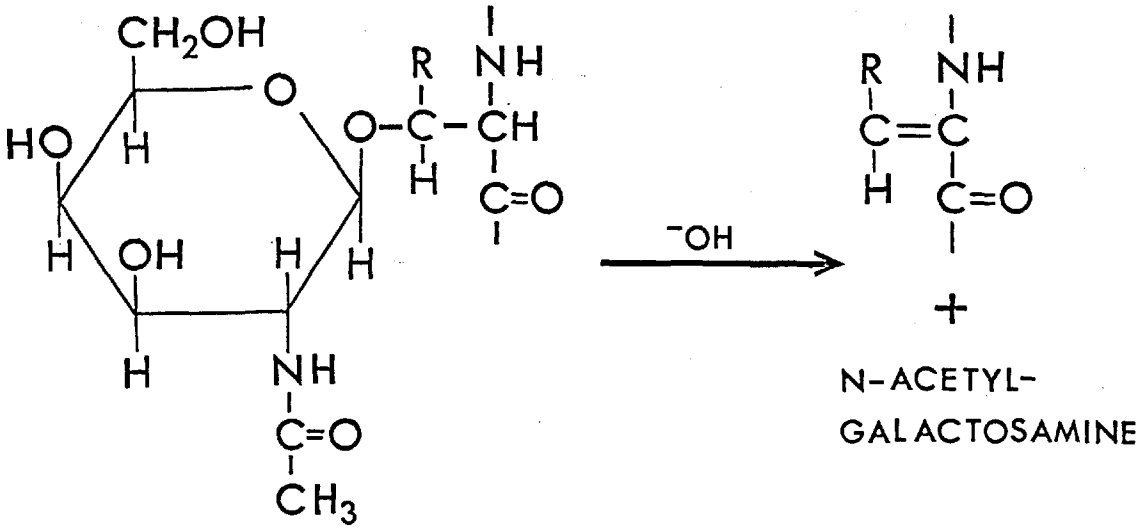


Fig. 2. The linkage of 2-acetamido-2-deoxy-β-D-galactopyranose to the hydroxyl group of a serine (R = H) or a threonine (R = CH_3) residue in epithelial glycoproteins, and its breakdown by β-elimination in the presence of alkali.

et al 1964)and in blood group substances (Anderson et al 1964, Adams 1965b). The sugar found up till now in this linkage is N-acetylgalactosamine (submaxillary gland glycoproteins and probably the blood group substances). The same type of linkage has been found in the glycosaminoglycans, chondroitin sulphate (Anderson et al 1963), keratosulphate (Anderson et al 1964) and heparin (Lindahl and Roden 1964). Xylose seems to be the sugar glycosidically attached to the hydroxyamino acid residues in these compounds. A characteristic property of this type of linkage seems to be its considerable lability in alkali, its breakdown occurring by a process of β-elimination (see fig. 2). It probably accounts for some of the "easily-split" linkages between carbohydrate and protein referred to as "ionic" or "salt-like" in some classifications (e.g. Meyer 1953, Winzler 1958 and Jeanloz 1960).

Another type of linkage, namely a glycosidic ester involving N-acetylgalactosamine and the ω-carboxyl groups of aspartic and glutamic acid, has been suggested earlier in submaxillary gland glycoproteins of ox and sheep, on the basis of the $LiBH_4$ reduction of the side chain carboxyl groups of the dicarboxylic amino acid residues in the glycoproteins (Gottschalk and Murphy 1961, Murphy and Gottschalk 1961). Release of the carbohydrate groups by alkali and hydroxylamine appeared to be in accord with this conclusion (Graham et al 1963). However, some of the data is explicable in terms of an O-glycosidic linkage to serine and threonine, but since later results, which were obtained using material from older animals, are not in accord with the original work, it has been suggested that some ester linkages may be present in young animals (Best et al 1965). Unequivocal evidence is required before a linkage of the ester-type can be considered to be established.

(v) The amino acid composition of glycoproteins. The amino acid composition of submaxillary and sublingual gland glycoproteins has been compared with those of the cervical glycoproteins and the blood-group substances (Hashimoto and Pigman 1962). An interesting feature is that these glycoproteins are particularly rich in serine and threonine, which together make up 40 to 50% of the amino acid residues. On this basis it was suggested that they might be called "Threoglycoproteins". The proline and alanine contents of all these glycoproteins are also high. A characteristic feature of the submaxillary gland glycoproteins of the species so far investigated (i.e. sheep, ox, dog and pig) is that glycine is the most abundant amino acid present (Hashimoto and Pigman 1962, Hashimoto et al 1964). The aromatic and sulphur-containing amino acids are either absent or present in very low amounts.

(e) A subdivision of glycoproteins

The various properties of glycoproteins, which have been considered above, show quite marked differences between two types of glycoprotein, exemplified on the one hand by the glycoproteins from plasma and on the other by the glycoproteins from epithelial secretions. They are referred to here as serum type and epithelial glycoproteins respectively. As referred to above the latter glycoproteins have often not been classified in the same category as glycoproteins of the serum type (e.g. Schultze 1963) and have been called "mucopolysaccharides" (e.g. Morgan 1963, Gibbons and Roberts 1963) and "mucins" (e.g. Blix 1963, Hashimoto et al 1963) due to their viscous nature, high carbohydrate content and their high content of threonine. The data available on some properties of representatives from the two types of glycoproteins has been outlined in

tables 1 and 2. The serum-type glycoproteins tend to have a lower molecular weight and less carbohydrate than the epithelial glycoproteins, although there are exceptions. However there does appear to be a distinctive difference in the number of heterosaccharide groups per mole and in the density of the carbohydrate units on the protein chain as shown by the polypeptide weight per carbohydrate unit. The heterosaccharide density is very high in the epithelial type glycoproteins, particularly for the blood-group substances, where practically all of the serine and threonine residues, which make up 40 to 50% of the number of amino acid residues present, must be substituted by carbohydrate groups.

On present information the type of linkage between carbohydrate and protein and the amino sugar involved in the linkage appear to be characteristic of each type of glycoprotein. In the glycosaminoglycan-protein complexes, xylose seems to be the sugar which joins the glycosaminoglycan chains to the protein by glycosidic linkage to serine and threonine.

Of interest is the difference in stability to alkali of the two types of carbohydrate-peptide linkage. In glycopeptides from ovine submaxillary gland the O-glycosidic linkages to serine and threonine are split in 0.5 N NaOH at 22° at a rate of about 10^{-3} min^{-1} (Carubelli et al 1965). The N-glycosidic amide linkage in egg albumin glycopeptide is cleaved in 0.2 N NaOH at 100° at a maximum rate of 7.2×10^{-3} min^{-1} (Marks et al 1963). Since the bovine cervical glycoprotein is readily degraded by mild alkali to low molecular weight material which gives a strong colour with Ehrlich reagent (Gibbons and Roberts 1963) it seems likely that the type of linkage involved is an O-glycoside to the hydroxy amino acids. The blood-group substance for which there is

evidence of this type of linkage, also behave similarly in alkali (Aminoff et al 1962).

A summary of the properties characteristic of the two types of glycoproteins is outlined in table 3. The glycosaminoglycan-protein complexes have been included for comparison. Since detailed knowledge about most glycoproteins is still fragmentary there will probably be exceptions in one or more of the criteria shown here and even if further study demonstrates a series of glycoproteins with a gradation in properties between the two types, it does demonstrate the widely differing substances covered by the term "glycoprotein".

Little is known about the mechanism of biosynthesis of glycoproteins such as whether any sugars are attached to any of the amino acid residues before the latter are incorporated in the peptide chain, or whether the sugars are added singly or in heterosaccharide units after the protein has been synthesized. In either case, the mechanism for the attachment of sugar to amino acid residue in the case of the serum-type glycoproteins, which have few of such linkages, is likely to entail considerable specificity, in comparison to the equivalent process in the epithelial glycoproteins, where most of the appropriate amino acid side chains become attached to carbohydrate (see Gottschalk and Neuberger 1966).

TABLE 1. SOME PROPERTIES OF SERUM-TYPE GLYCOPROTEINS

Glycoprotein	Molecular weight x 10^{-3}	Carbohydrate content %	Number of carbohydrate units per mole	Polypeptide weight per carbohydrate unit x 10^{-3}	Type of carbohydrate-protein linkage	Sugar residue in linkage
Egg albumin	45	3.2	1	44	amide (1)	GNAc[1]
Ovomucoid	28	24	3	7	amide[2]	GNAc[3]
α_1-Acid glycoprotein	44	40[4]	5-6	5	amide (1)	GNAc[5,6]
Fetuin	48	22	3	12	amide[7]	GNAc[8]
Transferrin	90	5.2	2	43	amide[9]	
Ceruloplasmin	150	7.9	9-10	14	amide[10]	
Haptoglobin	85	15.8	7	10[11]		
γ-Globulin (IgG)	160	2.7	2[12]	50*	asp.	GNAc[13]
Fibrinogen	330	2.5	7[14]	46	amide[14]	
Urinary glycoprotein monomer	28	26	2[15]	10		
Thyroglobulin	660	9.8	25[7]	24	amide[7]	
Thyrotropin	28	1.2	1[16]	24		
ICSH	16	14	2	7	asp.[17]	
Ribonuclease B	14.7	9	1	14	amide[18]	

(amide (1) - N-acetylglucosamine-asparagine compound was isolated.
(amide - ammonia was released from aspartic acid containing glycopeptides.
(asp. - aspartic acid was the main amino acid of the glycopeptides.

corrected for water of hydrolysis

* the light chains were excluded since they contain very little carbohydrate

1. Neuberger and Marshall 1966b
2. Montgomery and Wu 1963
3. Tanaka 1961
4. Eylar and Jeanloz 1962
5. Eylar 1962
6. Yamashina et al 1965
7. Spiro 1963
8. Spiro 1964
9. Jamieson 1965b
10. Jamieson 1965a
11. Cheftel et al. 1965
12. Clamp and Putnam 1964
13. Rothfus and Smith 1963
14. Mester et al 1963, Cynkin and Haschemeyer 1964
15. Maxfield and Stefanye 1962
16. Carsten and Pierce 1963
17. Papkoff 1966
18. Plummer and Hirs 1964

TABLE 2. SOME PROPERTIES OF EPITHELIAL GLYCOPROTEINS

Glycoprotein	Molecular weight x 10^{-3}	Carbohydrate content %	Number of carbohydrate units per mole	Polypeptide weight per carbohydrate unit x 10^{-3}	Type of carbohydrate-protein linkage	Sugar residue in linkage
Submaxillary gland glycoprotein of sheep	1,000	41	830	0.71	O-glycoside[1]	GalNAc
Submaxillary gland glycoprotein of ox	4,000	57	3,300	0.52	O-glycoside[2,7]	GalNAc
Human colloid breast carcinoma mucin*		56		0.24	O-glycoside[3]	GalNAc
Bovine cervical mucins	4,000	78			labile to alkali[4]	
Blood-group substances	500	85	350	0.21	O-glycoside[6,7]	GalNAc[5,7]

* isolated by digestion with pepsin so the peptide chain was probably fragmented.

1. Bhavanandan et al 1964
2. Tanaka et al 1964
3. Adams 1965a
4. Gibbons 1959
5. Watkins 1966
6. Anderson et al 1964
7. Adams 1965b

TABLE 3. COMPARISON OF THE PROPERTIES OF TWO TYPES OF GLYCOPROTEIN

	Serum-Type	Epithelial	Glycosaminoglycuronan-protein complexes [1]
Main sources	Blood plasma, egg white and tissues	Epithelial secretions	Body fluids and tissues
Viscosity in water	Most are not viscous	Often viscous solutions	Viscous solutions
Molecular weight	Most less than 1×10^5	Greater than 10^5 and of the order of 10^6	$0.3 - 10 \times 10^6$
Carbohydrate content	2% - 40%	40% to 85%	50%
Number of carbohydrate units per mole	1 to 25	300 to 3,000	
Weight of polypeptide per carbohydrate unit	5,000 to 50,000	200 to 700	
Type of linkage	N-glycosyl amide	O-glycosyl	O-glycosyl type [2]
Amino acid involved in linkage	aspartic acid	serine or threonine	serine [2]
Sugar residue involved in linkage	N-acetylglucosamine	N-acetylgalactosamine	xylose [3]
Amino acid composition	Similar to non-conjugated proteins	Rich in serine and threonine, very little S-containing or aromatic amino acids.	

1. Muir, 1964.

2. Chondroitin sulphates: Muir 1958; Anderson et al 1963, 1965; Gregory et al. 1964.
   Heparin: Lindahl et al. 1965.
   Both serine and threonine appear to be involved in the carbohydrate-protein linkage in the glycosaminoglycan, keratosulphate (Anderson et al. 1964).

3. Gregory et al. 1964; Lindahl and Roden 1965.

2. The Estimation of Sugars in Glycoproteins

(a) The sugar components of glycoproteins

Several types of sugars have been found in glycoproteins: the hexoses are represented by D-galactose and D-mannose, the 6-deoxyhexoses by L-fucose, the 2-amino-2-deoxy-hexoses by D-glucosamine and D-galactosamine and also present in many glycoproteins of diverse sources is sialic acid, an acylated 5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid. The sialic acids or acylated neuraminic acids are derived from N-acyl-D-mannosamine which is joined in aldol fashion to pyruvic acid to give the deoxy keto sugar acid with characteristic properties, i.e. its ready release by acid from glycosidic linkage and its lability to acid after being released (see monograph Gottschalk 1960). D-Glucose is occasionally found in glycoproteins e.g. ichthyocol (Blumenfield et al, 1963), glycoprotein from the fat/plasma interface of cows milk (Jackson et al 1962), bovine aorta glycoprotein (Berenson and Fishkin 1962, Radhakrishnamurthy et al 1965), pregnant mare serum gonadotropin (Bourrillon et al 1959), haptoglobin (Cheftal et al 1960) and glycoproteins in normal human urine (Bourrillon et al 1962). L-Rhamnose has been found in several glycoproteins, e.g. pregnant mare serum gonadotropin (Bourrillon et al 1959).

Although the sugars from a number of glycoproteins, notably the blood-group substances, egg white proteins and some of the submaxillary gland glycoproteins, have been completely identified by characterization of crystalline derivatives there are still many glycoproteins in which the component sugars, particularly the non-amino sugars, have been identified by such techniques as paper chromatography which does not

distinguish between the D- or L-forms. D-Mannose has been identified by formation of the phenylhydrazone in egg albumin (Fränkel and Jellinek 1927, Neuberger 1938) in ovomucoid (Chatterjee and Montgomery 1962) and in bovine seromucoid (Inazawa 1951) and as the p-bromophenylhydrazone in egg albumin (Neuberger 1938). D-Galactose has been converted to variously substituted phenylhydrazones after isolation from the blood-group substances (Annison and Morgan 1952a,b, Gibbons and Morgan 1954) from ovomucoid (Chatterjee and Montgomery 1962) and from bovine seromucoid (Inizawa 1951). Further confirmation of the D-isomer has been obtained from the identification of crystalline saccharides such as D-galactosyl-β-(1→4)-N-acetyl-D-glucosamine isolated from the blood-group substances (Kuhn and Kirchenlohr 1954, Côté and Morgan 1956) and from α_1-acid glycoprotein (Eylar and Jeanloz 1962). L-Fucose was identified in blood-group substances as the 2,3,4-trimethyl-L-fucose (Bray et al 1946) and as the diphenylhydrazone (Annison and Morgan 1952).

The amino sugars D-glucosamine and D-galactosamine have been identified as crystalline hydrochlorides after isolation from glycoproteins such as seromucoid (Zanetti 1903), frog spawn mucin (Folkes et al 1950), and submaxillary gland glycoproteins (Blix et al 1952), as crystalline Schiff's base derivatives of variously substituted aryl aldehydes (Neuberger 1938, Jolles and Morgan 1940), and as the dinitrophenyl derivatives from blood group substances (Annison and Morgan 1952a, b). N-Acetyl-D-mannosamine has been obtained by the action of an aldolase on N-acetylneuraminic acid (Comb and Roseman 1960). The D-mannosamine was identified as the hydrochloride and also converted to D-arabinobenzimidazole by ninhydrin oxidation followed by reaction with O-phenylenediamine.

The sialic acids, which differ only in their acyl substituents, have been obtained in a crystalline state from many glycoproteins (see Gottschalk 1960).

The specificity of action of purified enzymes has been employed in the identification of sugars. Neuraminidase has often been used to split off the terminal acylated D-neuraminic acids from glycoproteins (Gottschalk 1960). An α-fucosidase was induced in Klebsiella aerogenes by the α_1-acid glycoprotein (Barker et al 1962, 1963). A purified β-galactosidase from Diplococcus pneumoniae has been used to release D-galactose from α_1-acid glycoprotein (Hughes and Jeanloz 1964a). Similarly an N-acetyl-β-D-glucosaminidase from the same source released N-acetyl-D-glucosamine from an α_1-acid glycoprotein derivative which had been treated with neuraminidase followed by galactosidase (Hughes and Jeanloz 1964b). D-Galactose oxidase (Avigad et al 1962) was able to oxidize D-galactose non-reducing terminal residues in sialic acid-free α_1-acid glycoprotein (Barker et al 1962), and in tropocollagen (Blumenfield et al 1963). D-Glucose oxidase was found to oxidize D-glucose from tropocollagen (Blumenfield et al 1963).

(b) The colorimetric estimation of non-amino sugars in glycoproteins without prior hydrolysis

Many methods, which have been described for the estimation of sugars in polymeric substances, involve heating the substance in concentrated mineral acid to give products such as 2-furfuraldehydes, which produce coloured complexes with certain reagents, for example, phenolic compounds (α-naphthol, orcinol, resourcinol, phenol), nitrogen-containing aromatic compounds (tryptophan, diphenylamine, indole, pyrrole,

carbazole) or thiol compounds (cysteine, thioglycolic acid) (for reviews see Winzler 1955, Dische 1955, 1962 and Ashwell 1957). The colour intensity is compared with that given by solutions of free sugar of known concentration. Although such methods are usually reproducible and easily applied the results may be open to some doubts. It is not certain that sugars in glycosidic linkage will always give the same colour yield as that from free sugars. For instance, a mannan and dextran in the orcinol-sulphuric acid reaction required a longer heating time to give the same colour yield as the monosaccharides (Vasseur 1948). When different sugars are present, as in glycoproteins, the contributions of each sugar to the colour produced has to be assessed (Spik and Montreuil 1964). When the material to be assayed contains a high proportion of protein, amino acids, such as cysteine or tryptophan, may react with sugar derivatives to an extent not insignificant in comparison with that of the reagent (Hormann and Gollwitzer 1962, Marshall 1964). It is difficult to obtain a true "blank" value. To correct for colour produced by the reagents the assay solution can be replaced by water but then non-specific colour given by the protein part is not corrected for. Blanks obtained by omission of the reagent from the sulphuric acid reaction mixture does not allow for the possibility that the substances which otherwise react with the reagent now can react with other substances present, e.g. tryptophan and cysteine.

(c) The acid-catalysed hydrolysis of glycosides in glycoproteins

For the identification or estimation of the individual sugars occurring in a glycoprotein, it is necessary to liberate them from their glycosidic linkages, since practically all the chemical reactions used to

distinguish the monosaccharides makes use of the reactivity of carbon atom 1 of the reducing sugar. Many methods for the determination of sugars are based upon the ability of monosaccharides to reduce compounds in alkaline solution, for example, the reduction of ferricyanide to ferrocyanide (Hagedorn and Jensen 1923), of cupric salts to copper oxide (Somogyi 1937), of hypoiodite to iodide (Willstätter and Schudel 1918) and of 2,3,5-triphenyltetrazolium chloride to triphenyl formazan (Fairbridge et al 1951). In the estimation of glucose by glucose oxidase the glucose must be in free form and in the β-anomeric configuration (see Bentley 1963). Many colorimetric methods, which involve heating of the sugar or its glycoside in strong mineral acid in the presence of a reagent, requires cleavage of the glycosidic linkage prior to the conversion of the sugars to 2-furaldehyde derivatives which, by coupling with the reagent, form coloured complexes. The amino sugars are also determined only when they are free, by such methods as that of Elson and Morgan (1933) for the unsubstituted amino sugars, Morgan and Elson (1934) for the N-acylated amino sugars, and the Aminoff (1961) and Warren (1959) methods for the sialic acids.

However, galactose residues in non-reducing terminal position of a saccharide may be estimated without hydrolysis by galactose oxidase which oxidizes the hydroxyl group at C 6 to an aldehyde group (Avigad et al 1962). The sialic acid content may also be estimated by titration of its carboxyl group. In a glycoprotein the titration results are compared with that obtained when the sialic acid residues have been enzymically removed (Popenoe and Drew 1957, Spiro 1960).

The release of sugars from their glycosides is usually effected by aqueous mineral acid, since alkali is not effective in cleaving acetals

except in certain cases such as the glycosides of phenols, enols and hydroxyl groups which are in β-position to a negative group (Ballou 1954). Also the use of alkali is undesirable since by initial attack on the reducing part of a sugar, alkali brings about extensive degradation and rearrangement of the sugars (Pigman 1957). Although sugars are much more stable generally in acid than in alkali they are slowly degraded in acid conditions necessary for the cleavage of their glycosides. Different types of sugar vary greatly in the ease with which their glycosides are hydrolysed by acid and in their stability to acid, two properties which are related. For example, methoxy sialic acid is completely hydrolysed at pH 3 and 80° in 45 minutes, and in 0.1 N HCl at 100° sialic acid is slowly destroyed since after 1 hour it gives only 60% of the colour in the thiobarbituric reaction (Karkas and Chargaff 1964).

(d) The mechanism of the acid-catalyzed hydrolysis of glycosides

Studies which have been carried out on the acid-catalysed hydrolysis of simple glycosides give some information about the possible situations which may occur in the hydrolysis of the carbohydrate groups in glycoproteins. The mechanism suggested for the reaction of most synthetic glycopyranosides is shown in Fig. 3 (Edward 1955). The first step involves a rapid equilibrium-controlled protonation of the glycosidic oxygen to give the conjugate acid II. In the rate-determining step, the conjugate acid undergoes unimolecular decomposition to the alcohol and a cyclic carbonium ion III, which is thought to be stabilized by conjugation with the ring oxygen atom and to exist in the half chain form IV (Edward 1955). The subsequent rapid addition of water gives glucose and a hydronium ion.

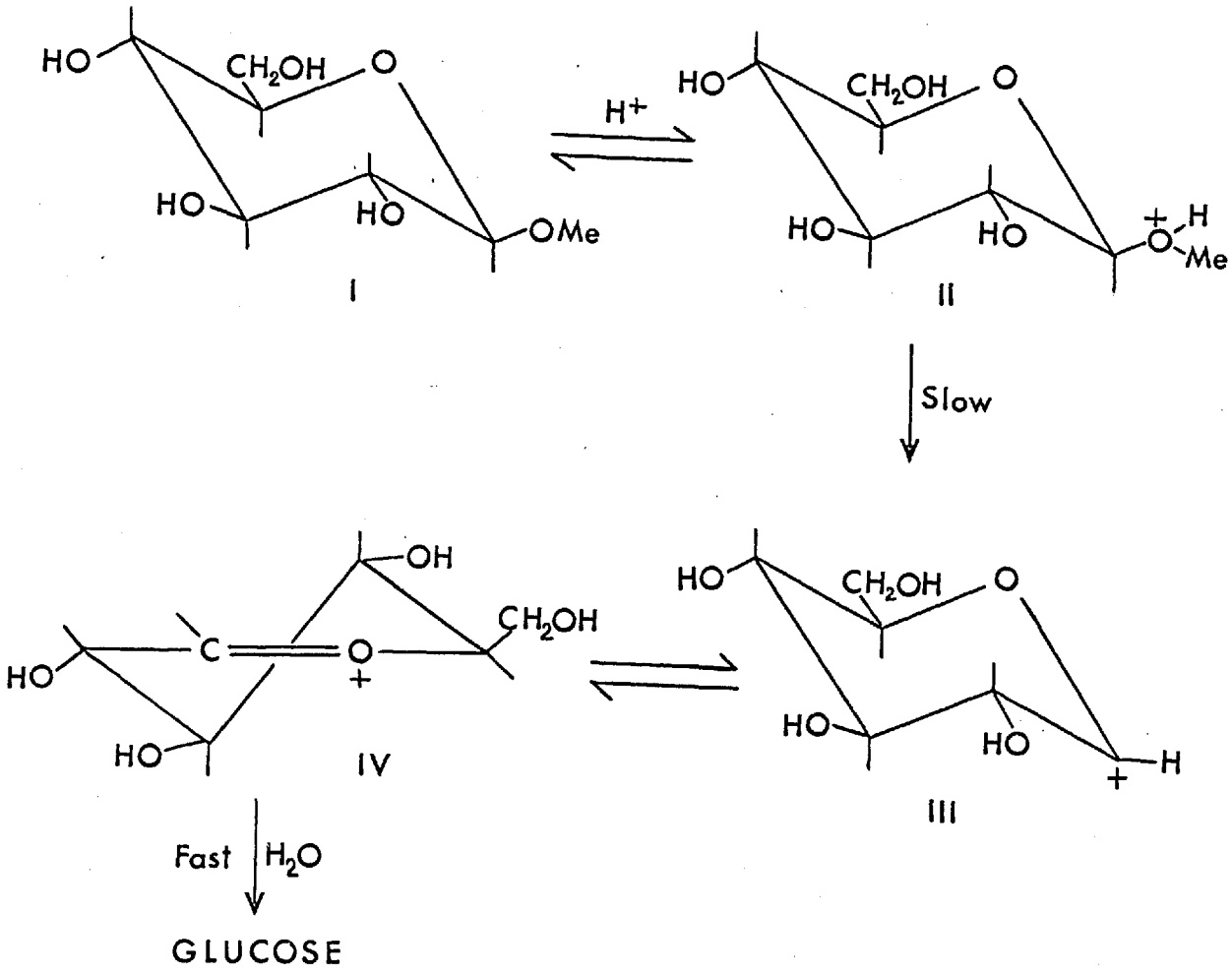


Fig. 3. The mechanism of the acid-catalysed hydrolysis of some glycopyranosides (from Edward 1955).

Evidence for the unimolecularity of the acid hydrolysis of many alkyl- and substituted phenyl-glycopyranosides has been based on linearity of the logarithm of the reaction rate with Hammett's acidity function (Bunton et al 1955, Armour et al 1961, Timell 1964, Semke et al 1964) and on the large positive values found for the entropy of activation (Capon and Overend 1960, Overend et al 1962, Timell 1964, Semke et al 1964) a characteristic feature of unimolecular mechanisms. In the case of a furanoside, ethyl-β-D-galactofuranoside, the entropy of activation was found to be negative suggesting a bimolecular reaction (Overend et al 1962).

Cleavage of the hexose-oxygen bond rather than the aglycone-oxygen bond was shown by isotopic examination of the products of hydrolysis of the methyl and phenyl glucopyranosides carried out in ^{18}O enriched water (Bunton et al 1955). A further indication is the formation of thiophenol and ethanethiol from phenyl and ethyl thioglucoside (Bamford et al 1962). This type of bond rupture occurs similarly in the hydrolysis of acetals (O'Gorman and Lucas 1950). An example of glycoside hydrolysis where alkyl-oxygen fission only has been shown to occur is in the hydrolysis of t-butyl β-D-glucopyranoside (Armour et al 1961) which occurs with great ease (1000 times the rate of methyl β-D-glucopyranoside in M $HClO_4$ at 25°). The different hydrolytic pathway is due to the great stability of the tertiary alkyl carbonium ion. Triethylmethyl β-D-glucopyranoside was hydrolysed at an even greater rate, due to the more stable triethylmethyl carbonium ion and possibly also to the greater release of steric strain on hydrolysis of the glycoside (Timell 1964).

An alternative mechanism of glycoside hydrolysis involving protonation of the ring oxygen, opening of the pyranose ring in a rate-limiting step, and rapid reaction with water to give the products was also considered by Bunton et al (1955). Against this mechanism are the following data (Banks et al 1961). Acid-catalysed methanolysis of phenyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranosides and of phenyl α- and β-D-glucopyranosides has been found to proceed with predominant inversion at C 1 which would not be expected if ring opening occurred. Acid hydrolysis of methyl α-D-glucopyranoside has been associated with an oxygen isotope effect which is explainable only if the glycosidic oxygen is involved in the rate-limiting step.

(e) <u>The effect of substituents of glycosides on the rates of their acid hydrolysis</u>

The main factors which are invoked to explain the widely different rates of acid hydrolysis of different glycosidic linkages may be roughly classified into steric and electronic effects (Shafizideh 1958, Capon and Overend 1960, Reichstein and Weiss 1962, Neuberger and Marshall 1966; Overend et al 1962). Steric effects are mainly concerned with the strain associated with the sugar ring, due to its size, conformation, and to the size and spatial position of groups attached to it. Electronic effects are concerned with the effect of groups in the glycoside on (a) the protonation of the glycosidic oxygen and (b) the cleavage of the bond linking the glycosidic oxygen to the anomeric carbon atom.

As with the cycloalkanes, the 6-membered sugar rings are usually more stable and thus less readily hydrolysed than the 5- and 7-membered rings. For example, the rates of hydrolysis of methyl α-D-galacto-

septanoside and methyl α-D-mannofuranoside is of the order of 100 times greater than their corresponding pyranosides respectively in 0.01 N HCl at 100° (Shafizedeh 1958). The ready hydrolysis of the furanosides has been attributed to the relief of internal strain due to decrease in non-bonded interaction in the furanoside on formation of the carbonium ion (Reichstein and Weiss 1962) in analogy to the reactions of the cyclopentanes (Brown and Borkowski 1952). However the effect of substituents in the ring can also predominate. Highly strained aldopyranoside rings such as methyl 3,6-anhydro-2,4-di-O-methyl-α-D-mannopyranoside can be hydrolysed even more rapidly than the corresponding furanoside (Foster et al 1954). The furanosides and pyranosides of fructose, which are very easily hydrolysed, differ by relatively little in their rates of acid hydrolysis (Heidt and Purves 1944).

The C_1 conformation of the chair form is the most stable arrangement for most of the D-aldohexoses and it was shown that the order of the ease of acid hydrolysis for the methyl D-aldohexopyranosides (idose > altrose > galactose > mannose > glucose) is parallel to that predicted from instability factors arising from the position of functional groups, such as axial substituents and proximity of oxygen atoms (Reeves 1950). The rates of hydrolysis of some methyl glycopyranosides in 2 N HCl at 100° are given in table 4.

The cyclic carbonium ion postulated by Edward (1955) in the mechanism of hydrolysis of glycosides was considered to be stabilised by conjugation with the ring oxygen, and for this to be so C2, C1, O and C5 must lie in one plane making a half-chair structure. The conversion, which will be hindered by opposition of substituents at C2 relative to C3 and C5 relative to C4, would account for the order of

TABLE 4

The Rate of Hydrolysis of Glycosides in 2 N HCl at 100°

Glycoside	$k^* \times 10^2$ min^{-1}	Activation Energy kcal $mole^{-1}$
Methyl α-D-fucopyranoside	210	33.9
Methyl α-D-galactopyranoside	51	34.0
Methyl β-D-galactopyranoside	60	32.3
Methyl α-D-glucopyranoside	10	34.1
Methyl α-D-mannopyranoside	25	31.9
Methyl N-acetyl-α-D-glucosaminide		28.4†
Methyl α-D-glucosaminide	0.1	36.0†

* calculated by Neuberger and Marshall (1966) from the data of Overend et al (1962).

† from Moggridge and Neuberger (1938).

stability: heptopyranosides > hexopyranosides > 6-deoxyhexopyranosides > pentopyranosides, and partly for the lability of the deoxy glucopyranosides (Edward 1955, Overend et al 1962).

The greater ease of hydrolysis of methyl β-D-glycopyranosides compared to the α-anomers has been ascribed to the higher free energy of the β-anomers caused by repulsive interaction between the glycosidic and ring oxygen atoms, a situation which will be removed on protonation (Edward 1955). For more bulky aglycones the steric effect of the α-axial group will predominate.

The electronic effects of the sugar substituents on the rate of hydrolysis will be to affect the protonation of the glycosidic oxygen and the heterolysis of the conjugate acid in the same way (Overend et al 1962).

The great difference between the rates of hydrolysis of variously 2-substituted methyl glucopyranosides has been explained in terms of an inductive effect and correlated with the effect of such groups on the hydrolysis of diethyl acetals (Shafizedeh 1958) and on the dissociation constants of substituted acetic acids (Marshall 1963). The replacement of a hydroxyl group by an amino group on C2 in methyl glucopyranoside is to reduce the rate of hydrolysis by over 100 times (Moggridge and Neuberger 1938). In this case the positively-charged amino group will provide an electrostatic shielding effect against protonation of the glycosidic oxygen. In methyl α-D-glucosaminide, the amino group is closer to the glycosidic oxygen, than is the case with methyl β-D-glucosaminide and therefore the α-compound is less readily hydrolysed (Neuberger and Pitt-Rivers 1939). Acetylation of the amino group gives a rate of acid

hydrolysis comparable to the methyl glucopyranoside. Overend et al (1962) have compared the rates of hydrolysis of the various methyl deoxy-α-D-glucopyranosides with methyl α-D-glucopyranoside. The relative rates for the 2-, 3- and 4-deoxy compounds are 2090, 20 and 40 respectively.

The effects observed with the 3- and 4-deoxy compounds together with a part of the effect in the 2-deoxy compound have been ascribed to a steric effect in the conversion of the chair form of the glycoside to the half-chair form of the carbonium ion. The 4-substituent would have a larger effect since it is in opposition to the more bulky 5 hydroxymethyl group.

The substituent at C5 has a small effect on rate of hydrolysis. For example the rate of hydrolysis of methyl 6-deoxy α-D-glycopyranosides is about 5 times that of the fully hydroxylated glycoside and about the same as that of methyl α-D-xyloside (Overend et al 1962, Timell 1965). Timell (1965) has investigated the effect of many different substituents at C5 on the rate of acid hydrolysis and concluded that neither the inductive effect nor steric effects alone could explain the different rates obtained. Methyl 6-amino-6-deoxy-α-D-glucoside was the most stable to acid of those 6-substituted glucosides tried, being 12 times more stable than methyl α-D-glucoside. The greater stability of the 6-amino compound was attributed at least partly to the electrostatic shielding effect of the ammonium ion. The substitution of a carboxyl group at C5 usually has a stabilizing effect on the glycoside in acid but in some cases it hardly affects, and in other cases even enhances, the rate of hydrolysis (see for example Semke et al 1964, Timell 1965).

The great ease of hydrolysis of the glycosides of sialic acid is predictable from its structure as a 2-deoxy and also a keto sugar. The mechanism of acid hydrolysis was suggested by Neuberger and Marshall (1966) to proceed predominantly through intermediates in which the carboxyl group (pKa 2.6) is ionised, since (a) the uncharged carboxyl group would inhibit protonation of the glycosidic oxygen, (b) protonation of the glycosidic oxygen will reduce the pKa of the carboxyl group and (c) the leaving group will be released more easily from the doubly charged glycoside. This would explain why the ester glycoside is less easily hydrolysed. Karkas and Chargaff (1964) originally suggested the difference was due to an intramolecular catalytic effect involving formation of a bond between the carboxyl hydrogen and the glycosidic oxygen.

The effect of the aglycone on the rate of acid hydrolysis of a glycoside is small for the alkyl and substituted-alkyl compounds (Timell 1964). The phenyl glycosides have a greater rate than the alkyl compounds but substitution of the phenyl group does not have a great effect (Nath and Rydon 1954, Hall et al 1961, Semke et al 1964). The rates of hydrolysis of reducing disaccharides depends on the nature of the glycone and its anomery and to some extent on the particular hydroxyl group substituted in the aglycone residue (Moelwyn-Hughes 1929, Timell 1964).

The rates of hydrolysis of some disaccharides and some polysaccharides, are listed in tables 5 and 6 respectively. In the glucose-containing disaccharides and polysaccharides, the saccharides having $1 \longrightarrow 6$ linkages are hydrolysed at an appreciably lower rate than the

corresponding 1 → 4 linked saccharides. This difference is not observed with the two galactosyl disaccharides shown. The 1 → 3 linked residues of lammarin are hydrolysed at a similar rate to the α-linked polysaccharides. The non-reducing terminal sugar residue seems to be released more rapidly than residues in non-terminal position. The 1 → 4 glycosidic linkage of maltose is hydrolysed 3 times more rapidly than those of sugar residues in non-terminal positions in the polymer chain but maltose may not be an accurate model for the two non-reducing terminal residues bound in a polysaccharide. However the rate of hydrolysis of a polysaccharide increased as the reaction proceeds (Szetjli 1965c).

TABLE 5

Rate of hydrolysis of some disaccharides in 0.5 M sulphuric acid at 80°

Disaccharide		$k \times 10^5$ sec^{-1}
Glucosyl	α(1 → 4) glucose	9.1
Glucosyl	β(1 → 4) glucose	4.0
Glucosyl	β(1 → 6) glucose	2.2
Mannosyl	α(1 → 4) mannose	6.8
Galactosyl	β(1 → 4) glucose	7.8
Galactosyl	β(1 → 6) glucose	8.8

Data from Timell (1964).

TABLE 6

Rate of hydrolysis of some polysaccharides in 1.23 N HCl at 100.8°

Polysaccharide	Sugar and linkate	k x 10^3 sec^{-1}
Starch	glucose α(1→4)	2.4
Dextran	glucose α(1→6)	0.8
Laminarin	glucose β(1→3)	1.8
Alginic acid	uronic* acid β(1→4)	0.4

* mainly mannuronic acid, some guluronic acid also.

Data from Szejtli (1965a,b,c,d).

(f) The release of sugars from glycoproteins by acid

The release of a sugar from a glycoprotein by acid will depend on the type of sugar and the number and nature of its substituents. Of the various types of sugar sialic acid is the most readily released from its glycosidic linkage. As it seems to be almost invariably in the non-reducing terminal position in glycoproteins it can usually be specifically released by very mild acid e.g. for fetuin 0.025 N H_2SO_4, 80° for 1 hour (Spiro 1960) for blood-group Le^a substance pH 2, 37° for 24 h (Pusztai and Morgan 1961a) and for α_1-acid glycoprotein 0.04 N H_2SO_4, at 80° for 1 h (Eylar and Jeanloz 1962a).

L-Fucose, 6-deoxy-L-galactose, is more easily released from its glycosides than galactose and since it occurs in terminal position in glycoproteins its complete hydrolytic release requires fairly mild conditions e.g. for blood-group Le^a substance N acetic acid at 100° for 24 hr (Annison and Morgan 1952) and for α_1-acid glycoprotein N HCl at 100°

for 15 min (Winzler 1958). For fibrocystic intestinal mucoid 0.1 N H_2SO_4 at 80° releases about 30% in 1.5 hr (Johansen 1963). In blood group B substance only 75% of the fucose is split off by N acetic acid at 100° despite prolonged treatment (Gibbons and Morgan 1954). In the trichloroacetic acid-precipitated fraction of serum the optimum conditions for the release of fucose was 0.6 N H_2SO_4 at 100° for 1 h (Gyorky and Houck 1965).

The neutral sugars D-galactose and D-mannose require stronger conditions of hydrolysis as their glycosides are less readily hydrolysed than those of fucose and as they are often substituted by other sugars thus needing 2 glycosidic linkages to be broken for their release. Also the glycosidic linkages within a chain are hydrolysed less rapidly than those of sugar residues in non-reducing terminal position. In graded acid hydrolyses of serum proteins galactose appears before mannose, as its substituents, fucose and sialic acid, are easily split off and since galactosides are cleaved a little more readily than mannosides (Winzler 1958, Graham 1961, Spiro 1962, Eylar and Jeanloz 1962a). Where the hexoses have N-acetylhexosaminyl substituents, any hydrolysis of acetamido groups which occurs prior to hydrolysis of the N-acetylhexosaminides, will result in acid-stable saccharides containing hexosaminidic linkages (Moggridge and Neuberger 1938, Kent and Whitehouse 1955, Gottschalk and Ada 1956 and Foster et al 1957).

A similar situation is also likely to occur when an amino group is adjacent to a glycosidically-substituted hydroxyl group. Such a structure may be formed during the acid hydrolysis of glycoproteins containing 3-O-glycopyranosyl-N-acetyl-hexosamine. Disaccharides of this type have been isolated from, for example, blood group substances (see Morgan 1963). The

amount of hexose not determined due to these causes will depend on the number of hexose residues with N-acetylhexosaminyl substituents and on the relative rates of cleavage of the amide and glycoside. The latter is a consequence of the conditions employed for the hydrolysis. High temperatures and concentrated acid were considered to favour glycoside rather than amide hydrolysis (Johansen et al 1960) but the hexoses are destroyed at a greater rate under more severe conditions. In practice, the conditions employed for the release of hexoses from a glycoprotein, are determined experimentally on the basis of maximum yield. Typical acid concentration and times of hydrolysis is at 100° giving optimum values for hexoses are, α_1-acid glycoprotein 1 N H_2SO_4 for 6 h (Eylar and Jeanloz 1962, Michon and Bourrillon 1961), fetuin 1 N H_2SO_4, 8 h (Spiro 1960), egg albumin 2 N HCL, 1 h (Francois et al 1962), thyroglobulin 2 N H_2SO_4, 6 h (Spiro and Spiro 1965), for blood-group substances 0.5 N HCl, 18 h (Annison and Morgan 1952a, b), and for several glycoproteins 0.25 N H_2SO_4 18 h (Gottschalk and Ada 1956).

The quantitative release of hexosamine from glycoproteins by acid hydrolysis is likely to be prejudiced by the resistance of hexosaminides to cleavage by acid even more acutely than in the case of the hexoses. For example, the hexosamine residues, which are glycosidically linked to the protein through the amide N of asparagine, are probably released at a lower rate than those glycosidically attached to other sugars. For egg albumin glycopeptide, the N-glycosylamide bond in N HCl at 100° is cleaved at a rate of 8.2×10^{-3} min^{-1} (Marks et al 1963) in comparison to a rate of 63×10^{-3} min^{-1} for methyl N-acetyl-α-D-glucosaminide (Johansen et al 1960). Also in some glycoproteins, at least, there occur

two N-acetylhexosamine residues linked together e.g. γ-globulin (Rothfus and Smith 1963).

The hydrolysis of methyl N-acetyl-α-D-glucosaminide was studied by Moggridge and Neuberger (1938) and shown to proceed by the two pathways in fig. 4. To achieve quantitative recovery of glucosamine hydrochloride

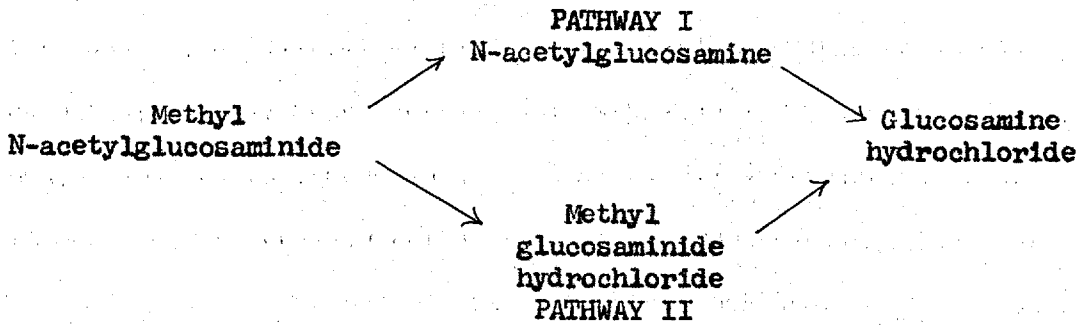


Fig. 4 Alternate pathways for the acid hydrolysis of methyl N-acetylglucosaminide (Moggridge and Neuberger 1938).

the hydrolysis must proceed via pathway I as the acid conditions required for cleavage of the glucosaminide in pathway II will degrade the product to a considerable extent. Predominance of glycoside to amide hydrolysis occurs with increase of both temperature and acid concentration as the activation energy of hydrolysis of α-methyl-N-acetylglucosaminide (36,000 k cal/mole) is greater for hydrolysis of the amide (19,000 k cal/mole) and increase in acid concentration causes a greater increase in the rate of glycoside hydrolysis than of amide hydrolysis (Moggridge and Neuberger 1938, Johansen et al 1960).

The recovery of glucosamine from a series of N-acylglucosaminides after hydrolysis in 1.16 N HCl at 100° varied from about 70% to 100% depending on the particular glycoside tested (Foster et al 1957, François

et al 1962). In the analysis for hexosamine in egg albumin (Johansen et al 1960), ovomucoid (Marshall and Neuberger 1960) and fetuin (Spiro 1962) it has been shown that the optimum acid concentration for hexosamine analysis is 4 N HCl for 3 to 6 h at 100°, lower values being obtained with 2 N HCl. In a hexosamine-containing polysaccharide Smith and Zwartouw (1956) showed increased yields of hexosamine with increased acid concentration.

(g) The destruction of sugars by acid

Different types of sugars are destroyed by acids at different rates. In glycoproteins, sialic acid is the least stable sugar and the hexosamines are the most stable. The stability to acid of the 6-deoxyhexoses and hexoses lie between the two extremes. N-Acetylneuraminic acid, when heated for 1 h in 0.1 N HCl, loses 17% of its colour at 84° and about half at 100° when assayed by the thiobarbituric acid method (Aminoff 1961, Karkas and Chargaff 1964). In 2 N HCl at 100° 23% of mannose is destroyed in 5 hours (Francois et al 1962). In 4 N HCl at 100° 6.6% of glucosamine is degraded in 15 hours (Boas 1953). It is generally considered that sugars, bound in glycosidic linkage, are not destroyed by acid (or alkali) without first rupture of the glycosidic bond. However the carbonium ion, formed as an intermediate in the acid-catalysed hydrolysis of glycosides, may well be more reactive than free sugars in solution (Neuberger and Marshall 1966). Some evidence for this is that acid reversion occurs more readily with glycosides than with free sugars (Overend et al 1962). The reactivity of the carbonium ion may also account for the observation that free sialic acid degraded in N or 2 N HCl gives up 22-25% of its N as NH_3 whereas under the same conditions the sialic acid bound in glycoproteins

yields about 50% of its N as NH_3 (see Graham et al 1963). Consequently one would expect less side reactions if hydrolyses are carried out in dilute solutions.

The effect of the presence of amino acids on the destruction of sugars in hot acid is still largely unknown. The loss of mannose in 2 N HCl at 100° for 5 h amounts to about 23% as measured by recovery of the phenylhydrazone, but if in addition cysteine is present at a concentration of 1 M the loss of mannose is greatly increased to 42% (François et al 1962). The increased acid destruction of some amino acids in the presence of carbohydrate is well known (Martin and Synge 1945) and if this is brought about by direct reaction with the carbohydrate the latter is also likely to be partly destroyed. Tryptophan, cysteine and methione suffer considerable losses when hydrolysed in large amounts of carbohydrate, even in dilute solutions (Schram et al 1953). Under similar conditions the other amino acids are not similarly affected (Dustin et al 1953). Hydrolytic conditions employed for carbohydrate estimation are much milder than those necessary for amino acid analysis so the released sugars will be in the presence of many peptides. If the amino acids react with sugars differently in peptide linkage, corrections for loss of sugar by this process will be difficult to obtain.

Oxygen causes increased destruction of sugars during acid hydrolysis. Glucosamine and galactosamine when heated in 2 N HCl at 100° for 10 h in sealed tubes, showed a loss of 5%. There was no loss if the tubes were flushed with nitrogen prior to selaing (Walborg and Ward 1963). If the acid hydrolysis of proteins is carried out in evacuated sealed tubes clear solutions are obtained (Smith and Stockell 1954). However, tryptophan, which normally contributes to humin formation, is still destroyed.

EXPERIMENTAL

II THE ESTIMATION OF GALACTOSE, MANNOSE AND FUCOSE IN GLYCOPROTEINS BY ISOTOPE DILUTION

1. Introduction

The method of estimation by radio isotope dilution involves addition to the assay mixture of a compound which contains one or more radioactive atoms in its structure and which is identical with the substance to be estimated. The dilution of radioactivity found in the compound after isolation gives a measure of the amount of that compound present. The conditions which must be met are that complete mixing occurs and that there is no isotope effect either during isolation or in any side reactions, such as degradation, that may occur.

The isotope dilution method is not usually any more sensitive than direct methods of assay because, although by addition of the radioactive compound, the amount available for assay is increased some of the compound is required for radio assay. The isotope dilution method is often less accurate, as further possible errors are introduced in the measurements of radioactivity. The method has found many applications(E.g. Broda, 1960).

The amounts of glucose and fructose, present in mixtures of sugars obtained during studies on the Lobry de Bruyn and Alberda van Ekenstein rearrangement, was determined by an isotope dilution method (Sowden and Schaffer 1952). Two components in "hydrol", glucose and gentiobiose, were determined by the dilution of the ^{14}C-labelled sugars added to the mixture (Sowden and Spriggs 1954). The method,which was developed for the estimation of mannose in egg albumin (François et al 1962), involved addition of radioactive mannose to egg albumin before acid hydrolysis so that as

mannose was released from the glycoprotein it mixed with the radioactive mannose. After acid hydrolysis, mannose was isolated as the insoluble mannose phenylhydrazone, which was recrystallized and counted at infinite thickness. From the difference in specific activity of mannose phenylhydrazone before and after hydrolysis the mannose content of egg albumin was calculated to be 2.0 $\pm$ 0.06% which is equivalent to 5 moles of mannose per mole of egg albumin (45,000 g). This result was considered to be close to the true mannose content of the glycoprotein for the following reasons.

1. Careful application of 2 modifications of the orcinol-sulphuric acid method, that of Johansen et al (1960) and Hewitt (1937), to egg albumin showed a mannose content of 1.83 to 2.02% (Johansen et al 1960). The absorption spectrum of the colour produced by egg albumin was identical to that produced from mannose.
2. Other colorimetric methods of assay such as the phenol-sulphuric acid method (Montgomery 1961) gave similar results.
3. Glycopeptide prepared from egg albumin was found to contain 5 moles of mannose per mole of aspartic acid present (Johansen et al 1961).
4. The molecular weight of egg albumin glycopeptide is only compatible with 5 moles of mannose per mole (Kaverzneva and Bogdanov 1962, Lee et al 1964, Fletcher et al 1963).
5. The greatest yield of mannose isolated as the phenylhydrazone from egg albumin was 1.77% which must represent a minimum value (François et al 1962).
6. A consideration of the relative rates of the reactions involved suggested that the amounts of radioactive mannose, preferentially

destroyed before dilution with most of the mannose released from egg albumin, was unlikely to be significant (Francois et al 1962). From the increase in reducing power of egg albumin glycopeptide in 1.5 N HCl at 100° (Neuberger 1938, Nuenke and Cunningham 1961) it was calculated that the half-life of mannose bound in egg albumin in the presence of 2 N HCl at 100° was about 10 min. The rate of destruction of mannose was assessed from the decreasing amounts of mannose phenylhydrazone isolated from hydrolysates of egg albumin after the maximum liberation of mannose had taken place in 2 to 3 h. A value for the half-life of mannose was about 120 min in 2 N HCl at 100°. The ratio of rate of liberation to rate of destruction therefore was about 12. Any preferential destruction of radioactive mannose will give too high an estimate.

The same method was used for the estimation of the mannose remaining after periodate oxidation of egg albumin glycopeptide (Fletcher et al 1963b). Colorimetric procedures such as the orcinol-sulphuric and anthrone-H_2O_4 cannot be used for the estimation of remaining hexose in periodate-oxidized carbohydrate as the products of oxidation give a colour comparable with that of the original material (Eylar and Jeanloz 1962b). The amount of egg albumin used in each experiment was about 2 g. and the minimum weight of mannose phenylhydrazone required for counting of radioactivity was about 25 mg. The aim of the present work was to extend this type of procedure to the estimation of other neutral sugars, such as D-galactose and L-fucose, in glycoproteins and to reduce considerably the amount of glycoprotein required for analysis.

2. Colorimetric Methods for the Estimation of Reducing Sugars

Of the many methods described for the assay of reducing sugars, one procedure seems to have certain advantages.

(a) The o-aminodiphenyl method (Timell et al 1956)

This method, which consists of heating a sugar with o-aminodiphenyl in glacial acetic acid requires only one reagent, is fairly sensitive, gives a stable colour, has a linear colour response from 20 to 500 µg/ml and avoids the use of concentrated sulphuric acid. All the hexoses tested gave a green colour with the same molar extinction coefficient (4,635) at 380 mµ, the absorbance maximum. The 6-deoxyhexose, L-rhamnose gave slighily lower molar extinction 4,535. Keto sugars such as fructose gave very little colour. Disaccharides, trisaccharides and also methylated sugars gave colours which differed in intensity and time of maximum development for each substance. A suggested mechanism of colour conformation is a condensation between free sugar and amine to give an equilibrium mixture of a glycosylamine and the corresponding Schiff base (Timell et al 1956). The method was found to be useful for estimating sugars eluted from paper chromatograms. In the method used here the volumes of all solutions were usually reduced to one half. The time of heating was extended from 45 to 50 minutes to ensure complete reaction (see Fig. 5). The procedure is as follows.

o-Aminodiphenyl was obtained from L. Light & Co. Although the material was recrystallized twice from aqueous ethanol using activated charcoal (BDH) the product was still coloured. White crystalline material was obtained if the o-aminodiphenyl was recrystallized as the hydrochloride from aqueous ethanol and then reconverted to the free base by the addition of sodium hydroxide mp 49.5 - 50°.

Acetic acid glacial. Laboratory reagent glacial acetic acid was distilled from 2% chromium trioxide, the fraction boiling between 118° and 119°C (uncorrected) was collected (Orton and Bradfield 1924).

Procedure: To the test solution (0.5 ml containing 10-200 µg of sugar) in ground glass stoppered test tubes was added freshly prepared solution of 0.4% o-aminodiphenyl in glacial acetic acid (2.5 ml). The tubes were stoppered and immersed in a boiling water bath for 50 minutes. The tubes were cooled and the absorbance at 380 mµ of the solutions was measured in 1 cm cells in a Unicam SP 500 spectrophotometer.

A distilled water blank in the assay gave an absorbance value of about 0.007 when measured against water. The response of galactose and fucose is shown in fig. 6.

(b) The ferricyanide assay of reducing sugars

As some glycoproteins contain less than 1% of a particular sugar component, the amount of material required for assay could be considerably reduced by a more sensitive method for estimating sugar. The most sensitive method available appeared to be that which involves the reduction of ferricyanide. Park and Johnson (1949) described a procedure which estimates sugars in the range of 1 - 10 µg/ml. The main disadvantage is that it is not specific and considerable care has to be taken to ensure reproducibility. Their procedure was used here with minor changes.

Consistent results were obtained with this assay only if certain precautions were taken. Assay tubes were cleaned with sodium dichromate in concentrated sulphuric acid after use. It was found necessary to increase the concentration of sulphuric acid from 0.05 N to 0.1 N in the ferric ammonium sulphate reagent to prevent the solutions from becoming

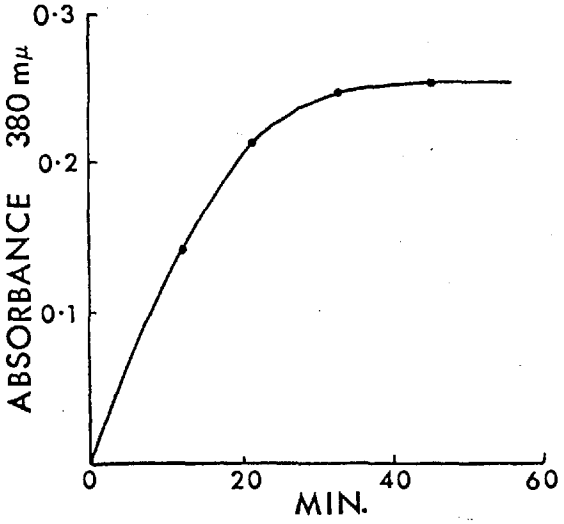


Fig. 5. Development of colour with time in the o-aminodiphenyl method. A solution (1 ml.) containing galactose (55.8 μg.) was heated at 100° with 0.4% O-aminodiphenyl in acetic acid (5 ml.).

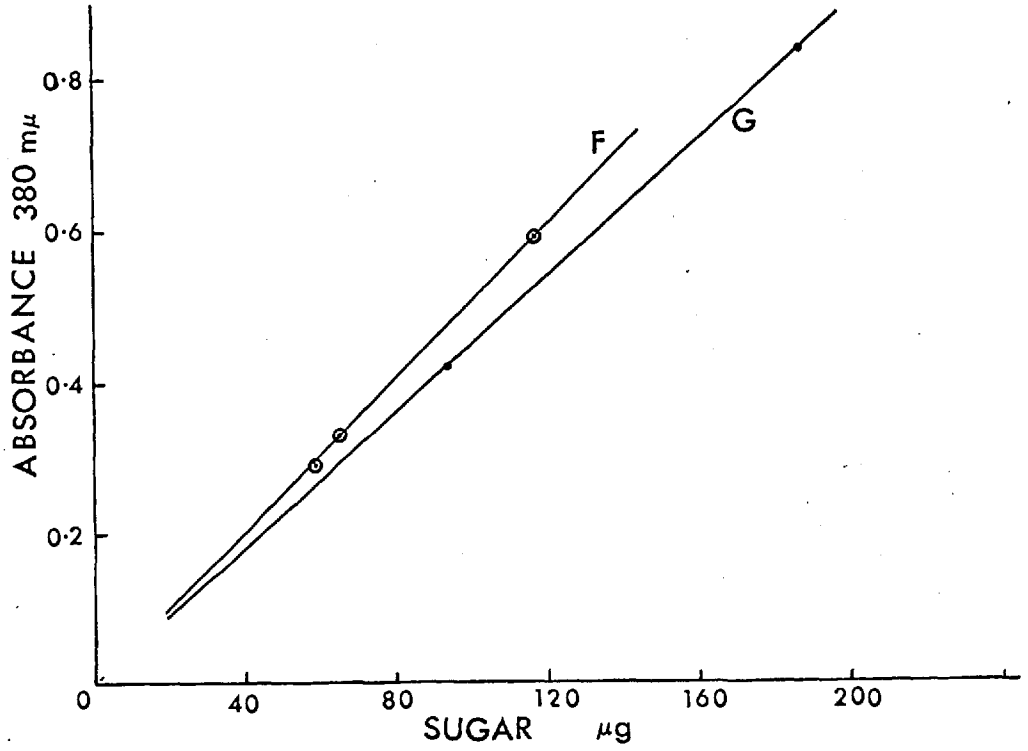


Fig. 6. Standard curves for L-fucose (F) and D-galactose (G) in the O-aminodiphenyl method (Timell 1956).

turbid. The volumes of all solutions were usually reduced by a half. Samples were assayed in triplicate since occasionally a tube gave an anomalous high value due presumably to contaminant reducing substances.

Solutions 1. Potassium ferricyanide (Analar, Hopkins and Williams), 0.5 g., was dissolved in water and made to one litre.

2. Sodium carbonate (anhydrous, A.R., British Drug Houses), 5.3 g., and potassium cyanide (A.R. British Drug Houses) 0.65 g., were dissolved in water and made to one litre.

3. Ferric ammonium sulphate (A.R., British Drug Houses), 1.5 g., and sodium dodecyl sulphate (L.R., British Drug Houses), 1 g., were dissolved in 1.l. of 0.1 N sulphuric acid.

To a solution (0.5 ml) containing from 1 to 5 µg sugar, was added solution 2 (0.5 ml) followed by solution 1 (0.5 ml). The tubes were stoppered with ground-glass stoppers and heated in boiling water for 15 minutes. The tubes were cooled and solution 3 (2.5 ml) was added. After 30 minutes the absorbance of the solutions were measured at 690 mµ in a Unicam SP500 spectrophotometer. The reading given by water alone was about 0.055. Control tubes taken from the Celite (see later) column gave readings usually about 0.005 higher. Standard curves for glucose, galactose, mannose and fucose are in fig. 7.

Estimate of Variation : The variation in the average value obtained from replicate measurements has been expressed as the standard error of the mean value, i.e. $\pm \left[\frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{n(n-1)}\right]^{1/2}$ where n is the number of values, x_i is any value and $\bar{x}$ is the mean of the values. The significance of this parameter is that such mean values will lie between the limits of $\pm$ 2 standard errors with a probability of 95%.

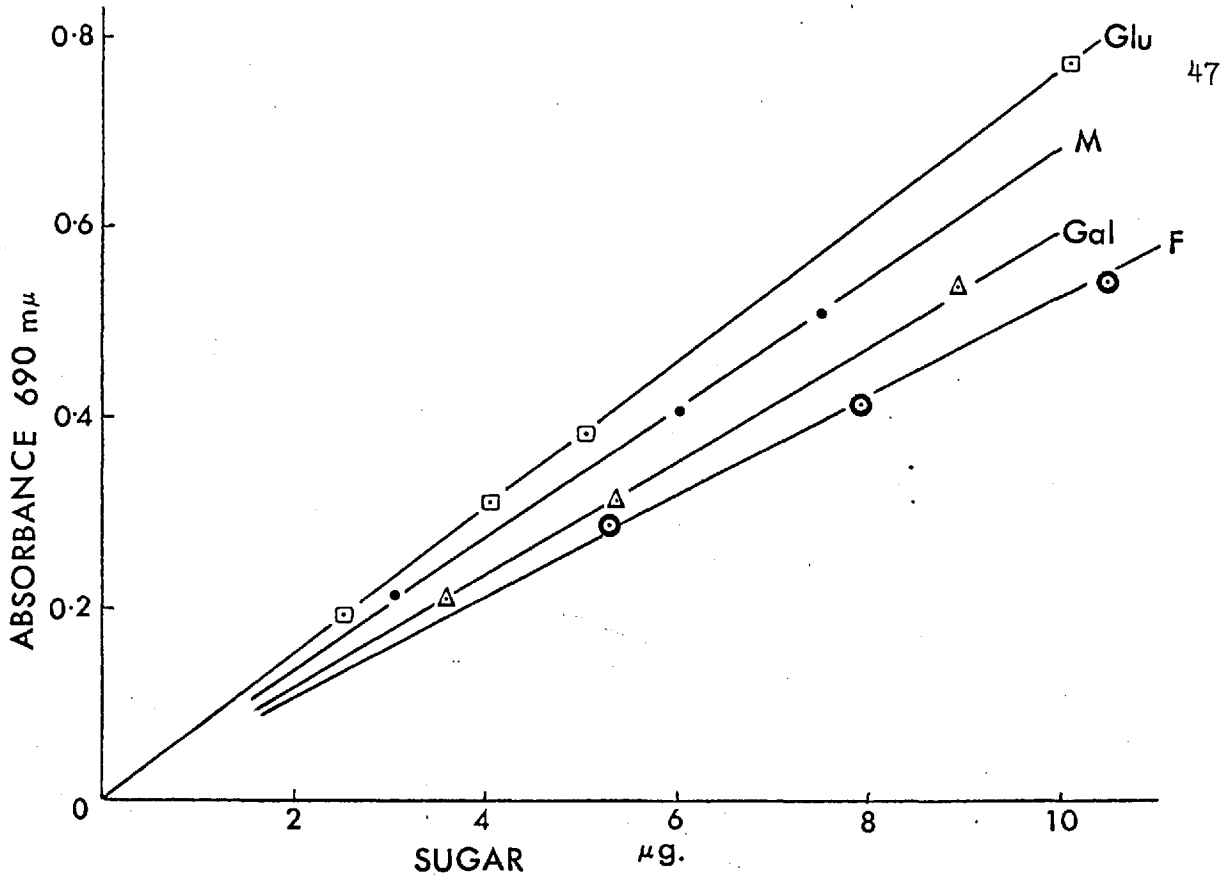


Fig. 7. Standard curves for sugars in the ferricyanide method (Park and Johnson 1949)

Glu - D-glucose; Gal - D-galactose;

M - D-mannose; F - L-fucose.

3. Conditions of Acid Hydrolysis of Glycoproteins for the Release of Hexoses

Various times and acid concentrations have been used in the hydrolysis of glycoproteins for the release of hexoses but the temperature of 100° is almost always used. For pleuromucoid Bourillon and Michon (1959) found N HCl for 6 h to be optimum. For the similar α_1-acid glycoprotein of serum, maximum values were obtained for galactose with 2 N HCl for 2 h and for mannose 2 N HCl for 3 h.(Montreuil and Scheppler 1959). The hydrolysis of thyroglobulin in 1 N H_2SO_4 for 6 h or 8 h, or in 2 N H_2SO_4 for 6 h or 8 h is common (Eylar and Jeanloz 1962, Chatterjee and Montgomery 1962, Spiro 1960, Walborg and Ward 1963).

For egg albumin François et al (1962) considered that half the mannose would be released in about 10 min. in 2 N HCl at 100°, or 98% in the first hour. Their results from isotope dilution experiments over a time period remained constant for periods of hydrolysis of 2, 3, 4 and 6 hours. The choice of 2 N HCl was a compromise between effective hydrolysis and destruction of the sugar, particularly at the start of the reaction before release of mannose from the glycoprotein had occurred. To minimise conditions whereby the acetamido groups might be preferentially cleaved, the radioactive sugar and glycoprotein solution were heated to 100° before addition of acid (François et al 1962).

Effect of heating galactose in HCl at 100°

Solutions (0.5 ml) of N or 2 N HCl containing galactose (1.12 mg) were heated in sealed tube in a boiling water bath for various times. The tube contents were made to 25 ml and aliquots were assayed in duplicate by the o-aminodiphenyl method.

Time	N HCl	2 N HCl
0 hour	0.200	0.207
1 "	0.198	0.206
2 "	0.197	0.198
3 "	0.202	0.195
Average standard error	± .004	± .002

It appears that galactose is fairly stable in 2 N HCl at 100° for up to 3 h, although the acid degradation products of galactose may also react with O-aminodiphenyl. This criticism is probably even more valid when applied to the estimation by the anthrone-sulphuric acid method of galactose destruction in 4 N HCl (Haab and Anastassiadis 1961), which showed a loss of 3% in 1 h and 14% in 2 h. The former value is compatible with that found here. However these conditions were not too destructive of galactose to be used with an isotope dilution method.

4. The Removal of Acid from Hydrolysates

The methods most commonly used for the removal of HCl from hydrolysates employ anion exchange resins. Weak anion-exchange resins have been used in various forms such as the hydroxyl form (François et al 1962, Chatterjee and Montgomery 1962) carbonate form (Pusztai and Morgan 1961a) and acetate form (Roberts and Gibbons 1966) and strong anion exchange resins have been used in such forms as the carbonate form (Eylar and Jeanloz 1962a) and the formate form (Spiro 1962). Reducing sugars are not quantitatively recovered from strong anion exchange resins in the hydroxyl form (e.g. Roseman et al. 1952).

In the first part of this work the hydrolysate was neutralised

with Amberlite IR-4B, ^{-}OH form and passed through a Dowex 50 H^{+} form column to remove positively-charged material, such as amino acids, peptides, amino sugars, inorganic cations and any soluble basic substances released from the anion exchange resin. The effluent was concentrated to dryness in a rotating flask under reduced pressure in a water bath of about 40°C.

The recovery of sugars from ion-exchange resins

Since low recoveries of radioactive sugars were observed the recovery of neutral sugars from ion-exchange resins was investigated. Each resin was regenerated by washing it into a column and passing through it in succession several column volumes of 2 N NaOH, water and 2 N HCl. The column was then washed with a 2 N solution of the compound, used for conversion of the resin to the form desired, until the effluent was free of the ion being removed. After exhaustive washing the resin was ready for use.

(a) Recovery of galactose from Amberlite IR-4B - hydroxyl form

A solution (0.5 ml) of 2 N HCl containing galactose (1.12 mg) was applied to a column of Amberlite IR-4B hydroxyl form (1 cm x 19 cm). Water was passed through the column and fractions of 25.0 ml were collected. Samples (0.5 ml) of each fraction were assayed by heating with 2.5 ml of O-aminodiphenyl for 50 min and reading the absorbance at 380 mμ.

A solution (0.5 ml) of galactose (1.12 mg) was applied to a Dowex 50 - H^{+} form column (1 x 4 cm) and washed with water. Fractions of 25 ml were collected and assayed in duplicate as above.

	Absorbance 380 mμ	Recovery %
Galactose original solution	0.214	
Water	0.010	
Amberlite 1R-4B fraction 1.	0.180	83
" " " 2.	0.041	15
" " " 3.	0.043	16
Dowex 50 fraction 1.	0.214	100
" " 2.	0.008	0

As a large volume was required to wash the sugars from the Amberlite 1R-4B resin and as other material was washed from the column, alternative anion-exchange resins were tried.

(b) Recovery of mannose from Dowex 2 - acetate

A solution (5 ml) of 2 N HCl containing mannose (1.79 mg) was applied to a column of Dowex 2 (x8, 200-400 mesh)-acetate form. Water was passed through the column and fractions of 25 ml were collected and assayed by O-aminodiphenyl method. Mannose (71.4 μg/ml) in 0.4 N acetic acid was the control.

	Absorbance at 380 mμ	Recovery %
Water	0.008 ± .001	
Fraction 1.	0.367 ± .002	101
Fraction 2.	0.016 ± .003	2
Control	0.365	

(c) Recovery of mannose from amino acids by passage through ion exchange resins

A solution, containing mannose (1.14 mg) (6.3 μmole) and a standard mixture of amino acids (0.4 μmole of each as used in amino acid analysis)

in 2 N HCl, was neutralized with Dowex 1-bicarbonate form and passed through small coupled columns of Dowex 1-bicarbonate and Dowex-50-hydrogen form. The columns were washed with water and the combined effluents were concentrated to dryness on a rotary evaporator at a temperature of less than 40°. The dried residue was made to 250 ml and compared with the original mannose solution by the ferricyanide assay method. The mean and standard errors of triplicate absorbances measured at 690 mμ are given.

Water	0.011 ± 0.001
Assay	0.628 ± 0.005
Standard	0.650 ± 0.006

As the recovery of mannose was about 97% this procedure was used for removing ions from hydrolysates.

5. The Counting of Radioactive Samples

Isotopes which emit β-particles, such as ^{14}C, can be directly counted with a Geiger counter by spreading the samples on planchets. The two main difficulties encountered in such a method for quantitative purposes, are the need for the symmetrical placing and even distribution of the samples on planchets and the problem of the absorption and scattering of the emitted particles by the sample itself (Calvin et al 1949, p.27). The first difficulty is overcome by reproducible procedures in placing the samples and, if in solution, in cleaning the planchets and in evaporation of the solvent. The effect of self-absorption may be overcome in several ways. If the sample is sufficiently thinly-spread on the planchet self-absorption is negligible("infinitely thin sample"). For ^{14}C-containing compounds, "thin" samples of 0.1 or 0.2

mg per cm^2 have been suggested (Calvin et al 1949). This method was applied to the counting of ^{14}C-labelled glucose and fructose in amounts of 0.2 mg per cm^2 dried on metal dishes (Sowden and Schaffer 1952). The response was linear for dilutions of the radioactivity by up to a factor of 5. For the assay of ^{14}C-labelled sterols up to 0.06 mg per cm^2 was used (Donna et al 1957).

For samples of known thickness self-absorption corrections can be applied. Glucose-1-^{14}C was counted in uniform films of known amounts of sodium O-(carboxymethyl) cellulose and corrected for self-absorption (Isbell et al 1960).

The third method involves the use of samples which are so thick that the number of particles emitted from the surface is independent of the thickness, "infinitely thick" samples. This method was used in the assay of ^{14}C-labelled mannose isolated as the phenylhydrazone (François et al 1962) and requires large amounts of material. A modification of this method, whereby the radioactive sugar was counted in a solution of the non-volatile solvents, ethylene glycol and formamide, had a counting efficiency of about 1%)(Schwebel et al 1954).

The first method was used here as the need was to count small amounts of pure sugars.

<u>^{14}C-Containing Monosaccharides</u>

(1-^{14}C) L-Fucose was obtained from Calbiochem, Los Angeles,U.S.A., (1-^{14}C) D-mannose and (U-^{14}C) D-mannose, (U-^{14}C) D-glucose and (1-^{14}C) D-galactose were obtained from The Radiochemical Centre, Amersham. Radioactive sugar (25 μC) was diluted with cold sugar (250 mg) in aqueous solution which after standing at 4° overnight was concentrated

to a syrup. Methanol and iso-propanol were added and crystallization occurred at 4° over several days. The sugars were dried at 78°, in vacuo over P_2O_5 for 1 to 2 h.

Counting of radioactive samples at infinite thinness

Aliquots (0.5 ml) in triplicate or quadruplicate, of an aqueous solution of the sample were placed in the centre of metal planchets which were either flat or which had concentric raised rings. The latter simplified symmetrical placing of the liquid. The area covered was about 2 cm^2. Planchets were washed with ethanol:acetone (1:1, by vol.) and wiped dry. The samples were dried under reduced presssure either at 70°, or at room temperature in the presence of H_2SO_4 and solid NaOH. The planchets were counted in a Nuclear-Chicago D47 gas flow Geiger counter with a Micromil window operating in the plateau region and fitted to an automatic sample-changing device. The gas used was 1.5% butane in helium. The efficiency of the counting was about 25%. Counting of samples was continued on each sample for usually much more than 10^4 counts which represents a relative standard error of $\pm$ 1%.

Counting of radioactive samples at infinite thinness implies no self-absorption by the sample and so a linear relation should exist between the quantity of sample applied and the number of counts recorded. Three aliquots (0.5 ml) each of dilutions of [1-^{14}C] galactose in water were dried on flat aluminium planchets at 70° in vacuo. The mean and standard deviations are given.

[1-^{14}C] Galactose µg	Activity* cpm S.E.	Specific Activity cpm/µg
28.1	1,320 ± 23	47.0
46.8	2,180 ± 57	46.6
93.6	4,370 ± 25	46.7
140.4	6,340 ± 24	45.2

* mean of triplicate corrected for background of 20 ± 1 cpm

A solution of [U-^{14}C] mannose (45 µg/ml was diluted with an equal volume of water and an equal volume of a solution of non-radioactive mannose of the same concentration. Duplicate samples (0.5 ml) of the three solutions were dried in vacuo at 70°, counted and corrected for background.

[U-^{14}C] Mannose µg	Activity cpm	Specific Activity cpm/µg
22.5	1,223, 1218	53.3
11.25	618, 611	54.4
11.25 + 11 µg mannose	611, 609	54.0

A solution of [1-^{14}C] glucose was diluted with water or with solutions of non-radioactive galactose and aliquots (0.5 ml) were placed on aluminium planchets having raised concentric rings and dried in a desiccator at room temperature and counted. (see below) With flat aluminium planchets the linearity between the counts obtained and the amount of sugar applied was usually more reproducible than that obtained with ringed planchets. The flat planchets were usually used.

[1-^{14}C] Galactose µg	Galactose µg	Activity* cpm	Specific Activity cpm/µg
22.25	0	1306, 1301	58.7, 58.5
22.25	22.9	1236, 1258	55.6, 56.5
11.13	0	664, 680	59.7, 61.1
11.13	11.45	670, 677	60.2, 60.8
11.13	22.9	657, 669	59.0, 60.1
5.56	0	335, 341	60.3, 61.3
5.56	5.73	338, 343	60.8, 61.7
5.56	11.45	340, 346	55.8, 62.2

* corrected for background 22 cpm.

Each of 12 planchets, giving measured counts of between 1,000 and 2,000 cpm was counted 6 times for 20,000 counts each time. The theoretical standard error for the total count of each planchet is $\sqrt{120,000} = 346$ or 0.29%. The average percentage standard error, calculated from the counts obtained from the 12 planchets was 0.34%.

The amount of radioactive sugar added to the glycoprotein before hydrolysis was about the same amount as that expected in the glycoprotein. The addition of radioactive sugar in amounts greater than that present produces much greater errors in the final results, calculated from percentage errors in the specific activities determined. However it is desirable not to have too small amounts of radioactive sugar in the presence of acid and glycoprotein at the beginning of the hydrolysis as the preferential destruction of the added sugar is likely to be greater on a percentage basis.

6. The Chromatography of Sugars on Celite Columns

Celite columns developed with organic solvents have been used to separate sugars and their derivatives (see review by Lemieux 1962). In comparison to cellulose, Celite is readily packed in columns, it gives better resolution, and faster flow rates. It does not release reducing substances which can happen with starch or cellulose columns.

Celite can be packed into columns either as a slurry in the organic phase used for later development (Neish 1950) or in portions as a dampened powder, each portion being tamped down with a plunger (Hall 1962a, 1962b). The latter method was used for the work described here as it was fairly rapid, convenient and gave good resolution. Hall (1962b) has shown that good separation of rhamnose, fucose, mannose and glucose can be obtained on a column of Celite-545 (50 g.)

Preparation of Celite

Celite-545 was a product of Johns-Manville, New York, and was purified largely according to Hall (1962a). Celite was slurried in 6 N HCl, poured into a large column and washed with 6 N HCl until the effluent was colourless. The acid was washed from the column with a large quantity of distilled water. The celite column was then washed with absolute alcohol followed by purified ethyl acetate. The Celite was dried in an oven at 110°.

The two-phase solvent system used by Hall (1962b) was used: ethyl acetate, n-propanol, water (4:1:2, by vol.). Ethyl acetate was purified as described by Vogel (1954). It was washed with an equal volume of 5% sodium carbonate, then with saturated calcium chloride solution. It was dried over anhydrous potassium carbonate and distilled through a Dufton column.

n-Propanol was refluxed with stannous chloride and distilled through a Dufton column.

Preparation of Celite columns

Celite 545 (10 g) was thoroughly mixed with the lower phase (4.4 ml) of a well-equilibrated mixture of ethylacetate-n-propanol-water (4:1:2, by vol). The damp mixture was firmly tamped in small portions into a precision-bore glass column (1 cm wide) using a close-fitting machined Teflon plunger (2 cm long) located at the end of a long stainless steel rod. The column had a water jacket maintained at a constant temperature which was close to that of the laboratory. The material to be chromatographed was dissolved in lower phase (usually 0.1 ml), mixed with dry Celite (200 mg for 0.1 ml of lower phase, or in the same ratio) and the mixture was tamped onto the top of the column. Upper phase was run onto the top of the column from the resevoir which was situated to give about 6 feet head of liquid. Thin teflon tubing was used to carry the solvent as it was not attacked by the ethyl acetate in contrast to other plastic tubing tried. Fractions of 10 ml were collected by an organic solvent siphon, by drop counting (400 drops) or by timing. Aliquots of the fractions were dried on planchets and counted in a gas flow counter.

Chromatography of galactose, mannose and fucose

Columns were freshly packed for each experiment since resolution was considerably decreased on a column used for the second time. Resolution of the sugars was also poor if the chromatography was carried out at 4°. For example a mixture of L-fucose (3.36 mg), D-galactose (3.41 mg) and D-mannose (3.39 mg) in 0.32 g. moist Celite were chromatographed on a column of Celite (6.25 g) at 4°. The fucose and mannose peaks overlapped although the galactose peak was separate.

In order to gauge the applicability of the method to sugars isolated from a glycoprotein hydrolysate, a mixture of L-fucose (2.43 mg), [1-^{14}C] D-mannose (0.97 mg), [1-^{14}C] D-galactose (1.13 mg), and egg albumin (51 mg) in 2 N HCl was heated at 100° for 2 h, de-ionised and dried. The residue in moist Celite (0.4 g dry) was submitted to chromatography on a column of Celite (10 g) jacketed at 25°C. Aliquots of 1 ml from each fraction were dried at 60° under reduced pressure and assayed using the O-aminodiphenyl method. The results are shown in fig. 8. Paper chromatography of dried 1 ml aliquots of each second tube in the same solvent showed the presence of fucose only in tubes 20, 22, mannose only in tubes 30, 32, 34 and galactose only in tubes 48 to 54 (aniline hydrogen phthalate spray).

In order to determine if the Celite columns could be used for several chromatographic runs L-fucose (1.35 mg), D-mannose-1-^{14}C (1.36 mg) and D-galactose-1-^{14}C (1.31 mg) was applied in the same way to the same Celite column used for the above experiment. Tubes 14, 16 and 18 showed, by paper chromatography, the presence of mannose and tubes 22 and 24 the presence of galactose. Tubes 1 to 25 were dried in vacuo at 60° and 2.5 ml of water was added to each. Aliquots (0.5 ml) were taken from each tube, one for O-aminodiphenyl assay and another for counting. The results are shown in fig. 9.

The recovery of L-fucose and D-mannose was about 95%. (Much of the D-galactose was lost due to a fault in the fraction collector).

The more rapid elution with consequent decrease in resolving power of the column indicated that columns should be freshly-packed for each experiment. Probably the flow of solvent slowly removes water

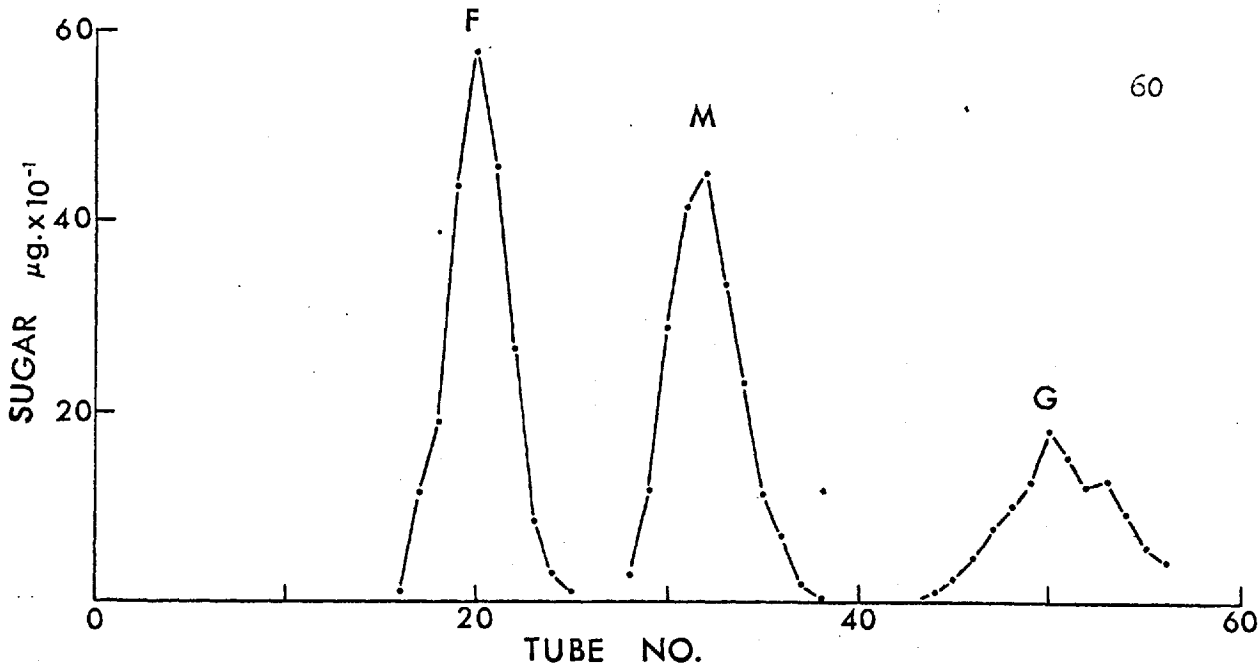


Fig. 8. The separation of fucose (F), Mannose (M), and galactose (G) on a column of Celite (10 g.) at 25°. Fractions of 10 ml. were collected.

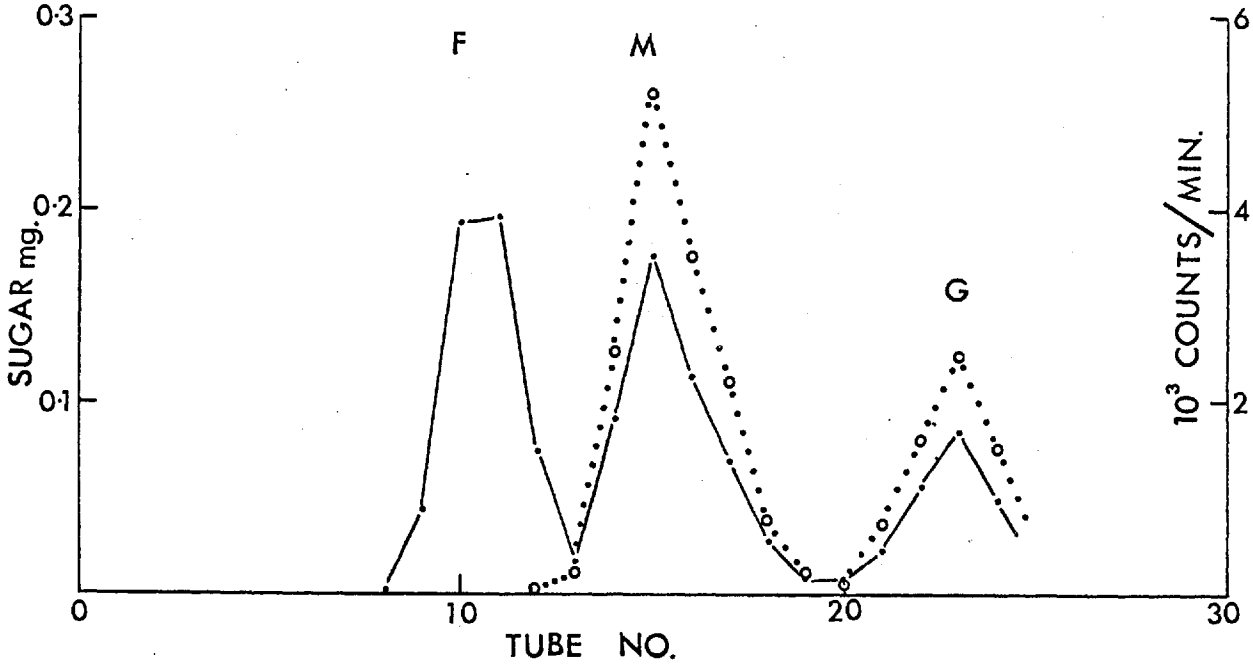


Fig. 9. The separation of fucose (F), mannose (M) and galactose (G) on the same column of Celite as that used in Fig. 8.

_____ Sugar content; ..... Radioactivity.

from the aqueous stationary phase, particularly as the ethylacetate could evaporate from the loosely-covered resevoir giving a less hydrophobic developing solvent.

Chromatography of radioactive sugars

[1-^{14}C] D-Galactose (1.24 mg) and [U-^{14}C] D-mannose (1.53 mg) in Celite (0.2 g) were chromatographed on a Celite column (8 g). The fractions (about 11 ml) dried in vacuo at 70°, were dissolved in 2.5 ml of water and aliquots (0.2 ml) from each second tube were dried on planchets and counted. The result is shown in fig 10. The contents of appropriate tubes were pooled and the specific activities compared with those of the sugars before chromatography, see Table 7.

The above experiment was repeated in a similar fashion except for the addition of L-fucose (206 µg). The results are shown in Table 7.

The change in specific acti vity of the mannose and galactose was small and reproducible.

7. The Estimation of Galactose and Mannose in Rabbit γ-Globulin by Radioisotope Dilution

The hexose content of rabbit γ-globulin, prepared by salt precipitation or DEAE-cellulose chromatography, was reported as 0.6% in the anthrone-H_2SO_4method using glucose as standard (Porter 1958). A value of 0.67% of galactose and mannose, in a molar ratio of 1:2, and 0.05% fucose was obtained with the orcinol-H_2SO_4 method after correcting for the relative amounts of the different sugars (Nolan and Smith 1962). A hexose content of 1.23% was estimated by the orcinol-H_2SO_4 method using mannose as standard and measuring the absorbance at 505 mµ instead of 540 mµ (Fleischmann et al 1963).

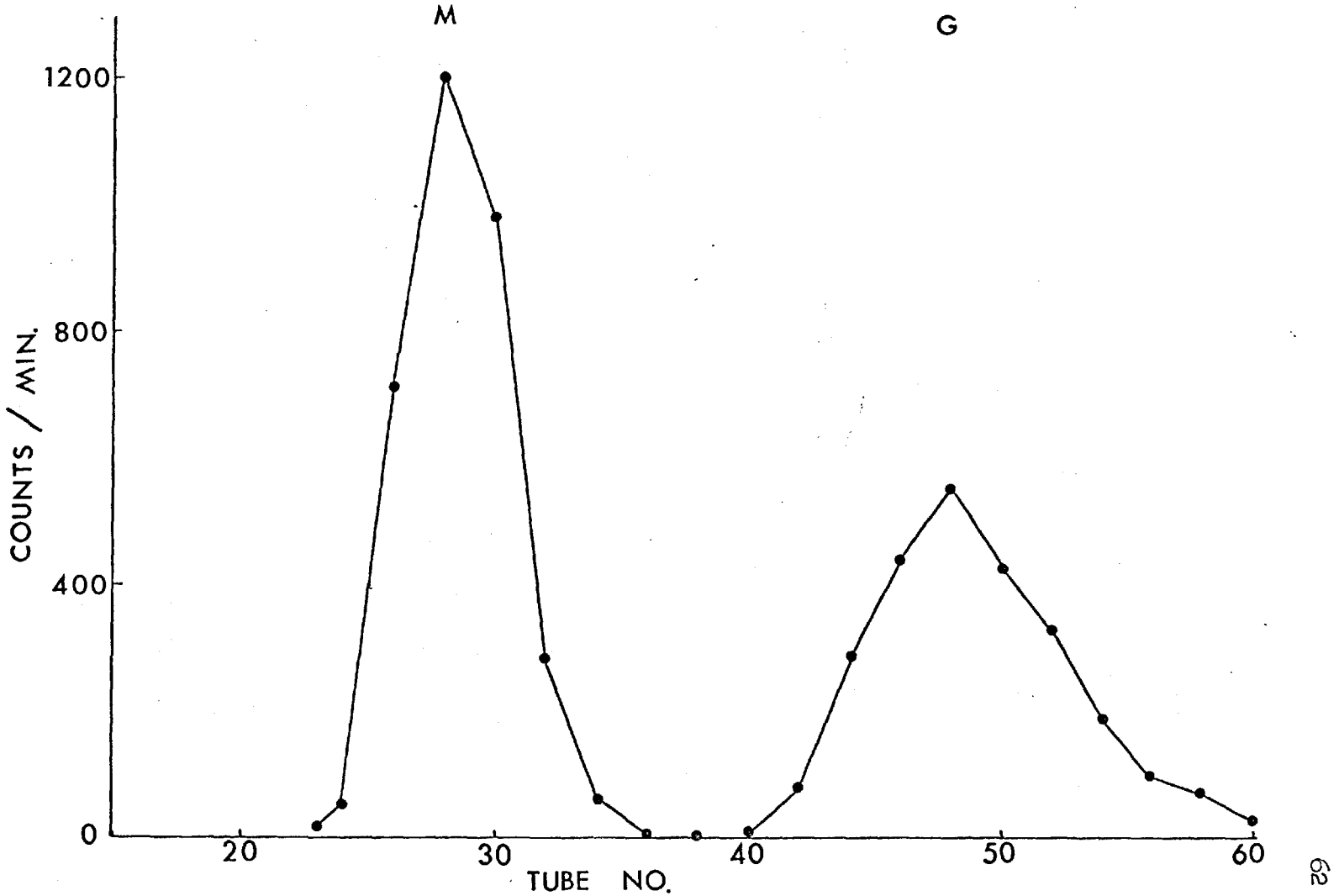


Fig. 10. The separation of ^{14}C-labelled mannose (M) and galactose (G) on a Celite column (8 g.).

TABLE 7

Change in Specific Activity of ^{14}C-labelled Sugars on Chromatography

Sample	Absorbance* x 10^3 at 380 mμ	Sugar μg	Activity† cpm	Specific activity cpm/μg	Change in Specific Activity %
Experiment 1					
Sugars before chromatography					
Mannose	393 ± 5	38.86	2,111 ± 14	54.3	
Galactose	311 ± 3	31.35	1,615 ± 4	51.5	
From column					
Tubes 8 - 14	4 ± 1				
Tubes 25- 32 (man)	364 ± 5	35.60	1,883 ± 8	52.9	- 2.6
Tubes 46 - 50 (gal)	248 ± 1	24.60	1,284 ± 8	52.2	+ 1.4
Tubes 70 - 73	4 ± 2				
Experiment 2					
Sugars before chromatography					
Mannose	1,358 ± 3	154.3	7,471 ± 54	48.5	
Galactose	1,068 ± 23	124.6	5,817 ± 74	46.5	
From column					
Tubes 2 - 8	36 ± 0				
Tubes 19 - 28 (man)	1,283 ± 6	141.6	6,689 ± 52	47.1	-2.9
Tubes 31 - 46 (gal)	994 ± 6	111.7	5,289 ± 121	47.2	+ 1.5
Tubes 53 - 65	38 ± 1				

* average of triplicate and corrected for water blank of 0.008

† average of triplicate and corrected for background of 20 cpm

For most of this work the rabbit γ-globulin was kindly provided by Miss E.M. Press, Department of Immunology, St. Mary's Hospital Medical School, London W.2. It had been prepared by precipitation with sodium sulphate according to Kekwick (1940) and contained about 15% NaCl. On starch gel electrophoresis at pH 8.6 (Smithies 1959) followed by staining with Amido Schwarz reagent or Eriochrome Black T, the preparation gave a single broad anodic band near the origin. The amount of protein present was determined by measurement of the absorption at 278 mμ (the wavelength of maximum absorption) of a solution of γ-globulin in 0.01 N HCl. To convert the absorbance to the weight of γ-globulin the value of 13.5 for a 1% solution of the protein (Crumpton and Wilkinson 1963) was used.

Rabbit γ-globulin (52.06 mg) was dissolved in 0.01 N HCl and made up to 10 ml. Dilutions of 0.504 ml to 5.0 ml and 10.0 ml gave readings of 0.543 and 0.273 respectively, corresponding to 76.6% and 77.1%, average 76.9%.

A hexose value for this preparation of rabbit γ-globulin was estimated by the orcinol-H_2SO_4 method described by Svennerholm (1956) with mannose as standard. The test solution (1 ml) was cooled in ice and a pre-cooled solution (2 ml) of 0.2% orcinol in concentrated H_2SO_4 was added slowly. After standing in an ice-salt mixture for about 15 min. the tubes were shaken and heated at 80° for 20 min. After cooling, absorbance of the solutions was measured at 505 mμ. (See Fig. 11).

Sample	Absorbance at 505 mμ
Water	0.060 ± .001
Mannose (35.7 μg)	0.638 ± .007
(59.5 μg)	1.007 ± .006
(89.3 μg)	1.45 ± .012
γ-globulin (2.02 mg)	0.515 ± .003

Therefore the "mannose" value of γ-globulin by this method is 28.1 μg/2.02 mg or 1.39%.

Method of hydrolysis

A solution containing a weighed amount of glycoprotein (up to 100 mg) and known amounts of radioactive monosaccharides was heated with shaking in a ground-glass stoppered tube in a boiling water bath until the temperature of the solution was at least 95°. A sufficient amount of redistilled hydrochloric acid (6.65 N) was added to make the final concentration 2 N (or 1 N in some experiments) in a volume of 5 ml. The tube was flushed with nitrogen, stoppered and heating was continued for 2 hours. The solution was cooled, filtered if necessary, and passed through a column of Amberlite IR-4B $-OH^-$ form coupled to a Dowex 50 - H^+ form column. The columns were washed with water and the effluent was concentrated to dryness or near-dryness in a rotating flask under reduced pressure with a water bath temperature of less than 45°. The residue was washed from the flask with a small amount of water which was then taken to dryness in a small vessel in vacuo over H_2SO_4 and NaOH.

The dried residue was submitted to chromatography on a column of Celite (8 g. or 10 g.). Fractions (about 10 ml) were collected and

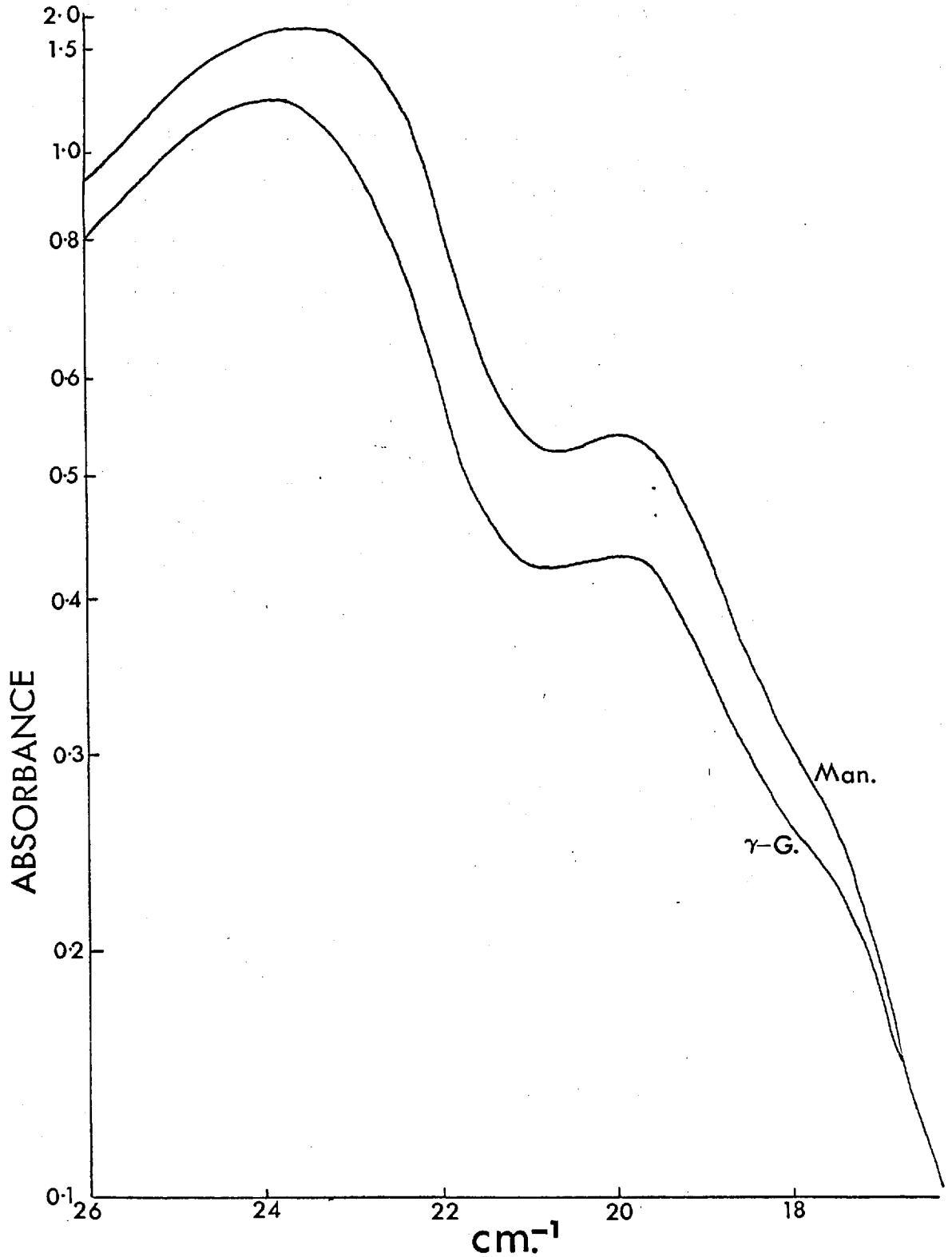


Fig. 11. The absorption spectra of mannose (Man.) and rabbit γ-globulin (γ-G) in the orcinol method (Svennerholm 1956).

taken to dryness at 70° in vacuo. Water (2.5 ml) was added to each tube and aliquots (0.5 ml) taken for assay and counting. Fractions comprising most of each peak were pooled and assayed colorimetrically by the O-aminodiphenyl method and by counting at infinite thinness.

The readings taken in a typical experiment (number 4 below) are given in detail in the table. The elution curve is given in fig. 12. The results of 4 experiments are summarized in table.

Sample	Absorbance* x 10^3 at 380 mμ	Sugar μg	Activity cpm	Specific activity cpm/μg
Galactose (28.07 μg)	254 ± 3	[illegible]	1516 ± 8	54.0
Tubes 48 - 54	130 ± 2	14.37	442 ± 4	30.8
Mannose (22.53 μg)	206 ± 2		1194 ± 12	53.0
Tubes 28 - 33	373 ± 2	40.8	1058 ± 10	25.9

* mean of triplicate corrected for waterblank of 0.006.

The mean and standard errors for the estimation of the galactose and mannose contents of this preparation of rabbit γ-globulin were 2.2 ± 0.2 and 4.3 ± 0.2 mole per 10^5 g. respectively. The maximum variation from the mean was 0.66 for galactose and 0.43 for mannose respectively. The values obtained in the experiments 2 and 3 where 1 N HCl was used for hydrolysis, appear to be higher than those found using 2 N HCl but the variation is too great for any real significance to be established. If it were so it may mean that the rate of destruction of sugar in comparison with that of glycoside hydrolysis is favoured for the more dilute acid.

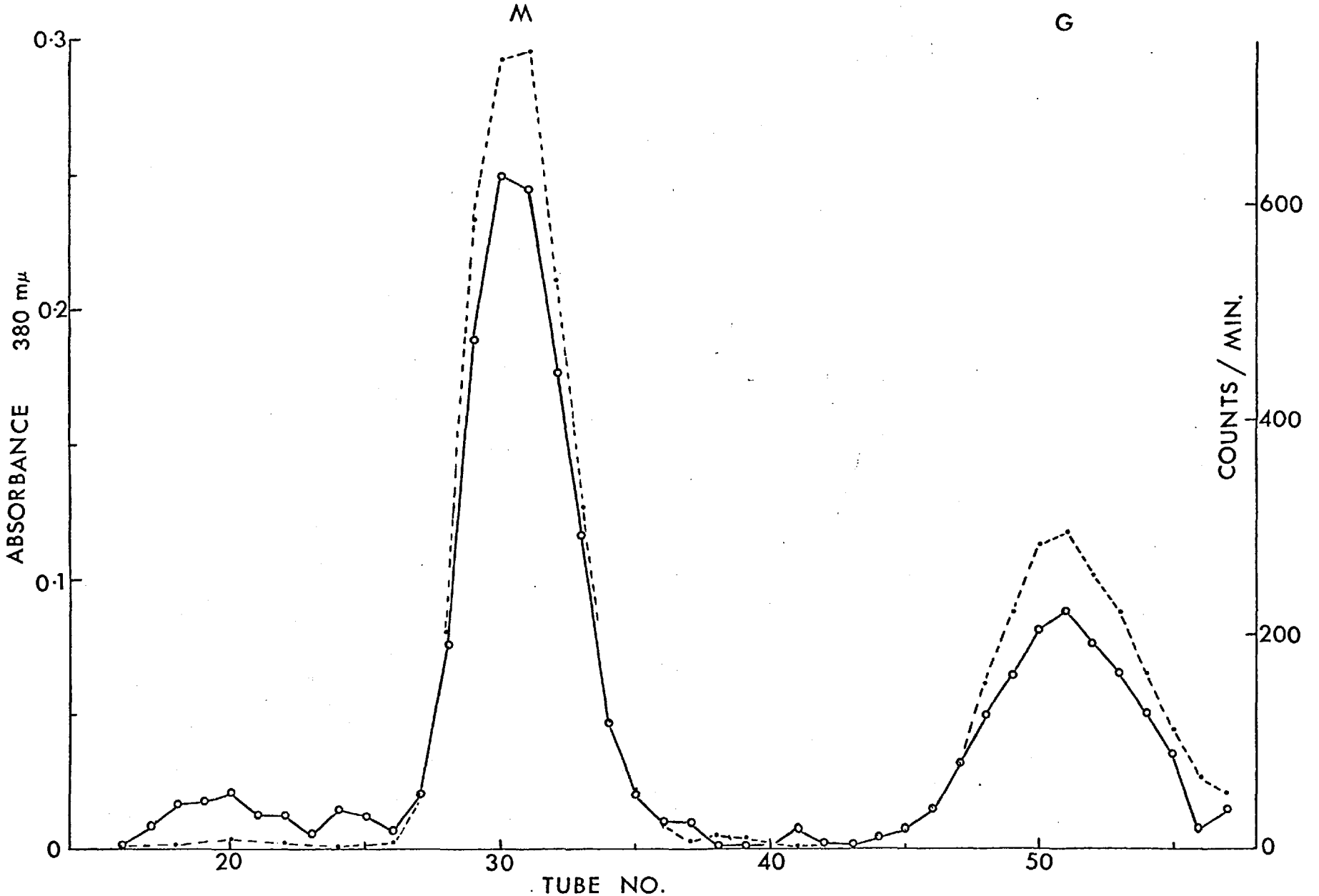


Fig. 12. Celite chromatography of the neutral sugars of rabbit γ-globulin after addition of ^{14}C-labelled mannose (M) and galactose (G). _____, absorbance; ------radioactivity.

TABLE 8

ESTIMATIONS OF MANNOSE AND GALACTOSE IN RABBIT γ-GLOBULIN

Experiment Number	Protein taken (mg)	Sugar added µg	Specific Activity Initial cpm/µg	Specific Activity Final cpm/µg	Sugar found µg	Sugar found mole 10^5g
1	22.5	man. 211.5	53.2	30.2	161	3.98
2	57.9	man. 451	54.2	27.1	451	4.33
		gal. 281	51.3	27.3	247	2.37
3	50.5	man. 451	53.0	26.5	451	4.96
		gal. 281	54.0	30.3	220	2.42
4	65.9	fuc. 82				
		man. 1,015	54.7	37.2	477	4.02
		gal. 491	49.7	34.7	212	1.79

Hydrolysis was carried out for 2 hr. at 100° with 2 N HCl (experiments 1 and 4) or with 1 N HCl (experiments 2 and 3) in a volume of 5 ml., except for experiment 4 where the volume was 25 ml.

8. The Estimation of Mannose in Egg Albumin by Isotope Dilution

The radioisotope dilution method was applied to the estimation of mannose in egg albumin. Egg albumin, five times crystallized from ammonium sulphate, was provided by Dr. R.D. Marshall of this department. He estimated the mannose content to be 1.96% by the orcinol-H_2SO_4 method.

Egg albumin (90.6 mg) was hydrolysed in 2 N HCl (25 ml) at 100° for 2 hr. in the presence of (U-^{14}C) D-mannose (1.53 mg) and (1-^{14}C) D-galactose (1.24 mg). The hydrolysate was cooled, filtered and de-ionized on columns of Dowex 1-acetate and Dowex 50 - H^+ form. The effluent was taken to dryness and submitted to chromatography on Celite (8g). The results are shown.

Sample	Mannose µg/0.5ml	Activity cpm/0.5 ml	Specific activity cpm/µg
Tubes 24 - 34 (man)	85.9	2,153	25.06
Mannose	71.85	3,943	54.88

From the above values the mannose found was 1.832 mg or 2.01% of the glycoprotein.

9. The Estimation of the Sugar Content of Rabbit γ-globulin after Performic Acid Oxidation

Since both tryptophan and cysteine have been implicated in the destruction of sugars under strongly acidic conditions it was thought that the oxidation of these residues by performic acid might considerably reduce or eliminate this source of sugar loss. Performic acid oxidation of protein results in rapid oxidation of the tryptophan to a number of products, one of which is kynurenine, and less rapid oxidation of the cyst(e)ine to cysteic acid (Toennies and Homiller 1942). Methionine is converted to the sulphone. As cysteic acid and methionine

sulphone can be satisfactorily determined in the presence of carbohydrate, (Sehram et al 1954, Moore 1963), it does not seem likely that they react with sugars.

Prior performic acid oxidation of egg albumin, fibrinogen and fibrin has been used to prevent interference by tryptophan in the anthrone-H_2SO_4 reaction for the estimation of hexose (Hörmann and Gollwitzer 1962, 1963). Hexose values found after oxidation agreed with those obtained from reading the anthrone colour as a wavelength when tryptophan did not interfere. Thus it appears that the oxidation products of tryptophan do not interfere in the colorimetric reaction and are probably less detrimental to any sugars present. It was reported that performic acid oxidation of fetuin at 0°C does not result in any loss of the carbohydrate components (Spiro 1963b). To confirm this finding two glycosides were treated with performic acid at 0° and assayed for sugar by the orcinol procedure.

Performic acid was prepared according to Moore (1963). Hydrogen peroxide (30% v/v, 0.25 ml) and formic acid (99%, 4.5 ml) was allowed to stand for 1 hour at room temperature. A few drops of methanol was added and the solution cooled to -2°. The mixture (0.2 ml) was added to each of methyl-α-D-glucopyranoside (2.28 mg) and α,α'-trehalose (1.63 mg) and left at 0° for 4.5 hr.

The acid was removed with Dowex 1 (bicarbonate form) and the effluent was taken to dryness in a flask in vacuo. The residues were each dissolved in 5 ml of water and aliquots (0.5 ml) were taken for assay by the orcinol method (Johansen et al 1960). The readings at 505 mμ corrected for a water blank of 0.05 were

	Treated	Untreated
methyl α-D-glucopyranoside	1.10	1.09
α,α'-trehalose	0.759	0.755

Under the conditions used the two glycosides investigated do not appear to be hydrolysed, because it is likely that any free sugar formed would be oxidized by the performic acid and become unavailable for colour formation.

The loss of tryptophan and cyst(e)ine from glycoproteins by performic acid oxidation

Rabbit γ-globulin after oxidation at 20° for 1.5 hr was not completely soluble in 0.01 N HCl. The soluble material gave no colour in the tryptophan assay (Opienska-Blauth 1963) and the absorption spectrum lacked the tryptophan "shoulder" at 289 mμ. Carbohydrate was shown to be present in both the soluble and insoluble material by the Molisch test.

The change in absorbance of p-chloromercuribenzoate at 255 mμ (Boyer 1954) was used to determine the sulphydryl groups in egg albumin after performic acid oxidation followed by borohydride reduction (Moore et al 1958).

Since glycoproteins are not completely soluble after performic acid treatment, the egg albumin was treated with pepsin before oxidation. p-Chloromercuribenzoate (15.64 mg; purified according to Boyer (1954)) was dissolved in 0.01 M acetate buffer at pH 5. The absorbance at 234 mμ (0.547) gave a molar extinction coefficient of 1.67×10^4 compared to the value of 1.73×10^4 found by Boyer (1954).

An egg albumin solution was heated at 100° for 5 min, the pH of the solution was brought to pH 2.5 with formic acid and pepsin added. After allowing digestion to proceed for 16 h at 40° the clear solution was freeze-dried. The dried material was treated with performic acid at 2° for 4 h, ice-cold water was added and the solution freeze-dried.

The treated egg albumin (15.8 mg) was dissolved in 5% $NaBH_4$ solution (2.5 ml) and kept at 50° for 1 h. Hydrochloric acid (0.33 ml. of 1 N) was added to make the solution pH 5. In two sets each of two cuvettes (No. 1 and No. 2) the following solutions were added.

Cuvette 1: 1 M acetate buffer pH 4.6 (0.9 ml), 0.1 mM p-CMB solution (0.45 ml) and water (1.65 ml).

Cuvette 2: 1 M acetate buffer pH 4.6 (0.9 ml), treated egg albumin solution (1 ml), and water (1.1 ml).

One set of cuvettes was placed in the reference holder of the SP700 recording spectrophotometer and the other set in the sample holder. After running a base line, the contents of the sample cuvettes were mixed and the absorbance, measured at 250 mμ, was allowed to reach its maximum value (in about 5 min). The difference spectrum was run. It showed a maximum absorbance of 0.38 at 248 mμ. Boyer (1954) found absorbance maximum at a wavelength of 255 mμ and a change in molar absorbance of 6.2×10^3 for cysteine. Using this value, it would mean that about 1.5 of the 7 potential sulphydryl groups of egg albumin (Neuberger and Marshall 1966) were not oxidized to cysteic acid by this treatment.

The estimation of galactose and mannose in performic acid-oxidized rabbit γ-globulin

Freeze-dried rabbit γ-globulin (55.0 mg.) containing 93% glycoprotein by ultraviolet absorption measurements, was freed of sodium chloride by dissolving in water (1 ml.) and precipitating with acetone (3 ml.). The precipitated material was washed with acetone-methanol (1:1 by vol.) and dried in vacuo. To assess the loss of glycoprotein the combined supernatants were taken to dryness and the residue was dissolved in 0.01 N HCl. The absorption at 276 mμ was 0.045 corresponding to 0.17 mg. of γ-globulin.

The dried γ-globulin was treated with performic acid (2 ml.) for 4 hr. at 2° giving a light purple-brown solution. Ice-cold water (about 30 ml.) was added, the solution was frozen and taken to dryness from the frozen state.

(1-^{14}C) Galactose (237 μg), [1-^{14}C] mannose (382 μg) and the dried, oxidized γ-globulin were made to 25 ml. in 2 N HCl and hydrolysed for 2 hr. in a boiling water bath. The hydrolysate was filtered through sintered-glass, de-ionised and taken to dryness as before. The residue was submitted to chromatography on a Celite 545 (10 g.) column and the sugar solutions obtained were assayed for radioactivity and for reducing power by the ferricyanide method. The results are shown in the table. The galactose and mannose values, 1.96 and 4.30 mole/10^5 g. respectively, found in this experiment are similar to the average values of previous experiments, 2.2 and 4.3 moles/10^5 g. respectively.

TABLE 9

The Estimation of Mannose and Galactose in Performic Acid-Oxidized Rabbit γ-Globulin

Sample	Absorbance	Sugar µg	content µg/0.5ml*	Activity cpm	Specific activity cpm/µg	Sugar µg	content mole/10^5g
Water	0.081 ± .003			21 ± 1			
Tubes 5 - 15	0.103 ± .001			35 ± 2			
40 - 48	0.570 ± .003	6.60	16.5	488 ± 3	27.5	396	4.30
76 - 86	0.311 ± .002	3.43	8.56	286 ± 0	29.3	180.4	1.96
Mannose	0.516 ± .004	6.15	19.2	1096 ± 11	56.0		
Galactose	0.370 ± .001	4.76	23.8	1248 ± 14	51.6		

* adjusted for dilution

10. Experiments Designed to Assess the Radioisotope Dilution Method

In these radioactive dilution experiments the rate of destruction of added free ^{14}C-labelled sugar during the course of hydrolysis of a glycoprotein is not known with any accuracy and whether such destruction has an appreciable effect on the values obtained from radioisotope dilution experiments is not certain. There are two methods which may be used to assess this effect. (a) One method is to determine during the hydrolysis of a glycoprotein both the rate of release of the sugar and the rate of destruction of the released sugar. If labelled sugar is added at the start of the hydrolysis and the specific activity and total amount of the free sugar present is measured at different time intervals and plotted against time the rate of release and the rate of destruction of the sugar at any particular time can be obtained from the curves. The kinetics of similar systems has been worked out by Neimann (1958) and used for such purposes as the identification of intermediates in the oxidation of methane and the measurement of the formation and consumption of aldehydes in cyclopentane oxidation (Horscroft 1963). (b) Another method is to employ an internal standard. Hydrolysis of the glycoprotein is carried out in the presence of a mixture of a ^{14}C-containing sugar, which does not occur in the glycoprotein, and a known glycoside of the sugar. The amount of sugar determined by isotope dilution can then be compared with that originally added in the form of the glycoside. A criticism of this procedure is that unless the rate of hydrolysis of the glycoside and the rate of destruction of its free sugar are comparable to the equivalent rates associated with the sugars occurring in the glycoprotein, the results will not be applicable to the estimation of the sugars in the glycoprotein.

(a) The rate of release of mannose from egg albumin in 2 N HCl at 100°

The application of the first method to the estimation of mannose in egg albumin was attempted. In this experiment the solutions obtained after different times of hydrolysis were frozen in alcohol-solid CO_2 and stored at -12°. It was later found that at this temperature partial thawing occurred resulting in significant destruction of the free mannose present so that the total amount could not be estimated. However the specific activity of the mannose was obtained over various time intervals.

Egg albumin was recrystallized for this experiment. It was dissolved in water. Material, insoluble in 50% saturated ammonium sulphate at pH 7, was removed and crystallization initiated by adding sulphuric acid to pH 4.8. The solid material was recovered by centrifugation and dissolved in water. The solution was dialysed three times at 4° with stirring and freeze-dried. Since in hot acid solution egg albumin quickly becomes insoluble forming lumps in which the heterosaccharide may not be as accessible to acid as in solution, the glycoprotein was first denatured and treated with pepsin. Egg albumin (494 mg.) was dissolved in 20 ml. of water, the solution was brought to pH 7.5 with ammonia and heated at 85° for 8 min. (Neuberger 1938). Formate buffer pH 2.5 (5 ml. of 2.5 M) and pepsin (1.7 mg.) were added to the opalescent solution which was then kept at 37° overnight. The solution was clarified by further treatment with pepsin (3.8 mg.) for 4 hours, and was then made to 50 ml.

Orcinol Assay (Johansen et al. 1960). Aliquots of 0.5 ml. were mixed with 4 ml. of 0.19% orcinol in 53% sulphuric acid and the solutions were heated for 15 min. at 80°. The egg albumin solution (0.5 ml.) gave an absorbance of 0.463 at 505 mμ compared to 0.216 given by 41.3 μg of mannose,

corresponding to a mannose content of 2.01% of the egg albumin.

Hydrolysis Aliquots (5 ml.) were mixed with a solution of [U-^{14}C] mannose (5 ml. containing 46.3 µg mannose) in stoppered test tubes. The tubes were heated in a boiling water bath to over 95° when 4 N HCl (10 ml), heated similarly, was added. The tubes were flushed with nitrogen, the stoppers were taped on and heating continued. Tubes were withdrawn at various times, cooled in water, frozen in solid CO_2/alcohol mixture and kept at -12°. The tube contents were thawed and made to a volume of 25 ml. An aliquot (10 ml.) was de-ionised with Dowex 1 (bicarbonate form) and Dowex 50 (H^+ form), evaporated to dryness and submitted to chromatography on a Celite (8 g.) column as described previously.

The release of mannose with time is shown in table 10 and figure 13. From the figure it can be calculated that the time required for release of 50% of the mannose from egg albumin is about 7 min. and for 75% release about 13.5 min. This value is comparable to the half-time of 10 min. for the release of reducing groups from egg albumin (see François et al 1962). First-order reaction kinetics are not applicable to the data as the hydrolysis would proceed through intermediates such as oligosaccharides of mannose and of mannose with N-acetylglucosamine.

(b) The estimation of glucose in methyl α-D-glucopyranoside

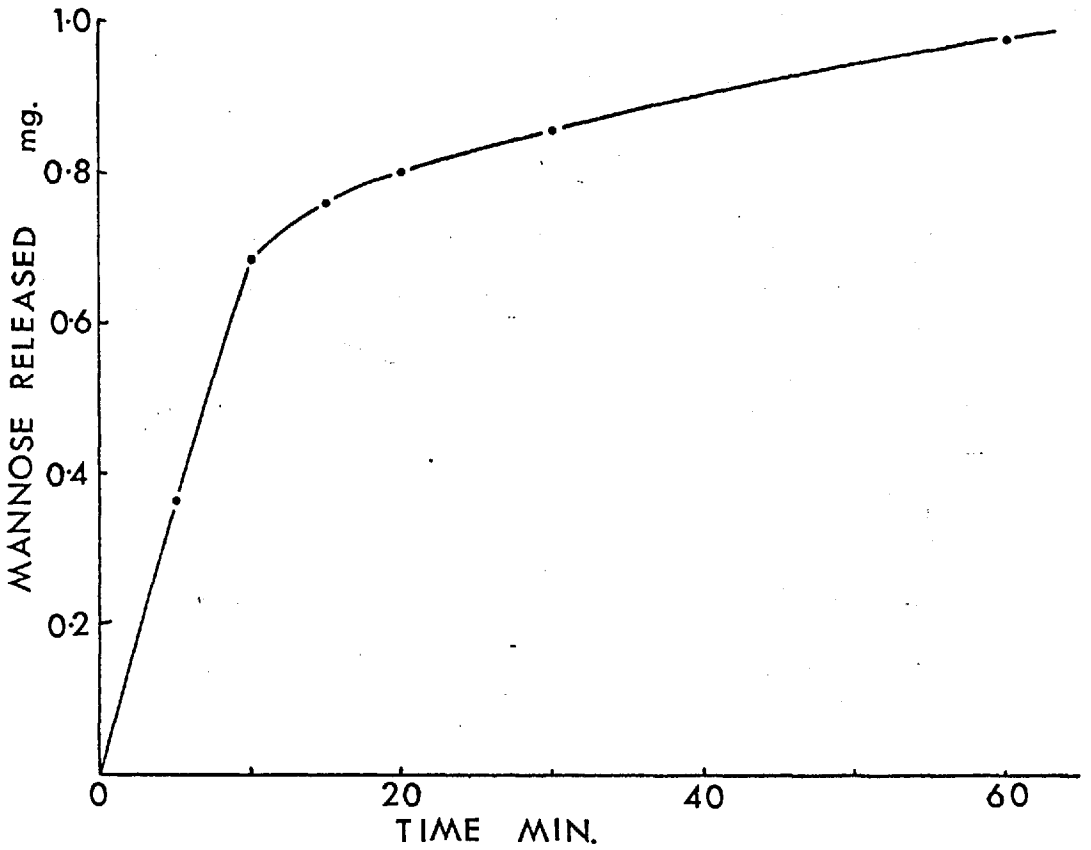
Methyl α-D-glucopyranoside was used as a model substance by estimation of the glucose content by isotope dilution. In 2 N HCl at 100° the half-life of hydrolysis is about 7 min. which is similar to that for the release of mannose from egg albumin.

TABLE 10

The release of mannose from egg albumin in 2 N HCl at 100°

Hydrolysis Time min.	Specific activity (cpm/µg) Recovered mannose	Standard mannose	Mannose released µg
0	78.0	80.4	
5	45.0	80.4	368
10	32.5	81.4	682
15	28.2	75.3	760
20	26.2	71.6	802
30	24.9	72.2	859
60	21.5	67.6	977

Fig. 13. The release of mannose from egg albumin in 2 N HCl at 100°



(i) The specific activity of [1-^{14}C] D-glucose after chromatography on Celite 545

[1-^{14}C] D-glucose (0.621 mg.) was applied in Celite (200 mg. to a column of Celite (10 g). Aliquots (0.2 ml.) of each second fraction (10 ml.) were counted and the results shown in Fig. 14. Fractions were pooled as in table below and taken to dryness. The residues were dissolved in 3 ml. of water. Aliquots (0.5 ml.) in triplicate were taken for counting at infinite thinness, and after dilution for assay by the ferricyanide method.

Sample	Absorbance* at 690 mμ	Glucose μg/ml	Activity† cpm	Specific activity. cpm/μg
Glucose (31.5 μg) Tubes 30 - 42	0.512 ± .006	31.5	755 ± 9	24.0 ± 0.3
Tubes 60 - 63	0.400 ± .001	24.6 ± .38	567 ± 6	23.0 ± 0.4
Tubes 64 - 67	0.576 ± .006	35.5 ± .49	853 ± 10	23.8 ± 0.4
Tubes 68 - 72	0.461 ± .009	28.4 ± .65	633 ± 6	22.3 ± 0.3

* corrected for blank value of .067

† background of 21 counts corrected.

Glucose, obtained from the central part of the peak from the column, had a similar specific activity to the original material. As the fractions at the outside gave a lower specific activity, in subsequent experiments only the main fraction was taken.

(ii) The estimation of glucose in methyl α-D-glucopyranoside

Methyl α-D-glucopyranoside (m.p. 166-168°) was prepared in this laboratory. Methyl α-D-glucopyranoside (2.13 mg) and [1-^{14}C] glucose (1.233 mg.) was heated in 25 ml. of 2 N HCl at 100° for 2 h. The solution was deionised with Dowex 1-bicarbonate and Dowex 50 - H^+ form, concentrated to dryness and the residue was subjected to chromatography on Celite (8 g.). Contents of appropriate tubes were pooled, taken to dryness and the residue was dissolved in 5 ml. Aliquots (0.5 ml) were taken for counting and after dilution for assay of reducing power.

Sample	Absorbance at 690 mμ	Glucose μg/0.5 ml	Activity† cpm	Specific activity cpm/μg
Water	0.130 ± .005			
Tubes 39-43	1.170 ± .002	124.5 ± 2.0	1,170 ± 6	9.33 ± 0.22
Tubes 60-90 (blank)	0.162 ± .001		7	
Standard	0.807 ± .015	20.9	509 ± 4	24.35 ± 0.19

† corrected for background of 20 counts/min.

The amount of glucose found in 2.13 mg. of methyl-α-D-glucopyranoside is $1.233\left[\frac{24.35 \pm .19}{9.33 \pm .22} - 1\right] = 1.985 \pm 0.080$ mg. compared to the calculated value of 1.976 mg., i.e. 101% with a standard error of ± 4.0%.

(iii) The estimation of glucose in methyl α-D-glucopyranoside in the presence of egg albumin

Egg albumin (99.3 mg.), methyl α-D-glucopyranoside (1.908 mg.) and [1-^{14}C] D-glucose (0.621 mg.) were heated in 25 ml. of 2 N HCl at 100° for 2 hr. The hydrolysate was processed as before and the neutral substances were submitted to chromatography on Celite (10 g). The result is shown in Fig. 15.

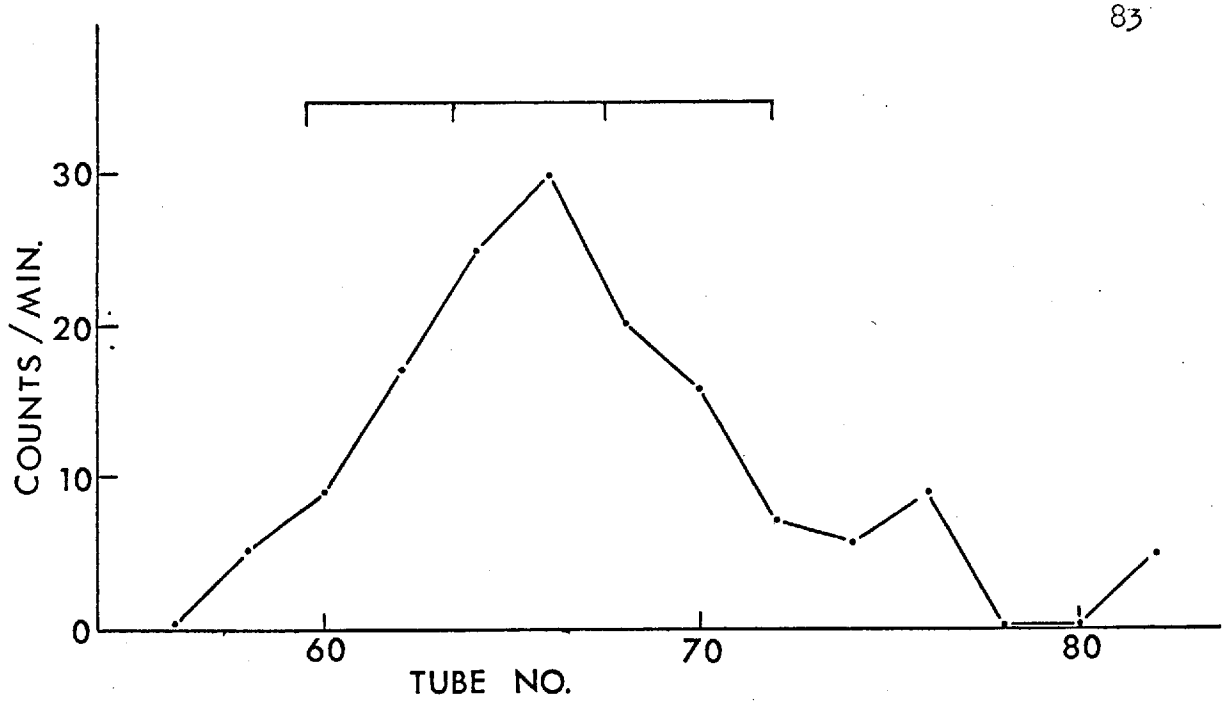


Fig. 14. The chromatography of [1-^{14}C] glucose in a column of Celite (10g.)

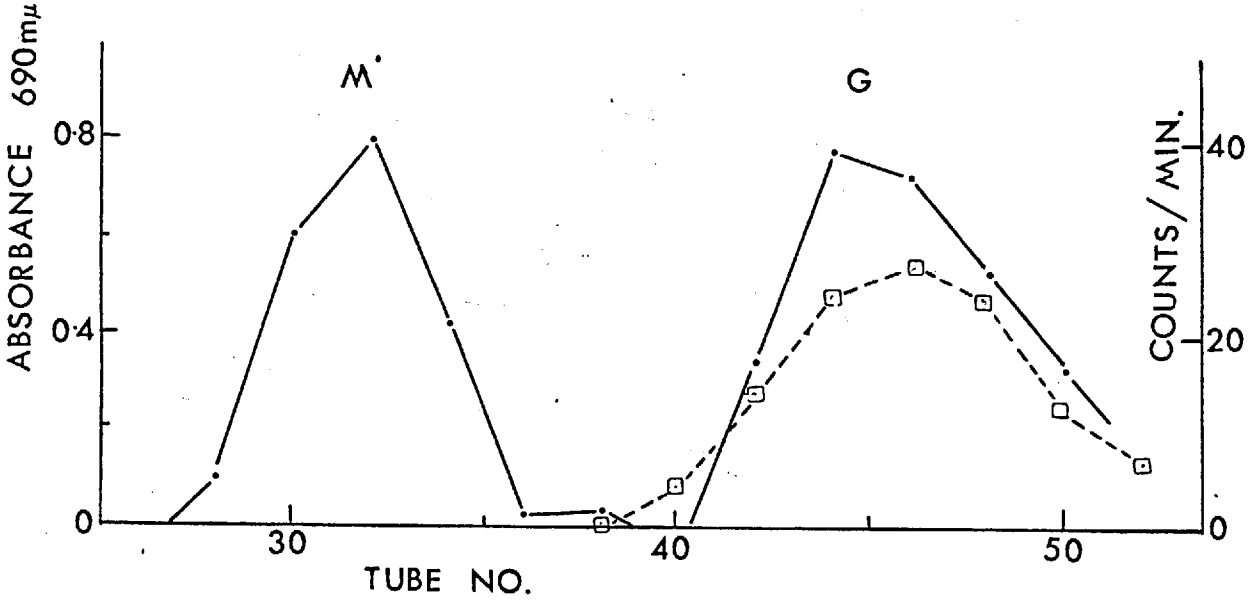


Fig. 15. The chromatography on Celite (10 g.) of mannose (M) from egg albumin together with [1-^{14}C] glucose (G);_____ absorbance; ------, radioactivity.

Sample	Absorbance* 690 mμ	Dilution factor	Sugar μg/0.5 ml	Activity† cpm	Specific activity cpm/μg
Tubes 5-20 (blank)	0.001±.001	19.7		1	
Tubes 41 - 53	0.300±.008	19.7	41.25±1.2	246±0.1	5.96± .19
Standard glucose	0.542±.006	3.31	12.51	324± 3	25.9 ± .24

* values corrected for water blank of 0.120 ± .005

† values corrected for background of 22 cpm.

The total amount of glucose present is $\left[\frac{25.9}{5.96} - 1\right] 621 = 2.08 \pm .09$ mg compared with 1.77 mg of glucose added as the methyl glycoside, and representing an overestimate of 18%. The overestimate obtained here suggested that the added [1-^{14}C] glucose was destroyed to a greater extent than the glucose released from the glycoside by an amount of about 13% (calculated on the specific activity of the recovered glucose). If the standard error of the method is about ± 4% the overestimate is unlikely to be due to chance. The ratio of protein to added sugar in the hydrolysate is about 160 to 1 by weight in this experiment which is nearly 3 times that of the usual ratio for the estimation of mannose in egg albumin.

(iv) <u>The estimation of glucose and mannose in a mixture of methyl α-D-glucopyranoside and performic acid-oxidized egg albumin</u>

If the observed preferential destruction of added [1-^{14}C] glucose in the presence of egg albumin is in some part due to the interaction between free sugar and tryptophan and/or cyst(e)ine, oxidation of the glycoprotein with performic acid would be expected to reduce the destruction. Consequently a similar experiment was carried out in the presence of performic acid-oxidized egg albumin and at the same time the mannose content of the egg albumin was similarly determined.

Performic acid was made according to Moore (1963). Hydrogen peroxide (30% v/v, 0.25 ml.) and formic acid (99%, 4.5 ml.) was allowed to stand for 75 min. at room temperature then cooled to 4°C. Egg albumin (128.5 mg.) was dissolved in formic acid (1 ml.) at 4°C containing a few drops of methanol and performic acid (1 ml.) at 4° was added. After standing 200 min. at 0°C, ice-cold water was added, the solution was shell-frozen and freeze-dried. To the protein was added methyl-α-D-glucopyranoside (2.133 mg.), [1-^{14}C] D-glucose (1.233 mg.) and D-mannose (2.28 mg.) and the mixture was heated in 2 N-HCl (25 ml) at 100° for 2 hr. The clear slightly yellow hydrolysate was treated as before and submitted to chromatography on a Celite (10 g.) column.

Sample	Absorbance* at 690 mμ	Sugar content μg/0.5 ml	Activity† cpm	Specific activity cpm/μg
Tubes 38 - 41	0.673 ± 7	46.17	1,263 ± 8	27.4
Tubes 57 - 63	1.102 ± 7	70.7	628 ± 13	8.88
Mannose standard	0.562 ± 5	19.34	1,083 ± 4	56.0
Glucose standard	0.654 ± 6	20.92	520 ± 3	24.86

* the standards are corrected for water blank absorbance of 0.070, and the column samples for a blank of 0.079.

† corrected for background of 22 cpm.

The value obtained for glucose was 2.22 mg. which, relative to the calculated value of 1.98 mg., corresponded to 112%. The mannose content was calculated as 2.38 mg. or 1.85% of the original egg albumin taken. The value obtained previously was 2.01%. Therefore the values obtained for glucose and mannose in this experiment are lower by 5% and

8% respectively than the values obtained previously from separate experiments using unoxidised egg albumin. The decrease in the glucose value may be ascribed to (a) the removal of interference by those amino acid residues, in the glycoprotein, which are oxidizable by performic acid; (b) the greater amount of reducing sugar present in comparison with that of the glycoprotein (i.e. a ratio of 1 to 37) which may decrease their relative destruction, and (c) the lower ratio (37 to 1) of glycoprotein to sugar. The lower mannose content, if not due to errors in the method may also be ascribed to the first two factors. There is also the possibility that some of the mannose is destroyed by performic acid oxidation.

13. The Estimation of Fucose and Galactose in Human Blood-group Substances

Introduction

Since the isotope dilution method, as used above, for the determination of neutral sugars was not very reproducible when applied to glycoproteins containing small amounts of carbohydrate, the method was applied to the estimation of neutral sugars in human blood-group substances. These substances contain up to about 85% carbohydrate which is made up of L-fucose, D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and N-acetylneuraminic acid (Morgan 1963). The latter component is usually only present in small amounts but a blood-group Le^a substance isolated from a human pseudomucinous ovarian cystadenoma contained 18% N-acetylneuraminic acid (Pusztai and Morgan 1961a). Blood-group A, B and H substances have a similar carbohydrate composition. The compositions of some blood-group substance preparations are given in Table 11.

TABLE 11. Published Analyses of Some Blood-group Substances*
(Values are expressed as g./100g.

Constituent	A(368)	B(376)	Le^a(350)
L-Fucose	17.0	17	8.4
D-Galactose			28.8
Hexosamine†	29.6	33	26.6
Sialic acid	2.2	2.4	17.9
Reduction as glucose†	52.0	59	51.3
N.	5.0	4.8	4.8
Aspartic acid	0.55	0.71	0.40
Threonine	3.97	4.72	3.66
Serine	2.20	2.61	1.69
Glutamic acid	0.80	0.90	0.59
Proline	1.90	2.31	1.66
Glycine	0.57	0.61	0.55
Alanine	1.10	1.26	1.05
Cystine/2	0.07		0.11
Valine	0.69	0.73	0.69
Isoleucine	0.40	0.37	0.31
Leucine	0.50	0.35	0.69
Tyrosine	0.12	0.10	0.31
Phenylalanine	0.28	0.18	0.69
Lysine	0.35	0.39	0.14
Histidine	0.74	0.44	0.41
Arginine	0.72	0.67	0.48

* Data was taken from Pusztai and Morgan (1963), except for carbohydrate analysis of A (Pusztai and Morgan 1961b) and B (Pusztai and Morgan 1961a

† After hydrolysis with 0.5 N HCl for 16 hr. at 100°.

The polypeptide part, which accounts for about 15% of the blood-group substances, is rich in threonine, serine, proline and alanine which account for over half of the amino acids (Pusztai and Morgan 1963). Aromatic and sulphur-containing amino acids are either absent or present in very low amounts. This includes the amino acids which have been implicated in contributing to the destruction of reducing sugars in acid solution, notably tryptophan and cyst(e)ine. Consequently the extent of loss of radioactive sugar, in the presence of such glycoproteins during acid hydrolysis is likely to be small. Also since all the carbohydrate components, apart from sialic acid, are present in large amounts the weight of added sugar relative to the weight of glycoprotein will be quite high and any destructive influence of the glycoprotein on the sugar should not be appreciable. Further, since the L-fucose is very easily released, radioactive sugar will be diluted with L-fucose very early in the hydrolysis.

The other source of error, i.e. the incomplete hydrolysis of glycosidic bonds due to hexosaminide formation, will probably only affect the release of D-galactose since L-fucose is in non-reducing terminal position.

Experimental

Radioactive sugars: The ^{14}C sugars were those used previously. Chromatography of a large amount of each sugar on Whatman No. 241 paper in ethylacetate/n-propanol/water (4:1:2, by vol.) revealed a single intense spot for each of mannose (R_G 1.5), fucose (R_G 2.5) and glucose (7 cm. from origin); for galactose, in addition to an intense spot (R_G 0.8), there was also a faint spot near the origin. Therefore, the sugars were

each submitted to chromatography on a large column of Celite 545 in the same solvent.

[1-^{14}C[- D-Galactose (117 mg., m.p. 168-169°) was dissolved in the aqueous phase of the ethylacetate/n-propanol/water (4:1:2) solvent (0.66 ml.), the solution was mixed with Celite 545 (1.5g.) and the mixture applied to a column of Celite 545 (12 g.). The column was developed in the usual way and aliquots (3 drops) of each fourth fraction (10 ml.) were counted. The fractions 30-54 were pooled and taken to dryness. The crystalline residue was redissolved in a minimum amount of water and recrystallized by the addition of methanol and ethanol. The galactose was dried in vacuo over P_2O_5 at 78° in vacuo. L-Fucose was similarly subjected to chromatography on Celite 545 and recrystallized from ethanol.

Blood-group substances

Blood-group substances were a gift from Professor W.T.J. Morgan,FRS, The Lister Institute, London, who has given the methods used in their preparation. The three samples, designated W, Y and Z, were each obtained from an individual cyst fluid. Each cyst fluid was dried from the frozen state and extracted with 95% phenol at room temperature (Morgan and King 1943, Gibbons et al 1955). The material insoluble in phenol and soluble in water had been fractionated as below.

Sample	Blood-group activity	No.	Solubility in sat. $(NH_4)_2SO_4$	Ethanol fractionation % ethanol	Number of peaks† on DEAE-cellulose chromatography
W	B	376	Sol.	48-55%	3
Y	B	413	Sol.	50-58%	
Z	Le^a	350*	Insol.	55-57%	1

* Pusztai and Morgan 1961a † Pusztai and Morgan 1964

Hydrolysis: For analysis, a weighed sample of blood-group substance was dried for periods of 1 to 3 hr., to constant weight over P_2O_5 at a pressure of 0.2 - 0.3 mm. Hg. and 78°. The dried material was dissolved in water and made to a known volume. Aliquots for hydrolysis were transferred to acid-cleaned pyrex test tubes, radioactive sugar added and sufficient acid and water added to make the required acid concentration in a total volume of 10 ml. (unless otherwise specified). The tube was constricted, flushed with nitrogen for a few minutes and sealed. The tube was heated in a boiling water bath. In some experiments the tube was evacuated to remove dissolved gases before flushing with nitrogen.

The tube was cooled and opened. Dowex 1-bicarbonate form resin was added until the evolution of CO_2 ceased, whereupon the slurry was poured onto a column, containing further Dowex 1-bicarbonate, coupled to a small column of Dowex 50-H^+ form. The columns were washed with about 100 ml. of water and the effluent was taken to dryness in a rotating flask under reduced pressure (provided by a water pump) and a waterbath temperature of less than 50°. The flask contents were dissolved in a small amount of water and the solution was taken to dryness in vacuo over NaOH and H_2SO_4.

Chromatography: The dried residue was submitted to Celite column chromatography as described earlier. Aliquots of each second fraction were dried on metal planchets and counted in a gas flow Geiger counter. The fraction making up the central part of each peak were pooled and taken to dryness in a rotating flask under reduced pressure in a waterbath of 40°. The residue was dissolved in water to make a sugar concentration of about 20 μg./ml. and aliquots (0.5 ml.) were taken for

counting. For assay by the ferricyanide method the solution was diluted to give a final concentration of between 4 and 8 μg./ml.

The estimation of L-fucose: A fairly specific colorimetric method often used for the estimation of fucose was described by Dische and Shettles (1948). In this method fucose is heated in fairly concentrated sulphuric acid at 100° for 10 min. After cooling cysteine hydrochloride solution is added and the fucose content of the solution is obtained by measurement of the absorbance at 400 mμ and 430 mμ.

The method was modified by replacing the cysteine hydrochloride by thioglycolic acid (Gibbons 1955) which gave faster colour development and slightly higher absorbances. The effect of amino acids and sugars other than methylpentoses on the colour given by fucose was investigated and only galactose was found to interfere with the determination to any extent by lowering the fucose value.

The thioglycolic acid procedure was used in the colorimetric estimations of fucose carried out here, except that the volumes were reduced by half.

Ice-cooled sulphuric acid (2.5 ml. of a mixture of 6 volumes of concentrated sulphuric acid and 1 volume of water) was allowed to run freely from a burette, down the wall of ground-glass stoppered test tubes containing the fucose solution (0.5 ml.) cooled in ice. The tubes were shaken in ice water, stoppered, and heated in a boiling water bath for 10min. After cooling, a solution of thioglycolic acid (0.05 ml. of 3.3%, by vol.) was added. The tubes were shaken and allowed to stand for 3 hr. in the dark. The difference in absorbances at 400 mμ and 430 mμ was taken as a measure of the fucose content.

Errors For the assays of reducing power by the ferricyanide method, three or four replicate aliquots were taken to allow for the occasional extreme value produced with this unspecific method. The mean absorbance readings had an average standard error of about ± 1.1%, with an average range between extreme readings of 2.4%. Similarly three or four aliquots of each solution were taken for counting since counting at infinite thinness by this method is dependent on geometry, i.e. on the size of the area covered by the radioactive material, on its position relative to the counting chamber and on the distribution of the material over the area. The average standard error of the mean of three or four planchets was ± 0.67% with an average range of 2.6%.

The thioglycolic acid in sulphuric acid method of Gibbons was more reproducible and gave an average standard error of ± 0.5% for 8 assays in triplicate.

The estimation of L-fucose Since fucose is easily split off from blood-group substances the hydrolysis was usually carried out with mild acid such as 0.2 N H_2SO_4 at 100° for 1 hr. After Celite chromatography fucose was assayed by the method of Gibbons (1955). The results obtained from several experiments are shown in Table 12. The different hydrolytic conditions gave similar results.

Fucose assays were carried out in the intact blood-groupsubstances by the colorimetric method (Gibbons 1955), see Table 13. In the absence of thioglycolic acid the absorbance at 430 mμ of the solution was greater than that at 400 mμ. This difference was not corrected for, as it was not known whether it was unrelated to the chromogene which react with the thioglycolic acid. The values for fucose by this method are higher than those obtained by the isotope dilution method particularly for sample W which was 13% higher.

TABLE 12

The Estimation of L-Fucose in Blood-Group Substances by Isotope Dilution

Blood-group substance	Conditions of hydrolysis	Dried sample taken mg.	L-Fucose added mg.	Specific activity of L-fucose cpm/μg. Added	Recovered	L-Fucose content mg.	%
W	0.1N H_2SO_4, 60 min.	1.527	0.222	55.3	24.2	0.285	18.7
W	1 N HCl, 10 min.	1.527	0.222	55.3	24.1	0.287	18.8
Y*		1.148	0.241	43.4	25.2	0.174	15.2
Y	1 N HCl, 70 min.	1.209	0.222	50.9	28.6	0.173	14.3
Z	0.2N H_2SO_4, 60 min.	3.59	178	42.6	20.9	0.185	5.15

* Assays by ferricyanide method

TABLE 13

Assay of L-Fucose in Blood-Group Substances by the Colorimetric Method

	Amount taken mg.	Absorbance at 400 mμ - absorbance at 430 mμ* With TGCA	Without TGCA	L-Fucose μg.	%
L-Fucose	0.0416	0.636 ± 0.0007	-		
L-Fucose	0.0831	1.266 ± 0.011	-0.007		
W	0.293	0.654 ± 0.005	-0.013	62.6	21.4
Y	0.396	0.938 ± 0.002	-0.020	61.6	15.5
Z	0.981	0.823 ± 0.009	-0.049	54.0	5.5

TGCA - thioglycolic acid

* corrected for a water blank of 0.015 which was the difference between the absorbance at 400 mμ and 430 mμ.

The estimation of galactose Galactose was estimated in the blood-group substance samples by addition of [1-^{14}C] galactose before hydrolysis which was carried out at 100° in 2 N HCl in a volume of 10 ml. for 3 hr After Celite chromatography the galactose was estimated by the ferricyanide method. The results are shown in Table 14.

In one experiment (number 2 of table 14) aliquots (0.42 ml.) of each fourth tube were taken for counting and tubes 41 to 52 were pooled and taken for the galactose analysis as described previously. The remaining fractions were investigated for the presence of L-fucose. The contents of each fourth tube was taken to dryness in vacuo at 70° and water (8 ml.) was added to each tube. Aliquots (1 ml.) were taken for assay by the ferricyanide method. The results are shown in fig. 16. The recovery of fucose from the sample of blood-group substance W (3 mg.) was of the order of 0.3 mg., i.e. more than 50% of the fucose originally present in the sample had survived the acid conditions (2 N HCl, 100°, 3 hr.). It was therefore considered feasible to determine both fucose and galactose after the same hydrolysis.

The estimation of both fucose and galactose Two experiments were carried out in which both [1-^{14}C] fucose and [1-^{14}C] galactose were added to the glycoprotein before hydrolysis which was carried out as before in 2 N HCl for 3 hr. The fucose and galactose were estimated by the thioglycolic acid-H_2SO_4 method and the ferricyanide method respectively. The values obtained are listed in table 15.

The fucose and galactose contents of the blood-group substance samples as determined in the above experiments have been summarized in Table 16.

TABLE 14

The Estimation of D-galactose in Blood-Group Substances

Experiment	Blood-group substance	Loss on drying* %	Dried* sample taken mg.	[1-^{14}C] Galactose added mg.	Specific activity of [1-^{14}C] galactose cpm/μg Added	Recovered	Galactose content mg.	%
1	W	11.2	2.624	0.942	47.3	27.7	0.665	25.4
2	W	11.9	3.054	0.89	53.1	28.7	0.754	24.7
3	Y+	16.0	6.77	1.78	48.7	23.8	1.86	27.5
4	Y	15.7	2.296	0.445	47.9	18.8	0.693	30.2
5	Z	15.9	3.45	1.035	51.0	27.5	0.884	25.6
6	Z†	15.9	3.45	1.035	54.7	32.75	0.694	20.1
7	Z	16.2	3.59	1.035	48.9	25.6	0.984	26.2

\+ The added [1-^{14}C] galactose had not been purified by Celite chromatography.
* After drying over P_2O_5 at 78° in vacuo.
† Hydrolysed for 2.5 hr. in 0.28 N HCl.

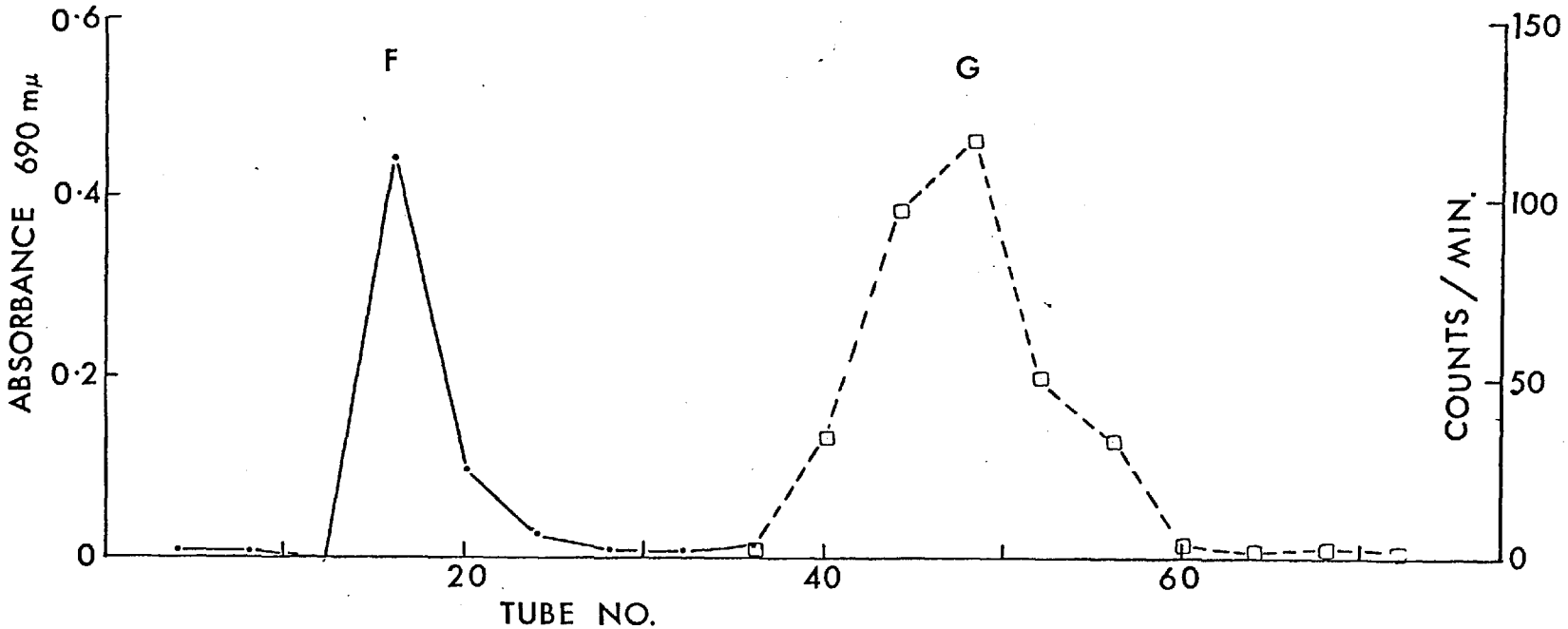


Fig. 16. Celite chromatography of fucose (F) and galactose (G) from a blood-group substance sample after addition of [1-^{14}C] galactose; _____absorbance; ------, radioactivity.

TABLE 15

The Estimation of both L-Fucose and D-Galactose in Blood-Group Substances

Blood-group substance taken mg.	Radioactive sugar added mg.	Specific activity cpm/µg. Added	Recovered	Sugar content in sample mg.	%
Y, 1.209	L-Fucose, 0.222	47.0	26.9	0.166	13.7
	D-Galactose, 0.338	66.6	30.0	0.412	34.1
Z, 3.924	L-Fucose, 0.177	47.0	20.5	0.229	5.8
	D-Galactose, 0.676	66.6	25.2	1.111	28.3

The values obtained from determining both sugars together in blood-group substance Y are comparable to those obtained in single experiments previously. The value for the galactose content of Y substance in particular was much greater than that from assays on the single sugar.

TABLE 16

Summary of the Neutral Sugar Estimations in the Blood-Group Specific Substances

Sugar estimated	Sample	Sugar content %				Values from Prof.W.T.J.Morgan,FRS	
		Colorimetric assay	Radioisotope dilution 1 sugar added	2 sugars added	Mean value	L-Fucose	Reduction as glucose
L-Fucose	W	21.4	18.7,18.8	-	18.8	17	59
	Y	15.5	15.2,14.3	13.7	14.4	16	50
	Z	5.5	5.15	5.8	5.5	8.4	51
D-Galactose	W		25.4,24.7		25.1		
	Y		27.5,30.2	34.1	30.6		
	Z		25.6,26.2	28.3	26.7		

The estimation of sugars after paper chromatography

Introduction. Since the chromatography of sugars on columns of Celite is a long process for the assay of several samples, paper chromatography was used as an alternative means of separation. Paper chromatography has often been used to separate monosaccharides for analysis (see Kowkabany 1954, Whistler and BeMiller 1962, Hough and Jones 1962).

Sugar mixtures have been analysed by spraying paper chromatograms with sugar reagents and comparing visually or instrumentally the intensity of the coloured spots of the unknown sample with those produced by known amounts of the sugar standards (e.g. McFarren et al 1951, Gottschalk and Ada 1956, Rosevear and Smith 1961). The coloured spots have also been eluted and the absorbance of the solutions measured spectrophotometrically (Leloir 1951, Wilson 1959, Pusztai and Morgan 1961). The other method used is to cut out the paper section containing the sugar and to elute the sugar from the paper with water (Flood et al 1947). A disadvantage of the method is that impurities eluted from chromatographic paper give high blank values. Prior washing of the paper at least partly overcomes this problem (Dubois et al 1956). Acid-washed paper (Bourrillon et al 1959) and paper washed with the developing solvent and water (Spiro 1960) have been found satisfactory with the Nelson-Somogyi method. The solvent system used for developing the chromatogram must be chosen so that residual solvent or solvent impurities left on the paper after drying, do not interfere with the method of estimation. Solvents used have included n-butanol, pyridine, 0.1 N HCl (5:3:2, by vol., Bourrillon et al 1959), n-butanol, ethanol, water (10:1:2, by vol., Spiro 1960; 4:1:5, Chatterjee and Montgomery 1962), n-butanol, acetic

acid, water (12:3:5, by vol., Clamp and Putnam 1964), ethylacetate, acetic acid, ethanol, benzene, water (Rosevear and Smith 1961) and ethylacetate, pyridine, acetic acid, water (7.5:5:3, by vol., Pusztai and Morgan 1961a).

Elution of sugars from the paper is usually carried out at room temperature either by immersing the paper in a known volume of water (Dubois et al 1956, Spiro 1960, Chatterjee and Montgomery 1962) or by developing the paper with water at room temperature (Laidlaw and Reid 1950) or at 0° (Bourillon et al 1959).

After elution from paper with water the sugar has been estimated by the phenol-H_2SO_4 method (Dubois et al 1956, Chatterjee and Montgomery, 1964) the Nelson-Somogyi procedure (Bourrillon et al 1959, Spiro 1960, Clamp and Putman 1964), the alkaline ferricyanide method (Montreuil and Scheppler 1959) or by O-aminobiphenyl in glacial acetic acid (Timell et al 1956). Aniline hydrogen phthalate was used to estimate sugars eluted from paper by refluxing methanol (Blass et al 1950).

Experimental. Aliquots (20 µl.) of an approximately 5% solution of mannose were applied in 2 inch lines to Whatman chromatography paper. Development of the paper was carried out overnight with the solvent ethyl acetate-n-propanol-water (4:1:2 by vol., upper phase). The paper was dried at room temperature and the position of mannose on the paper was found by treating guide strips with aniline hydrogen phthalate reagent (Partridge 1949) or 4% O-aminodiphenyl in acetic acid (Timell et al 1956). The appropriate areas of the unsprayed sheets were cut out and the mannose was eluted with 25% (v/v) ethanol at 4° in an apparatus similar to that described by Aminoff and Morgan (1951). Corresponding areas on blank

sheets were treated similarly for controls. Aliquots (0.5 ml.) were taken for counting at infinite thinness and for reducing power by the ferricyanide method.

The results obtained were unsatisfactory due to high blanks and variability of up to 10% in the specific activity of the recovered mannose. Various Whatman chromatographic papers were tried: No. 1 washed with water and ethanol, No. 540, No. 541 and No. 54 paper washed successively with 1% acetic acid, water and ethanol. Another solvent, n-propanol-water (4:1 by vol.), gave similar results. Washed paper gave lower blank values. The method of assay, although very sensitive, was thought to be too unspecific to be sufficiently reproducible for the estimation of small amounts of sugars eluted from paper. A method more specific for aldoses but less sensitive, the O-aminodiphenyl method (Timell 1956), was then used as it has been found applicable to the assay of sugars from paper chromatograms.

Accordingly [1-^{14}C] mannose (391 µg.) was submitted to chromatography as above and assayed by the O-aminodiphenyl method. The results are shown in Table 17. The specific activity of the mannose recovered from paper showed a variation of about 1%, but it was much higher than the specific activity of the mannose applied to the paper. In order to remove any non-radioactive impurities, [1-^{14}C] mannose (400 mg.) was submitted to chromatography on a Celite 545 (50 g.) column (38 x 2 cm.) which was developed with the upper phase of ethylacetate, n-propanol, water (4:1:2). Fractions (20 ml.) were collected and fractions numbered 46 to 60 were pooled, concentrated to dryness and the mannose was recrystallized from methanol and ethanol.

TABLE 17

The Specific Activity of [1-^{14}C] Mannose after Paper Chromatography

Sample	Absorbance*	Mannose µg/0.5ml	Activity cpm/0.5ml†	Specific activity cpm/µg
Experiment 1.				
Blank 1	.009			
Blank 2	.009			
Blank 3	.006			
Mannose 1	.218	19.15	1,555	81.2
Mannose 2	.302	26.8	2,161	80.6
Mannose standard	.214	19.5	1,424	73.0
Experiment 2.				
Blank 1	.013			
Blank 2	.009			
Mannose 1	.603	55.8	4,435	79.5
Mannose 2	.557	51.5	4,065	78.9
Mannose standard	.207	19.5	1,494	76.6

* Average of duplicates; absorbances read at 380 mµ and corrected for that given by 25% ethanol (.006 and .010 for the two determinations respectively).

† Average of four planchets, corrected where appropriate for either background or chromatographic blanks which amounted to 23 to 25 counts per min.

The destruction of mannose in 2 N HCl in the presence of various substances

In view of the possibility of preferential destruction of added radioactive sugars in the early stages of the acid hydrolysis of glycoproteins, it was of interest to investigate the effect of various substances on the destruction of mannose in 2 N HCl. Tryptophan is known to be destructive of sugars but no quantitative data are available. Ribose was included to see if it afforded any protection to the mannose. Poly-L-tryptophan was also included to slow the effect of bound tryptophan but since it is insoluble in water it was a poor model substance. Lysozyme was used to imitate the peptide part of glycoproteins.

Pyrex test tubes (16 x 150 mm.) were rinsed with sodium dichromate in sulphuric acid followed by water and dried at 100°. To each of six tubes was added 0.5 ml. of mannose solution (5.36 µmole/ml., 483 µg.) and 1 ml. of 6 N HCl. Further solutions were added as shown in the table.

Tube Number	Substances added before hydrolysis
1	None
2	Tryptophan (2.83 µmole, 575 µg.)
3	Tryptophan (2.83 µmole) and D-ribose (5.3 µmole, 797 µg
4	Poly-L-tryptophan (4.52 µmole, 840 µg.)
5	Lysozyme (7 mg.) which contains tryptophan (2.94 µmole) and cystine (1.96 µmole)
6	None. Not heated

Poly-L-tryptophan (0.84 g.) was added as the solid as it is insoluble in water. The contents of each tube was made up to 3 ml. with water and then frozen. The necks of the tubes were constricted, the tubes were evacuated to about 0.2 mm Hg and the frozen contents were allowed to thaw. When all

gas had been removed from the solutions the tubes were sealed. Tubes 1 to 5 were heated in a boiling water bath for 3 hr. 10 min., cooled and opened. [1-^{14}C]-Mannose (478 µg, 2.68 µmole in 0.5 ml.) was added to each tube. The contents of each tube was de-ionized in the usual way with Dowex 1 (bicarbonate form) and Dowex 50 (H^+ form) and taken to dryness in vacuo. Boiled distilled water (0.05 ml.) was added to each residue and 20 µl. aliquots were taken for chromatography as described above. The mannose on chromatography moved distances of 3 cm. to 5 cm. from the origin differing with each chromatogram. About 5 ml. of 25% ethanol was collected for each section of paper eluted.

The results are tabulated in Table 18. The specific activity of the [1-^{14}C] mannose, before (77.3 cpm/µg) and after (78.1 cpm/µg) chromatography, differed by about 1% which is probably not significant. The specific activity, calculted from the known dilution with cold mannose in tube 6 which was not heated, was similar (78.2 cpm/µg). Since the contents of tube 6 were subject to all the processes of the other tubes, except heating, this specific activity was used to calculate the recovery of mannose.

Discussion. The destruction of mannose when heated alone in 2 N HCl at 100° for 3 hours in an evacuated sealed tube was about 4% which is less than would be expected from the figure of 23% reported by François et al. (1962) for a 5 hr. heating period. The difference may be due to the absence of oxygen in the present experiment. The effect of adding tryptophan in equimolar amount to the mannose resulted in 15% destruction of mannose. In the presence of more tryptophan the destruction of mannose is likely to be greater since Francois et al (1962) found a

TABLE 18

The Recovery of Mannose Heated in 2 N HCl in the Presence of Various Substances

Tube No.	Absorbance* at 380 mμ	Mannose g./0.5ml	Activity† cpm/0.25ml.	Specific activity cpm/μg	Mannose+ recovered μg	Recovery of mannose %
1	0.305±.001	29.6	586 ± 1	39.6	466	96.5
2	0.492 ± .002	47.7	1,005 ± 4	42.1	410	84.9
3	0.559 ± .003	54.2	1,092 ± 6	40.3	450	93.1
4	0.349 ± .002	33.8	668 ± 2	39.5	468	97.0
5	0.554 ± .001	53.7	1,077 ± 8	40.1	454	94.0
6	0.466 ± .002	45.2	880 ± 5	38.9	483	100
Man.	0.496 ± .003	48.1	1,858 ± 4	77.3		
Man. (chrom.)	0.260 ± .001	25.2	984 ± 7	78.1		

* Average of triplicate with standard error of the mean. Readingshave been corrected where appropriate for 25% ethanol blank of 0.005 or for the paper blank of 0.007 (average of 0.008, .007, .007, and 0.005 for each from a blank section of paper).

† Values are means and their standard error from 3 or 4 planchets each counted for 30,000 counts. Corrections have been made where appropriate for a background count of 23 cpm or for a blank paper count of 24 cpm.

\+ The mannose recovered = $478 \left(\frac{78.2}{\text{spec.act.}} - 1 \right)$.

destruction of 42% of mannose in the presence of a vast excess of cysteine. It is interesting that the addition of two molar equivalents of ribose reduced the destruction of mannose by tryptophan from 15 to 6%.

Since the poly-L-tryptophan remained insoluble during the course of the heating period it is not surprising that it did not affect the destruction of mannose. The presence of lysozyme did not have much affect on the destruction of mannose. The tryptophan content of the lysozyme amounted to 2.94 μmole and the cystine content to 1.96 μmole (Canfield 1963). It would appear that the destruction of sugar caused by the peptide-bound amino acids, tryptophan and cysteine, are less than that caused by the free sugars, as predicted by François et al (1962).

The estimation of L-fucose in blood-group substances by paper chromatography

The eisotope dilution method was applied to the estimation of fucose in a blood group substance after separation of the sugars by paper chromatography. In contrast to the previous experiments with the blood group substance samples the dry-weight of the glycoprotein was determined on a separate sample. Hydrolysis was carried out in an evacuated sealed tube. Blood group substance Z (8.37 mg.) was dissolved in water (4.96 ml.) An aliquot (2 ml.) was taken to dryness in vacuo over NaOH and H_2SO_4 and the residue was further dried over P_2O_5 in vacuo at 92° for periods of about 2 hr. until constant weight was obtained (3.097 mg., 3.092 mg.). The concentration of sample in solution was therefore 1.546 mg/ml.

A mixture of sample (1 ml.) and [1-^{14}C] fucose (0.118 mg. in 0.1 ml.) was hydrolysed in 2 ml. of 0.2 M H_2SO_4 for 2 hr. in an evacuated, sealed tube as described previously. Chromatography of the sample and standard fucose was carried out as before and the fucose was eluted from

the chromatogram by suspending the excised paper in boiled distilled water (5 ml.) at 2° overnight. After centrifuging the solutions, samples (0.5 ml.) were withdrawn for assay (Gibbons 1955) and counting at infinite thinness.

	Absorbance 400-430 mμ	Fucose content μg/0.5 ml	Activity cpm	Specific Activity cpm/μg
W	.009 ± .002			
Bl.1	.008 ± .001			
Bl.2	.009 ± 0			
Z	.298 ± .001	9.00	281 ± 2	31.2
Fucose	.577 ± .003	17.69	1034 ± 7	58.4

The recovery of fucose was $(\frac{58.4}{31.2} - 1) = 102.9$ μg. or 6.66% fucose in the sample.

III THE ESTIMATION OF HEXOSAMINES IN GLYCOPROTEINS BY RADIOISOTOPE DILUTION

1. Introduction

(a) Hydrolysis

Methods for the estimation of hexosamine in glycoproteins require prior hydrolysis of (i) the glycosidic linkages and (ii) the acetamido groups (in that order) to give the free amino sugars. Incomplete release of hexosamine due to hydrolysis of the acetamido groups of N-acetylhexosaminides and conditions favourable to this pathway have been discussed earlier. Maximum values for hexosamine content of several glycoproteins have been obtained by the use of 4 N HCl at 100° for 3 to 6 hr. Hydrolysis of egg albumin in 5 N HCl for 3 and 6 hr. (Sorensen 1938) and 5.7 N HCl for 3 hr. (Johansen et al 1961) gave similar values. With 5.7 N HCl for 6 hr. a lower value was reported probably due to some destruction of the released amino sugar. The method of isotope dilution is much less influenced by destruction of the released sugar than by incomplete release of the sugar, so it should be applicable to the estimation of hexosamine in glycoproteins after hydrolysis under strongly acid conditions. In this work constant boiling HCl was used at 100° for 6 hr.

(b) Methods for estimation of 2-amino hexoses

The original method of Elson and Morgan (1933) which involves heating the amino sugar with acetylacetone in alkaline solution followed

by reaction in acid with p-dimethylaminobenzaldehyde to give a red colour, has had many modifications and is the most widely used method. The main chromogen formed is 2-methylpyrrole (Cornforth and Firth 1958) which is steam volatile (Schloss 1951). The principal change, which was made to the method, involved distillation of this chromogen from the alkaline reaction mixture into Ehrlich's reagent (Cessi 1952). This modification is more specific for hexosamine as less interference is caused by amino acids in the presence of hexoses, and muramic acid and 3-methylglucosamine do not give a colour (Johansen et al 1960, Cessi and Piliego 1960).

Other methods for hexosamine estimation include (a) deamination of the amino sugar by nitrous acid, and estimation of the 2,5-anhydro-hexose formed, by reaction with indole in ethanolic hydrochloric acid (Dische and Borenfreund 1950) or with other reagents, e.g. phenol-H_2SO_4 (Lee and Montgomery 1961a); (b) estimation of the ammonia released from 2-amino sugars in alkali (Tracey 1952); (c) estimation of N-acetyl-hexosamine by the Morgan-Elson method after N-acetylation (Morgan and Elson 1934, Aminoff et al 1952, Reissig et al 1955, Roseman and Daffner 1956, Levvy and McAllan 1959).

(c) <u>The estimation of hexosamines in mixtures</u>

As both glucosamine and galactosamine are often present in glycoproteins, methods of differential estimation have been developed based on (i) different reactivities in colour reactions; (ii) specific enzymatic conversion of one hexosamine to a derivative; (iii) separation before estimation.

(i) Glucosamine and galactosamine give about the same colour in the Elson-Morgan reaction but in the Morgan-Elson reaction glucosamine gives four times the colour of galactosamine (see Foster and Horton 1959). A colorimetric estimation of the two amino sugars in a mixture was based on these findings (Roseman and Daffner 1956). The method was improved by adding borate to the alkaline acetylacetone solution of the Elson-Morgan reaction; the resulting colour intensities were reduced but galactosamine then gave 4 to 5 times more colour than glucosamine (Tracey 1955; Good and Bessman 1964). The Cessi modification of the Elson-Morgan reaction has been changed in order to determine D-galactosamine in the presence of D-glucosamine (Cessi and Serafini-Cessi 1963). If the coupling of the hexosamine with acetylacetone is carried out under anhydrous conditions in the presence of methylamine at 55° for 16 hr. D-glucosamine forms almost quantitatively 3-acetyl-2-methyl-5-tetrahydroxybutylpyrrole which neither reacts with Ehrlich's reagent nor forms volatile chromogens on heating at 100° in water or alkaline solution. D-Galactosamine, however, behaves differently giving rise to only about 30% of the corresponding isomer of the pyrrole above, and in addition to material which, on heating under mild alkaline conditions, produces the volatile 2-methylpyrrole. The yield of 2-methylpyrrole is greater than in the original method.

(ii) The principle of several differential estimations of a mixture of D-glucosamine and D-galactosamine relies on specific enzyme systems. A hexokinase acting on a mixture of the N-acetylated amino sugars has been used to specifically convert N-acetylglucosamine to the 6-phosphate (Slein 1952) which can be removed by cation-exchange

chromatography (Johnston 1963). Another method involves enzymatic conversion of D-glucosamine to N-acetyl-D-glucosamine-6-phosphate which is then estimated by the Morgan-Elson reaction (Luderitz et al 1964). Galactosamine, mannosamine and muramic acid are not substrates for the enzyme. Other methods include removal of D-glucosamine by a mixture of baker's yeast (Pogell and Koenig 1958), oxidation of D-glucosamine to D-glucosaminic acid by Pseudomonas fluorescans (Imanaga 1963) and estimation of D-galactosamine or N-acetyl-D-galactosamine in mixtures with galactose oxidase (Sempere et al 1965).

(iii) The separation of amino sugars for quantitative analysis has been achieved by chromatography on paper (Aminoff and Morgan 1951) and on cation-exchange resins (Gardell 1953). The best solvents for paper chromatography of the amino sugars appear to be pyridine: ethyl acetate: acetic acid: water (5:5:3:1 by vol, Fischer and Nebel 1955), n-butanol: pyridine: 0.1 N HCl (5:3:2 by vol, Masamune and Yosizawa 1956, and Bourrillon and Michon 1959). Glucosamine and galactosamine are commonly separated with practically quantitative recovery on sulphonated resins eluted with 0.30 - 0.34 N HCl (Gardell 1953, Rondle and Morgan 1955, Crumpton 1959) or with salt solutions (e.g. Pearson 1963).

Derivatives of the amino sugars have also been separated. The N-2,4-dinitrophenyl derivatives of glucosamine and galactosamine were separated at 2° on columns of Hyflo-SuperCel containing borate buffer (pH 9.7) and eluted with organic solvents (Annison et al 1951). The recovery of about 80% was improved by $NaBH_4$ reduction of the amino sugars before dinitrophenylation (Leskowitz and Kabat 1954). The phenylisothiocyanate derivatives of the two common hexosamines have been estimated

after separation by electrophoresis in molybdate buffer at pH 5 or tungstate buffer at pH 7 (Scott 1962). Gas chromatography of the tetra-O-trimethylsilyl-N-acetylhexosamines, with penta-O-trimethylsilyl-2-acetamido-2-deoxy-D-glucitol as internal standard, has been successfully applied to the determination of glucosamine and galactosamine in mixtures (Perry 1964).

2. [1-^{14}C] Labelled Hexosamines

The preparation of [1-^{14}C] D-galactosamine

Since [1-^{14}C] D-galactosamine was not commercially available it was synthesized from D-lyxose by the procedure of Kuhn and Kirschenlohr (1956) outlined by Brossmer (1962), and based on the method first used for the synthesis of D-glucosamine (Fischer and Leuchs 1903). [1-^{14}C] D-Galactosamine has been prepared by this method (Kuhn et al 1959) and this procedure was followed. The synthesis involves the formation of lyxosylamine from lyxose in ammonia-saturated methanol and addition of H^{14}CN to form the nitrile, which is hydrogenated in dilute acid in the presence of a palladium catalyst to give galactosamine hydrochloride. D-Lyxose was synthesized from calcium-D-galactonate by a Ruff degradation or purchased from Sigma London Chemical Co., London.

D-Galactosamine hydrochloride

Dried lyxose (1 g.) was dissolved in 1 ml. of anhydrous ammonia-saturated methanol. After three days the crystalline material was freed of supernatant, washed with methanolic ammonia and dried in vacuo. Pyridine (6 ml.) and anhydrous HCN (1 ml.) were added and the suspension was shaken overnight. The white insoluble material was centrifuged down, washed with methanol and dried in vacuo. Yield 430 mg., m.p. 109-110° Dec.(uncorr.). (cf. m.p. 107-109°, Brossmer 1962).

The nitrile was dissolved in 0.5 N HCl (10 ml.) and hydrogenated with Pd-$BaSO_4$ catalyst (600 mg., prepared by the method of Kuhn and Haas (1955) and hydrogenated prior to use, taking up 6 ml. H_2). The hydrogen uptake was about 54 ml. (see Fig. 17). The catalyst was removed by centrifuging and the solution was taken to near dryness in a rotary evaporator. Methanol (2 ml.) was added to the crystalline residue and the insoluble crystals were filtered off. Addition of ethanol gave crystalline material (about 400 mg., m.p. 179-181°; Brossmer (1962) gives 178° for galactosamihe HCl).

On paper chromatography in the solvent system ethylacetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (Fischer and Nebel 1955) followed by spraying the chromatogram with ninhydrin the galactosamine HCl gave one spot at 22.5 cm. (authentic galactosamine HCl 22.3cm, glucosamine HCl 24.3 cm.). One peak only was observed in the amino acid analyser.

[1-^{14}C] D-Galactosamine

The procedure for the preparation of [1-^{14}C] galactosamine HCl was similar except that H^{14}CN, generated from a small amount of Na^{14}CN and saturated citric acid was passed as a gas into the suspension of lyxosylamine in pyridine and shaken for a day before addition of HCN. Since little incorporation of ^{14}C was obtained by bubbling the gas into the solution, the generation of H^{14}CN was carried out in a closed system under reduced pressure in the presence of lyxosylamine suspended in pyridine (see Fig. 17).

Lyxosylamine (120 mg.) was suspended in dry pyridine (0.8 ml.) and stirred magnetically in an evacuated tube having two side arms (see Fig.18)

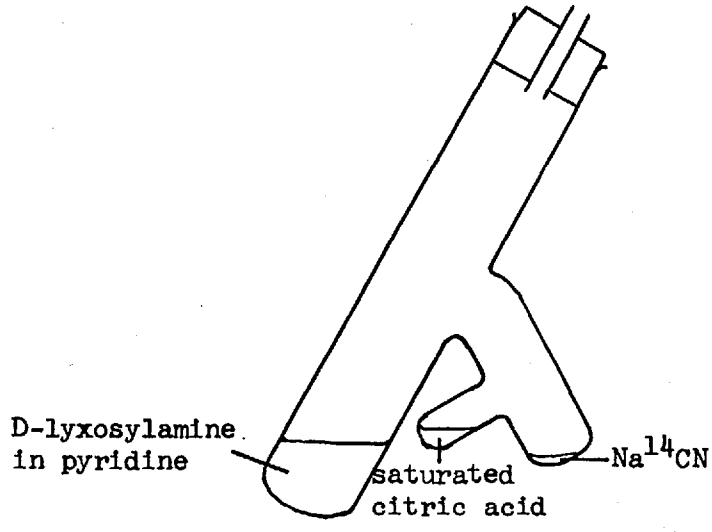


Fig. 18 The vessel used for the reaction between $H^{14}CN$ and D-lyxosylamine.

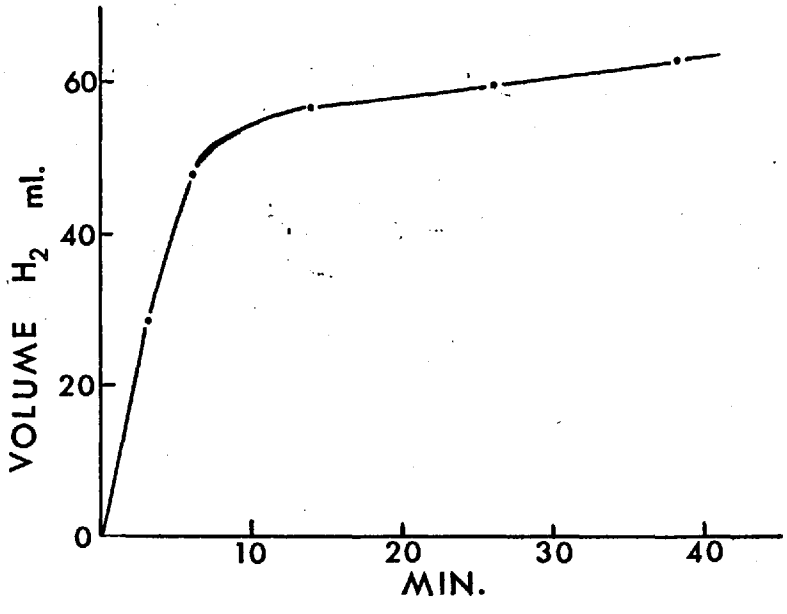


Fig. 17. The consumption of hydrogen by D-galactosaminic acid nitrile in the presence of a palladium catalyst.

containing saturated citric acid and $Na^{14}CN$ respectively. $H^{14}CN$ was generated by tilting the tube so that the citric acid solution flowed into the $Na^{14}CN$. After stirring the tube for 3 days HCN (0.4 ml.) was added. After stirring for another day the nitrile was isolated and hydrogenated as above. After taking the HCl solution to dryness, the residue was dissolved in water and applied to a Dowex 50 (200-400 mesh) H^+ form column (1 x 17 cm.). The column was washed with water, and developed with 0.5 N HCl. Fractions of about 2.5 ml. were collected and tested by the Elson-Morgan method for hexosamine. The most intense colour was obtained from tubes 10 and 11 which were accordingly pooled and dried. The crystalline residue was twice recrystallized from water and ethanol (13 mg., m.p. 169-174°).

[1-^{14}C] D-glucosamine hydrochlorde was obtained from New England Nuclear Corporation, Boston, U.S.A. It was diluted with D-glucosamine HCl, 0.05 mC to 250 mg., recrystallized by dissolving it in a minimum volume of water and adding ethanol and dried in vacuo over P_2O_5.

3. The Chromatography of Aminosugars on a Cation Exchange Resin

The most commonly used method for the separation of glucosamine and galactosamine obtained from hydrolysates of glycoproteins, is chromatography on columns of sulphonic acid resins developed with a constant concentration of dilute HCl usually about 0.3 N (Gardell 1953, Crumpton 1959. The concentration of HCl used gives good resolution with a reasonably rapid rate of elution. Although glucosamine and galactosamine can be separated on these columns without overlap, the peaks lie close together and other amino sugars, i.e. mannosamine and muramic acid, are eluted between them (Crumpton 1959). Also glucosamine and allosamine

are not separated from galactosamine. The order of elution of the 2-aminohexoses, glucosamine, mannosamine and galactosamine, cannot be correlated with their pK_a values, 7.85, 7.3 and 7.7 respectively (Neuberger, Marshall and Gottschalk 1966a) which is hardly surprising as in 0.3 N HCl the charged forms are the only species present. The separation found is likely to depend on factors which govern a very much weaker absorption of sugar to resin than that involving the charged amino group, and the acid concentration chosen is that which gives a sufficiently slow movement of the amino sugars on the column so that small differences in adsorption between the sugars can be expressed in their elution rates.

Chromatography of a mixture of [1-^{14}C] glucosamine and [1-14C] galactosamine on Dowex 50

A mixture of [1-^{14}C] glucosamine (0.182 mg.) and [1-^{14}C] galactosamine in 0.33 N HCl (0.2 ml.) was applied to a column (1 x 46 cm.) of Dowex 50 (200-400 mesh, x 4) well equilibrated in 0.33 N HCl. The column was eluted with 0.33 N HCl and 24 drop fractions were collected. Aliquots (0.05 ml) of the fractions were dried and counted. Fractions 19 to 22 (blank), 48 to 51 (A, glucosamine) and 54 to 57 (B, galactosamine) were pooled separately, taken to dryness in vacuo at 45° and the residues were dissolved in 5 ml. of water. Aliquots (0.5 ml.) were taken in triplicate for assay by the Elson-Morgan method and dried for counting at infinite thinness. The results are listed below.

Sample	Absorbance at 530 mμ	Absorbance	Hexosamine HCl μg/0.5 ml.	Activity cpm	Specific activity cpm/μg
Water	.031 ± .002	.001	-	30	0
Blank	.029 ± .000	-.001	-	30	0
A	.368 ± .003	.328	15.6	2,427 ± 25	154
Glucose HCl	.412 ± .002	.382	18.2	3,108 ± 13	169
B	.381 ± .001	.351	19.4	1,946 ± 3	99
Gal N.HCl	.277 ± .003	.247	13.7	1,469 ± 15	105.5

As the specific activity of the [1-^{14}C] D-glucosamine was 10% lower after chromatography it was submitted to chromatography on a larger column of Dowex 50.

Chromatography of [1-^{14}C] D-glucosamine

[1-^{14}C] D-Glucosamine (31 mg.) was submitted to chromatography on a Dowex 50 (200-400 mesh, X4, H^+ form) column (2.2 x 59 cm.) and eluted with 0.33 N HCl. Fractions (10 ml.) were collected and aliquots (.05 ml) of each fourth tube were dried and counted. The peak consisted of fractions 91 to 105 inclusive. Fractions (number 96 to 102) were pooled and taken to dryness. The yellow residue was redissolved in water and passed through a short column of Dowex 1-bicarbonate. The effluent was acidified with HCl, dried and crystallized from aqueous ethanol, m.p. 190° (decomp.)

The estimation of glucosamine in methyl β-N-acetylglucosaminide

Methyl β-N-acetyl-D-glucosaminide was separated by Celite chromatography from a mixture of the α- and β-anomers ($[\alpha]_D$ + 34°, m.p. 178-180°) kindly provided by Mr. V.D. Bhoyroo of this laboratory and

prepared according to Zilliken et al (1955). The anomeric mixture (424 mg.) was dissolved in the lower phase (1.12 ml.) of ethyl acetate-n-propanol-water (4:1:2, by vol.), mixed with Celite 545 (2.54 g.) and packed onto a Celite 545 column (65 g.) prepared as described previously. The column was developed with the organic phase of the solvent and aliquots (0.3 ml.) of each fourth fraction (10 ml.) were taken to dryness. The residues were dissolved in 3 ml. of water and the absorbances at 205 mμ were measured. The two main peaks, fractions 72 to 100 and 130 to 175, were pooled separately and taken to dryness. Crystallization was achieved with dry n-propanol giving the α-anomer (111 mg., $[\alpha]_D^{24^\circ}$ + 128° (c = 0.83, water), m.p. 194-195°) in the first peak and the β-anomer (173 mg., $[\alpha]_D^{24^\circ}$ -45.0° (c = 1, water), m.p. 197-200°) in the second peak. An elution diagram obtained from a small column of Celite is shown in Fig. 19.

Methyl β-N-acetylglucosaminide (0.5 ml, containing 0.543 mg.) and [1-^{14}C] D-glucosamine HCl (0.453 mg.) were dried in a pyrex test tube. Constant boiling HCl (2 ml.) was added, the solution was frozen and the tube was evacuated. The contents were thawed, the tube was sealed and heated in a boiling waterbath for 6 hr. The tube was cooled and opened and the contents were taken to dryness in vacuo over solid NaOH and concentrated H_2SO_4. Water (5 ml.) was added to the dried residue.

An aliquot (0.5 ml.) was taken to dryness and the residue was subjected to chromatography in n-butanol-pyridine-water (1:1:1 by vol.). The ninhydrin reagent revealed a single spot of large size at 26.6 cm. (galactosamine HCl 22.6 cm., glucosamine HCl 25.0 cm.).

For assay the solution was diluted 5 times and portions (0.5 ml.) taken for counting at infinite thinness and for assay by the Elson-Morgan

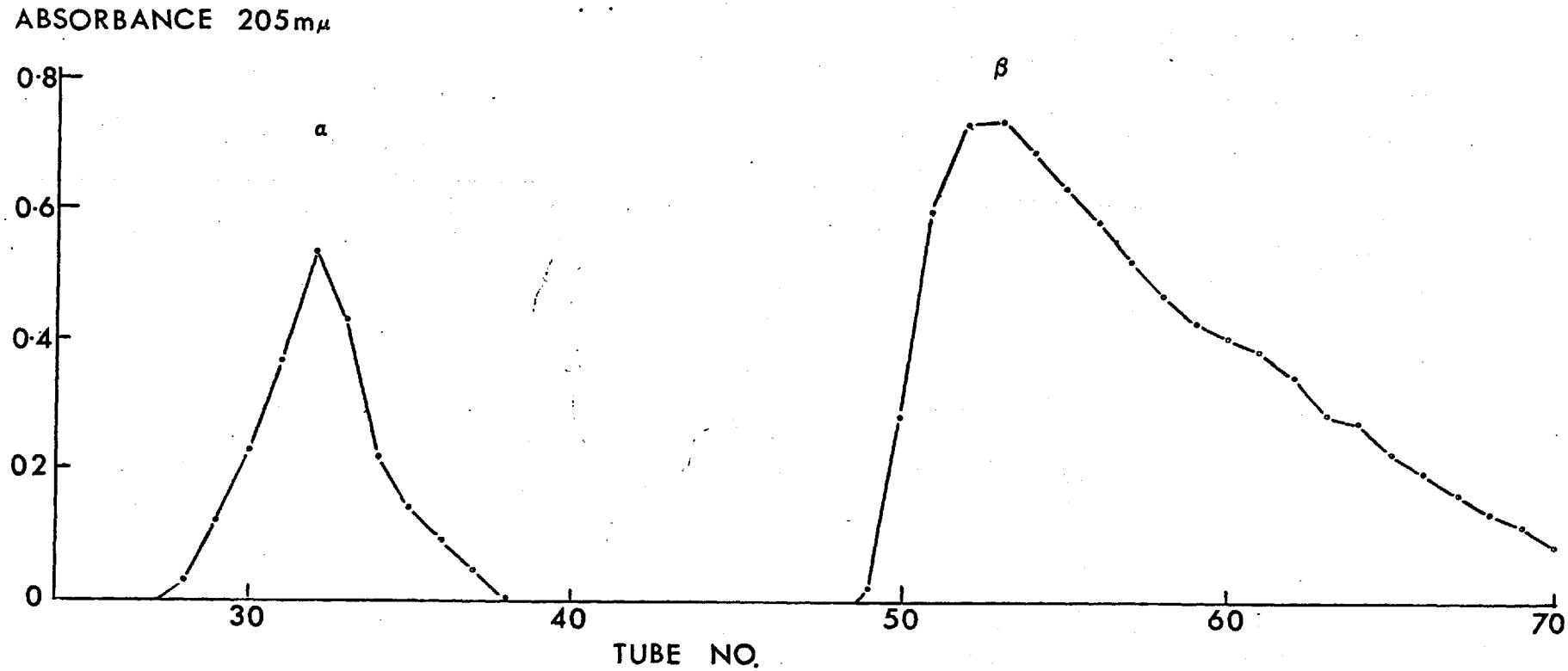


Fig. 19. The separation of the anomers of methyl N-acetyl-D-glucosaminide (0.4 mg.) on a column of Celite (14 g.). Fractions of 5 ml. were collected.

assay as modified by Kraan and Muir (1957). The results are shown in the table.

	Absorbance ± SE 530 mμ	Hexosamine HCl μg/ml	Activity ± SE cpm	Specific activit cpm/μg
Water	0.029 ± .001	0	27 ± 1	
Assay	0.396 ± .000	36.9 ± 0.7	1540 ± 11	41.0 ± 0.8
Standard	0.389 ± .005	36.2	3147 ± 24	85.7 ± 0.7

The amount of glucosamine HCl obtained from the methyl glycoside is $(\frac{85.7 \pm 0.7}{41.0 \pm 0.8} - 1)\ 453 = 494 \pm 20$ μg. (calculated 497.8 μg), i.e. an estimate of 99.2 ± 4.0%.

The total recovery of glucosamine as the hydrochloride = 36.9 x 25 = 922.5 μg (calculated 951 μg.), i.e. 97% recovery.

4. The Estimation of Hexosamines by Reaction with Naphthylisothiocyanate

The disadvantages of column chromatography are that only one or two columns can be run concurrently and that the running of the column and the testing of fractions takes considerable time. A recently described method for the determination of glucosamine and galactosamine in mixtures was based on electrophoretic separation of their arylisothiocyanate derivatives and seemed to offer the advantages of rapid separation and estimation of several samples concurrently.

The reactions between amino sugars and phenylisothiocyanate give rise to derivatives which may be determined by their absorption at 240 mμ. (Sjoquist 1959). The derivatives of glucosamine and galactosamine, although not separable by paper chromatography with the usual solvents, may be separated by paper electrophoresis in molybdate buffer at pH 5 or in

tungstate buffer at pH 7 (Scott 1962). The substances were originally thought to be the phenylthiocarbamoyl (PTC) derivatives but since complex formation with either molybdate or tungstate requires a cis-cis -1,2,3 triol system in cyclic sugars and at least four adjacent hydroxyl groups in acyclic sugars (Weigel 1963) the sugars were probably in the open chain form. As they also gave a red colour with dichloroquinone-dichloroimide reagent (Heath and Wildy 1956) unlike the yellow colour given by PTC derivatives and similar to the colour produced by thiol-imidazoles, a 4-tetrahydroxybutyl-1-phenyl-2-thiolimidazole structure was suggested (Scott 1962). However ultraviolet and infrared data were more in accord with a phenyl imidazolidine-thione structure, and, as $NaBH_4$ reduction of the phenylthiohydantoin of PTC-glucosaminic acid gave the same compound, the structure was given as 4-hydroxy-3-phenyl-5-tetrahydroxybutylimidazolidinethione (Scott 1964). See Fig. 20

The method of determination of a mixture of glucosamine and galactosamine as the phenylimidazolidinethione derivatives involved reaction with phenylisothiocyanate in pyridine-collidine buffer, electrophoresis in 0.05 M molybdate buffer pH 5, elution of the spots with 10% barium acetate and measurement of the absorption at 240 mμ (Scott 1962).

It was applied to the estimation of the amino sugars in hydrolysates of mucopolysaccharides. Derivatives of neutral and acidic amino acids were removed by anion exchange resins in the bicarbonate form and those of the basic amino acids migrated in the opposite direction to the amino sugar compounds on electrophoresis. Improvements in the methods were made by Dr. J.E. Scott of the Canadian Red Cross Memorial Hospital, Taplow and by Dr. A. Barrett, Strangeways Research Laboratory, Cambridge,

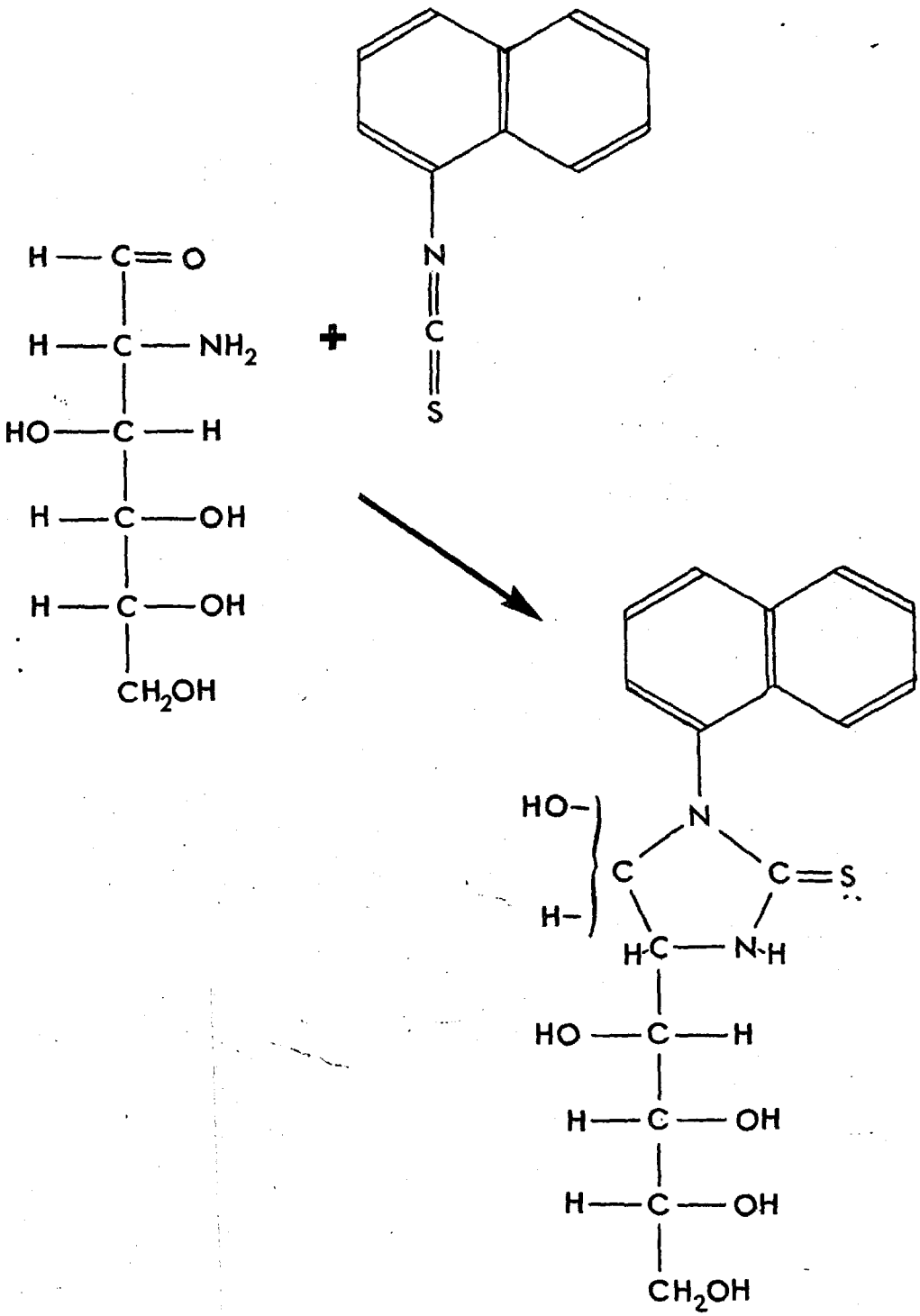


Fig. 20. The reaction between D-glucosamine and naphthylisothiocyanate.

who kindly made available the unpublished details. The sensitivity of the procedure was increased by formation of the naphthylisothiocyanate derivatives which had a higher molar absorbance at 240 mμ and an even greater absorbance at a peak at 222 mμ. Collidine in the buffer system was replaced by dimethyldodecylamine (DM 12D) which enabled the neutral and acidic amino acids to be extracted by benzene after the reaction. The reaction temperature was raised to 56° and the electrophoresis was carried out in tungstate buffer at pH 7. By heating the paper at 105° after electrophoresis the derivatives gave a yellow-green fluorescence which simplified detection. The method is as follows.

The hexosamine HCl solution (0.1 ml.) and the reagent (0.2 ml.), consisting of 4% (w/v) naphthylisothiocyanate in 5% (v/v) dimethyldocylamine (DM 12D) in pyridine are heated at 56° for 15 to 30 min., cooled and extracted twice with 1.2 ml. of benzene containing 1% DM 12D. Aliquots of the aqueous phase are submitted to paper electrophoresis in 0.05 M sodium tungstate-sulphuric acid buffer at pH 7.0. The paper is dried and the spots are marked out under a low wavelength ultraviolet lamp. The areas of each spot are cut out and eluted either by immersion in 3 ml. of 1% barium acetate at room temperature with occasional shaking or by heating at 100° for 10 min. in a solution containing barium acetate (1%) and barium chloride (1%). After centrifugation the absorbance is measured at 240 mμ or at 222 mμ.

The buffer system, used for coupling the naphthylisothiocyanate reagent, had a pH of 10.3 which is probably higher than that necessary for the reaction as glucosamine has a pK_a value of about 7.8. In the Edman method for amino-terminal amino acid analysis the phenylisothio-

cyanate reagent combines quantitatively with the peptide at pH 9 and 40° in 1 hr. (see Laver 1961). However for the preparation of the phenylthiohydantoins of the hydroxyamino acids serine and threonine, the reaction mixture was maintained at pH 8.6 at room temperature for several hours (Ingram 1953) as under the usual conditions a mixture of products is obtained, one of which for threonine is the dehydroderivative.

As dodecylamine (DM 12D) was not available for some time, other organic bases such as collidine (pK 7.5), triallylamine (pK 8.3) and the liquid anion exchange resins Amberlite LA 1 and LA 2 were tried as buffers and for extracting naphthylthiocarbamyl amino acids from the aqueous phase.

An amino acid mixture (0.1 ml.) containing ammonia and 17 amino acids at a concentration of 2.5 mM, was mixed with a reagent (0.2 ml.) consisting of 4% naphthylisothiocyanate and 5% Amberlite LA 1 in pyridine, and the solution (pH 9.3) was heated with shaking at 56° for 45 min. The solution was extracted with 1.2 ml. of benzene and taken to dryness. The residue was dissolved in water and subject to electrophoresis. A trail of material was observed up to 5.5 inches, the glucosamine derivative gave a spot at 3.4 in. After extraction of the solution with 5% LA 1 in n-heptane, followed by n-heptane, no spots were observed on electrophoresis. After similar extractions of a solution of the glucosamine derivative the recovery was about 92%. Using LA 2 similarly, nearly quantitative recovery was achieved.

It was not appreciated at the time that the liquid anion exchangers Amberlite LA 1 and LA 2 are in fact secondary amines which will presumably react with the naphthylisothiocyanate reagent. While they are useful in extracting excess reagent and naphthylthiocarbamyl amino acids from the

aqueous phase their use as buffers in the reaction will lead to loss of reagent.

(a) Electrophoresis of the Napthylisothiocyanate Derivatives of D-Glucosamine and D-Galactosamine

Naphthylisothiocyanate originally obtained from Hopkins and Williams Ltd., Chadwell Heath, Essex, was recrystallized from ethanol. It is now available from Kodak Ltd., Kirkby, Lancs. Pyridine was refluxed over KOH and distilled. Collidine was purified similarly. Benzene was allowed to stand for several days over 3 changes of concentrated H_2SO_4. It was washed twice with water, dried over KOH and distilled. n-Heptane was treated as for benzene. D-Glucosamine HCl (1.2 mg.) and D-galactosamine HCl (1.3 mg.) were each dissolved in water (0.1 ml.) in stoppered centrifuge tubes. To each tube was added the reagent (0.2 ml.), consisting of 4% naphthylisothiocyanate in 10% v/v collidine in pyridine. The tube was flushed with N_2, stoppered and heated at 56° for 30 min. Benzene (1.2 ml.) was added and the tube was shaken and centrifuged. The benzene layer was removed by a Pasteur pipette and an aliquot of the residual aqueous solution was taken for paper electrophoresis in 0.05 M sodium tungstate-H_2SO_4 buffer at pH 7.0. After electrophoresis at 3,000 to 2,500 volt for 1 hr. the paper was dried and examined under an ultraviolet lamp with maximum emission at 2570 Å. The glucosamine derivative showed a spot at 15 cm. and the galactosamine a spot at 8 cm. The spots were excised and eluted with 3 ml. of 1% barium acetate. The solutions were examined in the SP 700 Unicam recording spectrophotometer. Typical spectra given by the glucosamine and galactosamine derivatives after elution from paper are shown in Fig. 21

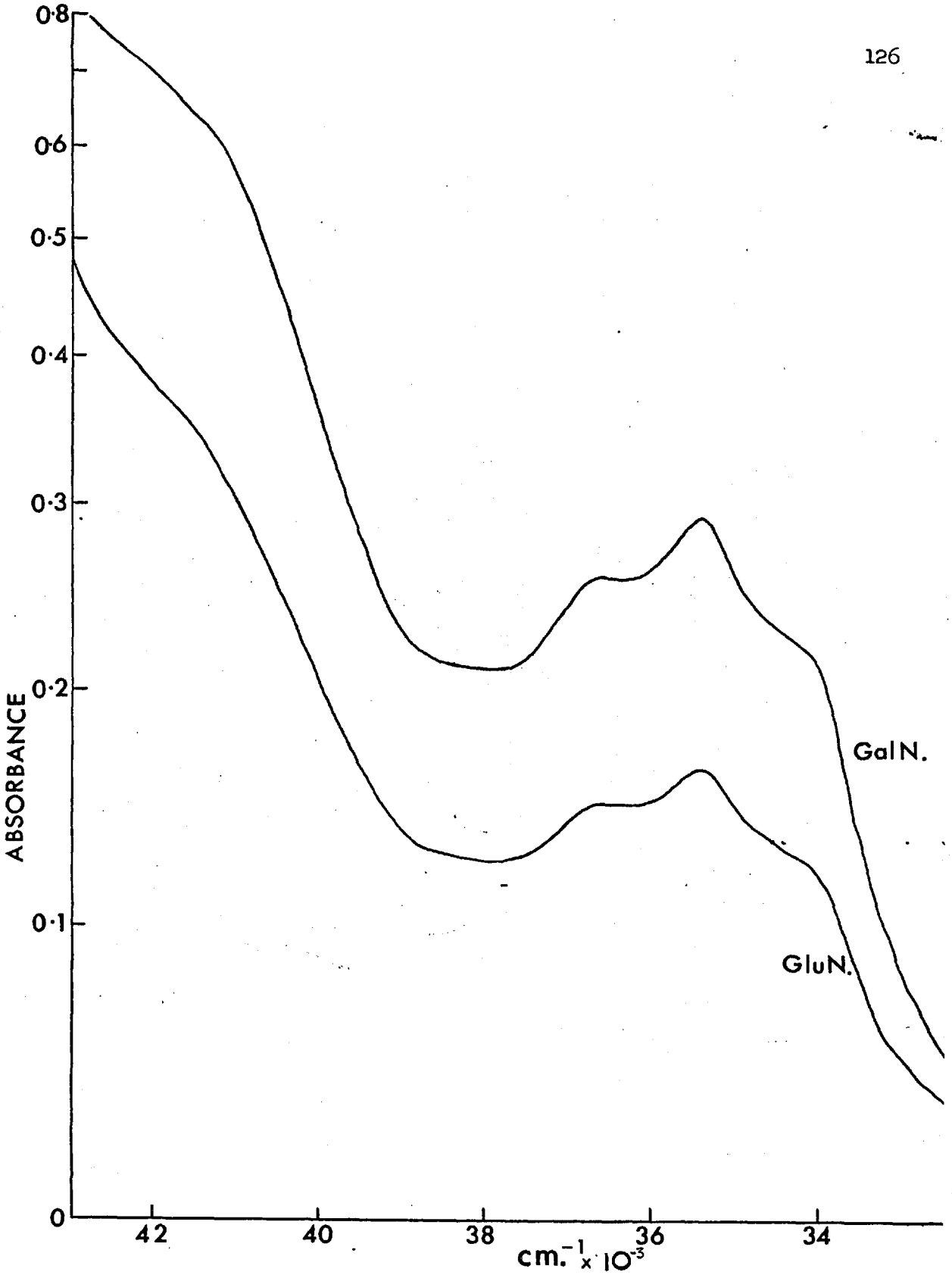


Fig. 21. Absorption spectra of the napthylisothiocyanate derivatives of D-galactosamine and D-glucosamine after elution from electrophoresis paper.

(b) Preparations of the Naphthylisothiocyanate Derivatives of D-Glucosamine

Recovery of D-glucosamine from Dowex 1-bicarbonate

As Dowex 1-bicarbonate effectively removed anions from hydrolysates of glycoproteins and gave quantitative recovery of the neutral sugars it was of interest to assess the recovery of amino sugars from this resin. A mixture of D-glucosamine HCl solution (2 ml. containing 562 µg) and 6.25 N HCl (2 ml.) was neutralized with Dowex 1-bicarbonate and passed through a column of the same resin. The effluent and washings were acidified with a drop of 0.33 N HCl and made to 25 ml. Aliquots (1 ml.) were assayed in triplicate by the Elson-Morgan method. The readings at 530 mµ were as follows: Water blank 0.025, column effluent 0.213 ± .003, glucosamine HCl (22.5 µg/ml.) 0.214 ± 0.001. Therefore complete recovery was obtained. Hartree (1964) obtained high yields of hexosamine when Dowex 1-bicarbonate was used for the removal of HCl from hydrolysates.

(1) D-Glucosamine HCl (0.64 g.) was dissolved in water and excess Dowex 1-bicarbonate was added. The suspension was filtered and the filtrate was mixed with naphthylisothiocyanate (640 mg.) in n-propanol. Pyridine (10 ml.) and collidine(2 ml.) were added and the solution was left at room temperature for 16 hr. The volume was reduced to 3 ml. on a rotary evaporator and extracted twice with benzene (8 ml. followed by 4 ml.). The aqueous solution was taken to dryness in a rotary evaporator giving off-white crystalline material which was recrystallized from a mixture of ethanol, n-propanol and acetone. After

drying over P_2O_5 in vacuo, material (0.74 g.) was obtained with melting point 154-157°.

The material (10.11 mg.) was dissolved in 50% v/v ethanol (10 ml.) and diluted by a factor of 10 with 1% barium acetate for spectrophotometry. The absorbance at 238 mμ and at 282 mμ was 0.471 and 0.193 corresponding to molar absorbance of 17,100 and 6,900 respectively. The material was sparingly soluble in methanol, ethanol, water and dioxane and very soluble in pyridine.

(ii) D-Glucosamine HCl (0.5 g.) and naphthylisothiocyanate (0.9 g.) in a mixture of water (10 ml.), pyridine (19 ml.) and DM 12D (1 ml.) were heated at 54-58° for 20 min. The solution was twice extracted with 1% DM 12D in benzene (180 ml. and 100 ml.) and once with n-heptane (100 ml.). The aqueous phase was taken to dryness and methanol (about 10 ml.) was added. Removal of the yellow methanol extract left white material which was recrystallized by dissolving in a small amount of pyridine and adding methanol, substance B (168 mg.). m.p. 165-170° (uncorr.) on Kofler block, $[\alpha]_D$ = +35.7° (C 1.16 in pyridine). After recrystallization from ethanol the material melted partly at 167° and completely at 215°.

The methanol extract standing for 2 days at 2° gave rise to coarse crystalline material which was filtered off, washed with ethanol and dried in vacuo, substance A (226 mg.) m.p. 215-225°, $[\alpha]_D$ = 101.5° (C = 1.15 in pyridine). The crystalline material was unexpectedly not very soluble in methanol. After recrystallization from methanol the melting point was 248-249°, and after a further recrystallization from ethanol the melting point was 230-232° (uncorr.) and $[\alpha]_D^{24}$ = 105.2° (C = 1.2 in pyridine).

Before recrystallization substances A (3.66 mg.) and B (4.49 mg.) were each dissolved in hot methanol (B.D.H., A.R.) and the volumes were made to 2,500 ml. with water to give a methanol concentration of 16%. The absorption spectrum of each solution was measured on a Unicam SP 700 recording spectrophotometer and found to be similar with molar absorbances at 222 mμ of 75,300 and 72,500 respectively, and at 240 mμ 18,900 and 18,900 respectively. After 2 recrystallizations the absorption spectrum of substance A in 1.1% methanol (2.60 μg/ml) was similarly obtained (see Fig. 22). The molar absorbances at 222 mμ, 240 mμ and 282 mμ were 73,000, 17,700 and 7,400 respectively.

5. Counting of Radioactivity

(a) Counting by liquid scintillation

The scintillators used in these experiments were a mixture of 2,5-diphenyloxazole (PPO) 1% and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) 0.05% or 2,5-bis-(5^1-tert.butylbenzoxazolyl(2^1))-thiophene (BBOT) (0.4% as recommended by the makers Ciba Limited, Basle, Switzerland). The composition of the scintillating fluid was described by Bruno and Christian (1961). This mixture (XDC) will take addition of an aqueous sample in amounts up to 16% of the scintillating fluid with an efficiency for ^{14}C of 55%. It is as follows: dioxane 429 ml., cellosolve 429 ml., xylene 143 ml., naphthaline 80 g.

Scintillation counting was done in a Tritomat 6020 automatic liquid scintillation counter (Isotope Developments Ltd., Reading) unless otherwise specified. Aliquots (0.5 ml.) of the aqueous solution containing the sugar to be counted were mixed with the scintillator fluid (6.5 ml.) in the counting vials.

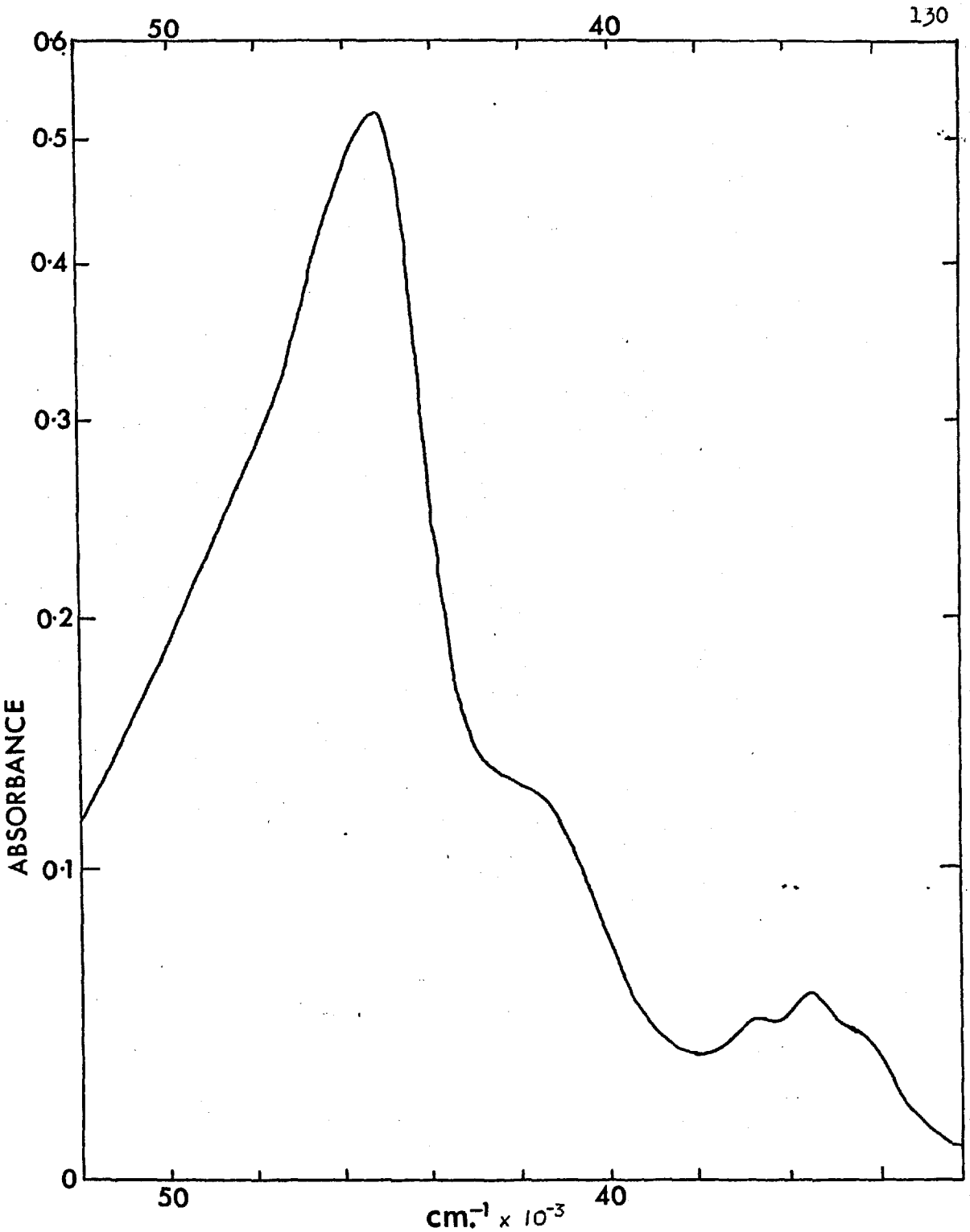


Fig. 22. The absorption spectrum of a purified product of the reaction between D-glucosamine and naphthylisothiocyanate.

Portions (0.5 ml.) in triplicate of a set of dilutions of [1-^{14}C] galactose in water and in 0.5% barium acetate were counted in the scintillation counter. The results are shown in the table.

[1-^{14}C] Galactose µg/0.5ml.	Water		0.5% w/v Barium acetate	
	Activity* cps	Specific Activity cps/µg	Activity cps	specific activity cps/µg
3.74	3.86 ± .05	1.039	3.75 ± 0.04	1.003
7.48	7.70 ± .14	1.032	7.54 ± 0.08	1.008
11.22	11.66 ± .04	1.029	11.21 ± 0.07	0.999
19.42	19.42 ± .04	1.039	18.69 ± 0.21	1.000

* corrected for background of 2.026 ± 0.032.

The average specific activity of the [1-^{14}C] galactose in barium acetate solution was 1.003 cps/µg compared to 1.036 cps/µg in water, i.e. a decrease of about 3.3%.

(b) Counting at infinite thinness

When the liquid scintillation counter was not available counting was performed at infinite thinness on the gas-flow counter. Since the 0.5% w/v barium acetate was used for elution of the naphthylisothiocyanate derivatives from paper after electrophoresis the effect of the salt on the counting of ^{14}C-labelled sugars was investigated.

Dilutions of [1-^{14}C] galactose were made in 0.5% barium acetate. Aliquots (0.5 ml.) were taken in quadruplicate, dried and counted for 45,000 counts each. The results are shown in the table.

[1-^{14}C] Galactose concentration µg/0.5 ml	Activity cpm	Specific activity cpm/µg	Deviation from mean %
18.7	745 ± 9	39.8 ± 0.5	0
11.22	438 ± 2	39.0 ± 0.2	- 2
7.48	302 ± 2	40.4 ± 0.3	+ 1.5
3.74	149 ± 2	39.8 ± 0.5	0

The average value was 39.8 cpm/µg and the deviations from this value are given in per cent. Therefore [1-^{14}C] galactose, when in a layer of about 1 mg. per cm^2 of barium acetate on a planchet, gives a linear relation between amount of sugar present and the number of counts registered.

To determine to what extent the barium acetate reduces the level of counting solutions of [1-^{14}C] galactose (15µg/ml) were made up in various concentrations of barium acetate and aliquots (0.5 ml.) were dried and counted for 36,000 counts each. The results are shown in the fig.[23] The efficiency of counting [1-^{14}C] galactose in samples obtained from 0.5% barium acetate solution is about 84% of that in the absence of salt.

6. <u>The Estimation of Galactosamine and Glucosamine in Blood-Group Specific Substances by Radioisotope Dilution</u>

Solutions of the samples of blood-group specific substances were prepared as described previously. For hydrolysis an aliquot of each solution was added to a test-tube containing a solution of [1-^{14}C] glucosamine HCl (143.9 µg) and [1-^{14}C] galactosamine HCl (92.7 µg.) and the solutions were taken to dryness in vacuo. Glycoprotein was not added to one tube. Twice distilled 6 N HCl (2 ml) was added to each tube and

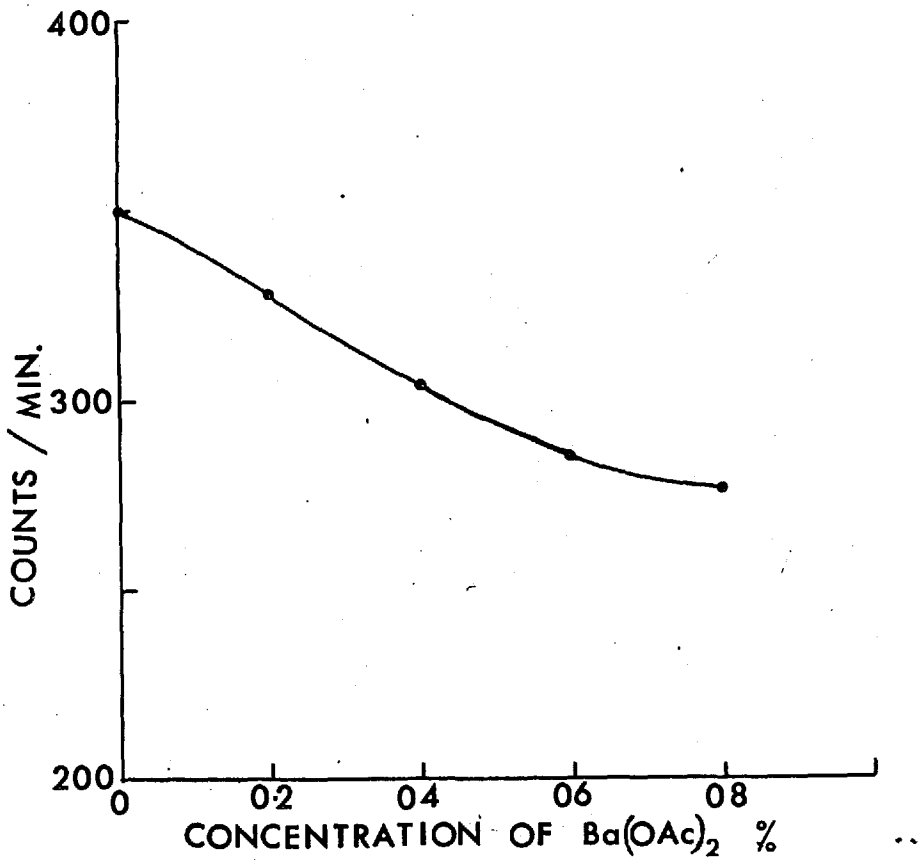


Fig. 23. The effect of barium acetate concentration on the counting of ^{14}C-labelled sugars at "infinite thinness".

the solutions were frozen. The tubes were evacuated and the solutions were thawed and refrozen. The tubes were sealed and heated in a boiling waterbath for 6 hr. After cooling the tubes were opened and the contents were taken to dryness in vacuo over H_2SO_4 and NaOH.

The residues were dissolved in 0.2 ml. of 50% pyridine and the solutions were transferred to stoppered centrifuge tubes. The reagent (0.1 ml.) consisting of 8% naphthylisothiocyanate and 10% Amberlite LA 1 in pyridine was added and the tubes were heated for 30 min. at 56°. After cooling the solutions were extracted successively with 1 ml. each of benzene, 5% LA 1 in n-heptane, and n-heptane. Aliquots (40 µl.) of the aqueous solution were applied as lines to Whatman No. 4 paper which was sprayed with 0.05 M tungstate buffer pH 7.0 and submitted to electrophoresis for 1 hr. at 2,400 volt falling to 1,500 volt with a current of 70-75 m.amp. The paper was dried in air and spots were observed under ultraviolet light at 8 cm. and 12.5 cm. respectively. For the samples containing blood-group substance spots also appeared at about 2 cm. from the origin. The areas indicated were cut out and eluted in 2.5 ml. of 1% barium acetate for 30 min. The tubes were centrifuged and aliquots (0.5 ml.) of the supernatant were mixed with 6.5 ml. of POPOP scintillator fluid and counted in a scintillation counter. To an aliquot of each supernatant was added 1 ml. of water and the absorbance at 240 mµ measured. The results, together with those from a repeated electrophoresis run, are shown in Tables 19, 20 and 21.

TABLE 19

The Estimation of Galactosamine in Blood-Group Substances

Sample	Wt mg.	Absorbance* 240 mμ	Activity† cpm	Specific activity cpm/abs	Galactosamine μg	Galactosamine %
Experiment I						
Gal N	0.0771	0.631	251.1 ± 9.0	398		
W_1	0.816	1.685	316.8	188	86.1	10.55
Y_1	0.6045	0.985	244.4 ± 0.9	248	46.4	7.68
Z_1	0.4905	0.793	189.9 ± 0.9	240	50.8	10.36
Experiment II						
Gal N	0.0771	0.612	242.7 ± 1.4	397		
W_1	0.816	1.464	273.0 ± 1.5	187	86.5	10.60
Y_1	0.6045	0.840	200.0 ± 4.5	238	51.3	8.49
Z_1	0.4905	0.440	110.4 ± 5.2	251	44.76	9.12

* corrected for 3 and 2 times dilution and blanks of 0.066 and 0.051 for experiments I and II respectively.

† corrected for blank counts of 133 cpm and 130 cpm for experiments I and II respectively.

TABLE 20

The Estimation of Glucosamine in Blood-Group Substances

Sample	Amount mg.	Absorbance 240 mμ	Activity cpm	Specific activity cpm/abs	Glucosamine μg.	Glucosamine %
Experiment I						
Glu N	0.1196	0.931	1,130 ± 7	1,214	[illegible]	[illegible]
W	0.816	3.09	1,488 ± 5	481.6	182	22.3
Y	0.6045	1.911	314.5 ± 1.0	164.6	128	21.2
Z	0.4905	1.458	277.3 ± 1.0	190.2	94.7	19.3
Glu N	0.1196	0.931	317.3 ± 5.8	340.8		
Experiment II						
W	0.816	1.192	332.7 ± 3.6	279.1	161.1	19.7
Y	0.6045	0.832	258.6 ± 6.5	310.8	132.5	21.9
Z	0.4905	0.387	150.7 ± 6.6	389.4	81.58	16.6
Glu N	0.1196	0.414	271.8 ± 1.6	655		

In experiment I, the first two items Glu N and W, the counting was carried out on a TriCarb liquid scintillation counter at 0°. The blanks had an absorbance of 0.092 and a background count of 28 cpm. The last three items of experiment I had blanks with an absorbance of 0.075 and a background of 132 cpm.

In experiment II, the blanks had an absorbance of 0.055 and a background count of 130 cpm.

TABLE 21

The Hexosamine Content of Blood-Group Substances

Sample	From present data				From Professor W.T.J. Morgan,F.R.S.	
	Galactosamine %	Glucosamine %	Total Hexosamine %	Ratio GluN/GalN	Total Hexosamine %	Ratio GluN/GalN
W	10.5, 10.6	22.3, 19.7	31.6	2.0	33	1.3
Y	7.7, 8.5	21.2, 21.9	29.7	2.7	28	4.0
Z	10.4, 9.1	19.3, 16.6	27.7	1.8	27	

The results obtained for total hexosamine in these samples are in general agreement with the values supplied by Professor W.T.J. Morgan,F.R.S. The greatest difference lies in the ratios of glucosamine to galactosamine.

7. The Estimation of Glucosamine in Rabbit γ-Globulin

(a) The Cessi Method

Rabbit γ-globulin (2.017 mg.) in 4 N HCl (1.5 ml.) was heated in sealed tubes at 100° for 4, 6 and 8 hr. The solutions were transferred to stoppered test tubes and taken to dryness in vacuo over H_2SO_4 and NaOH. For hexosamine analysis the Cessi method was reduced in scale to one half and the alkaline acetylacetone reagent of Kraan and Muir (1957) was used. The dried residues were dissolved in water (0.5 ml.) and a solution (2.5 ml.) of 4% acetyl acetone in 0.5 M Na_2CO_3 (pH 9.7) was added. The tubes were heated in a boiling water bath for 20 min. The contents of each tube was washed into a 10 ml. flask, which was fitted to a condenser, and distillation was commenced. Distillate was collected to the 5 ml. mark in a volumetric flask containing 3.5 ml. of 1% p-dimethylaminobenzaldehyde and 5% (v/v) concentrated HCl in redistilled ethanol. After about 30 min. the absorbance was read at 547 mμ in 1 cm. cells. In this method 14 μg of glucosamine gave a reading of 0.255 and the blanks were negligible (0.000 to 0.002).

Time of heating hr.	Glucosamine μg	Glucosamine mole/10^5g.
4	22.8, 21.4	6.32, 5.91
6	19.9, 21.5	5.51, 5.94
8	20.8, 20.9	5.74, 5.79

(b) The glucosamine content of rabbit γ-globulin by radioisotope dilution

Rabbit γ-globulin (200 mg.) was dissolved in water (about 5 ml.) and the solution was dialysed against 2 lots of 0.01 N HCl (2 l.) at 4°

during 3 days. The dialysed solution was frozen. An aliquot (0.1 ml.) was diluted with 0.01 N HCl to 10 ml. and the ultraviolet absorption spectrum was run. At 279 mμ the solution had an absorption of 0.410 corresponding to a γ-globulin concentration of 30.4 mg/ml. in the original solution.

A mixture of γ-globulin (0.5 ml.), [1-^{14}C] glucosamine HCl (180 μg. in 0.25 ml.) and 6.25 N HCl (18 ml.) was frozen in two pyrex tubes. The tubes were evacuated and the contents were allowed to thaw. The tubes were sealed, placed in a boiling water bath for a few minutes and transferred to an oven at 100° for 6 hr. One of the solutions boiled vigorously when it was first put in the boiling water bath, and at the end of the hydrolysis this solution was a pale golden colour. The other solution, which presumably had not been evacuated as thoroughly, was a light brown colour. No insoluble material was evident in either tube. The tubes were opened and connected to a rotary evaporator. When the volumes had been reduced to about 0.5 ml. the solutions were transferred to small open vessels and taken to dryness in vacuo over H_2SO_4 and NaOH. The residues were dissolved in water (0.75 ml.) and the solutions were transferred to stoppered centrifuge tubes.

Three other tubes were set up containing

(a) a solution (0.1 ml.) of [1-^{14}C] glucosamine HCl (72 μg.);

(b) a mixture of [1-^{14}C] glucosamine HCl (72 μg.) and glucosamine HCL (20.8 μg.) in water (0.35 ml.);

(c) water (0.15 ml.).

Naphthylisothiocyanate reagent (2 vol.) was added to each tube, the tubes were stoppered and heated at 56° for 15 min. The solutions were

extracted successively with 6 volumes each of 1% DM 12 D in benzene, benzene and n-heptane (1:1 by vol.), and n-heptane. During the second extraction some white insoluble material collected at the interface. Addition of 0.1 ml. of ethanol rendered it soluble. Aliquots were applied to Whatman "Benchcote" (Hemmens, 1965) previously washed with water and dried, and electrophoresis was carried out for 1 hr. at 2,500 volt which decreased to 1,500 volt with a constant current of 75 m amp. The paper was dried, heated to 100° for 2 or 3 min. and examined by ultraviolet light. All samples showed spots at the origin. The glucosamine samples showed fluorescent spots at 4 in. but the γ-globulin samples showed a fluorescent streak to 3 in. and a dark streak continuing on to about 5 in. The fluorescent areas were cut out and re-submitted to electrophoresis under the same conditions. For electrophoresis the excised pieces were placed across the paper over gaps cut out at the origin. Two bands were observed, one at 8 in. and one at 5 in. Areas of paper containing each spot were cut out and eluted each with 2.5 ml. of 1% barium acetate for 20 min. with shaking. Absorbances were measured at 222 mμ and counting was done at infinite thinness. The less mobile band gave a UV spectrum similar to that of naphthylisocyanate derivatives but was devoid of ^{14}C.

As considerable trailing was observed on the electrophoretic strips from the rabbit γ-globulin experiment mixtures of mannose (0.54 μmole), amino acids (0.125 μmole of 17 amino acids and ammonia) and glucosamine HCl (0.31 μmole) were taken to dryness, redissolved in water and treated with naphthylisothiocyanate reagent as before. The

TABLE 22

The Estimation of the Glucosamine Content of Rabbit γ-Globulin

Sample	Absorbance 222 mμ	Absorbance corr.	Activity cpm*	Activity/ abs.cpm/μg	Glucosamine μg.	res/10^5g.
Blank	0.393	0				
A	0.678	0.285	53 ± 1	186	156	5.7
Blank	0.394	0				
B	0.922	0.528	98 ± 3	186	156	5.7
Glucosamine	0.526	0.256	97 ± 1	379		
Glucosamine diluted	1.095	0.825	268 ± 3	325	25	
Blank	0.270	0	0			

* corrected for blank value of 21 cpm.

aqueous solutions obtained were applied to paper and submitted to electrophoresis. The electrophoresis strip is reproduced in Fig.24.

No spots were observed with either water or mannose alone. Glucosamine alone gave a single spot at 4.9 in. In the presence of mannose and amino acids the spot was much smaller and in addition two other spots were observed, one at 1.2 in. and a spot which had moved 0.5 in. toward the cathode. These two spots were also observed in a similar mixture without glucosamine.

Therefore in a mixture containing neutral sugars, amino acids and amino sugars the reaction with naphthylisothiocyanate produces substances of low electrophoretic mobility. These substances may be naphthylthiocarbamyl derivatives of the glycosylamines which could be formed in neutral and alkaline solution from mannose and amino acids. This may explain the origin of some of the trailing in the γ-globulin experiment, and the spots near the origin in the experiments in the blood-group substances which contain large amounts of neutral sugars. With γ-globulin it is also possible that peptides in the hydrolysate produce acidic naphthylthiocarbonyl derivatives which also interfere.

Therefore it seems desirable to remove neutral sugars from hydrolysates before the reaction with naphthylisothiocyanate. This can be done by absorption of amino compounds onto a cation exchange resin followed by elution with dilute HCl as used by Boas (1953) and by Dorfman et al (1955).

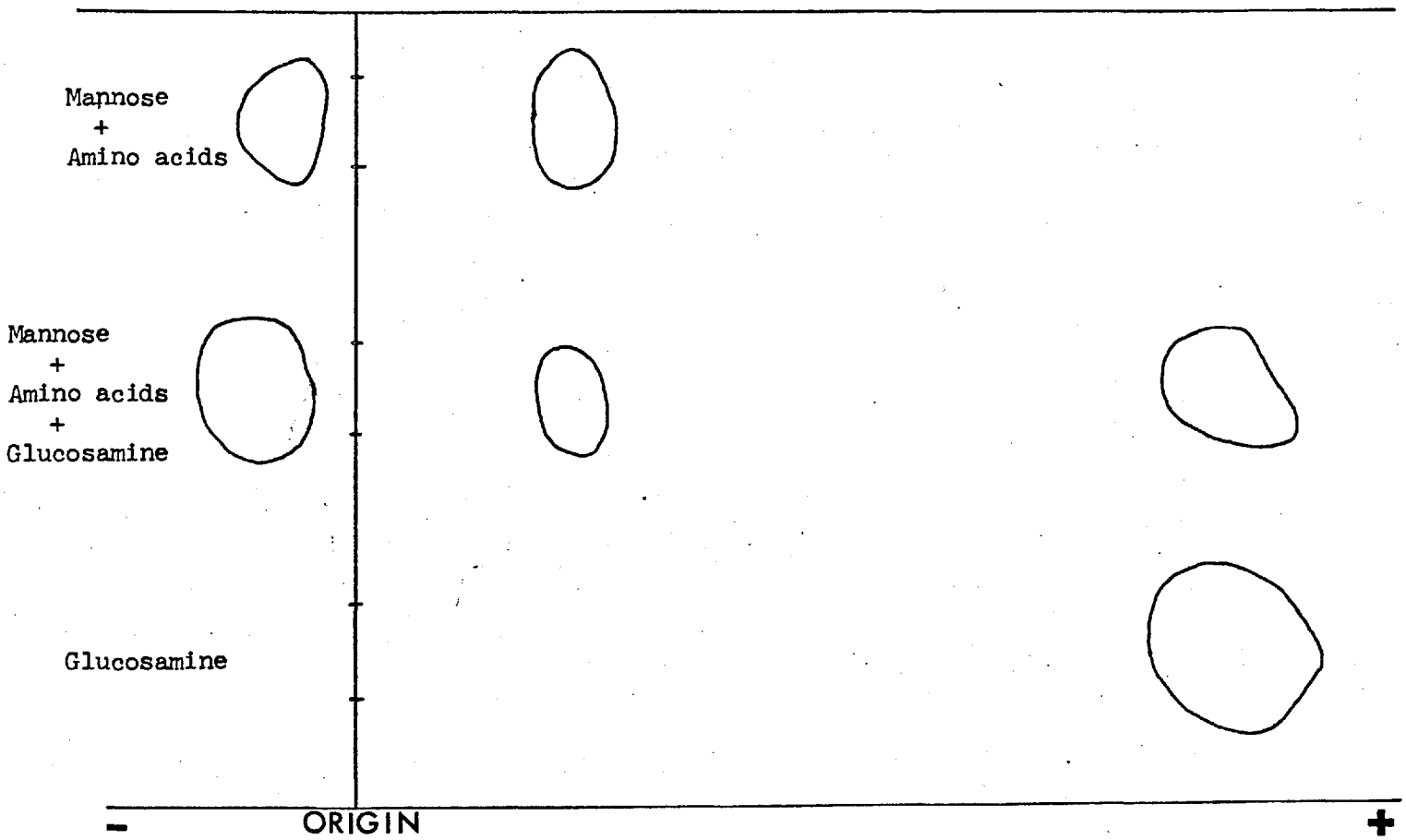


Fig. 24. Tracing of a strip after electrophoresis in .05 M tungstate buffer (pH 7) of the products of reaction between various compounds and naphthylisothiocyanate. Spots were revealed in ultraviolet light.

DISCUSSION

As all methods which are generally applicable to the estimation of sugars in glycosides require the sugars to be in the free state, acid hydrolysis is an essential preliminary operation. In the heterosaccharide units of glycoproteins, the four types of sugar which are commonly present i.e. hexose, 6-deoxyhexose, 2-amino-2-deoxyhexose and nonulosaminic acid, are each released by acid at a different rate due to (a) the different stabilities to acid of the glycosidic bonds of each sugar and (b) the location of the sugar residue in the carbohydrate chain, e.g. the mannose and N-acetylglucosamine residues in many serum-type glycoproteins occupy internal positions in the heterosaccharides and therefore require the cleavage of at least two glycosidic linkages for their release. The 2-amino-2-deoxy-hexoses occur in glycoproteins almost solely as N-acetylated residues and under the conditions of acid hydrolysis required for hexoside cleavage the acetamido group will also be split. Hydrolysis of an amide group adjacent to a glycosidic bond will greatly enhance the acid stability of the glycoside (Moggridge and Neuberger 1938), and therefore more severe conditions of hydrolysis are required for complete splitting of these linkages. The formation of acid stable hexosaminidic bonds during hydrolysis is less favoured with increase of acid concentration and temperature (Johansen et al 1960). For example, higher yields of hexosamine from glycoproteins are obtained after hydrolysis with 4 N HCl at 100° for several hours than after similar treatment with 2 N HCl (e.g. Johansen et al 1960, Marshall and Neuberger 1960, Spiro 1962). The use of acid more concentrated than 4 N HCl leads to increased destruction of the released amino sugars so even

if more hexosamine is released the yield is not greater. The destruction of the hexoses is appreciable even at 4 N HCl so that the maximum yields of hexose are obtained when acid concentrations of 2 N HCl or lower are used. Under such conditions it is possible that the release of hexose is not complete and that the residual bound hexose is joined to hexosamine residues. The amount of hexose which is not released will also depend on the number of glycosidic linkages involving both hexose and hexosamine. Therefore it seems that high acid concentrations are necessary for maximum release of hexose and 2-amino-hexoses from glycoproteins but unless the problem of destruction of the released sugar can be overcome, too high an acid concentration during hydrolysis is not practicable for quantitative purposes.

This problem is overcome for the hexoses in the colorimetric procedures in which the carbohydrate-containing substance is heated in concentrated sulphuric acid in the presence of a reagent such as a phenol, certain nitrogenous compounds, or a sulphydryl-containing compound (see Ashwell 1957, Dische 1962). In this method the hexoses are released and degaded by the acid to products which on formation give a colour with the reagent. The mechanism of the reaction is not well understood. The main chromogen produced from a hexose appears to be 5-hydroxymethyl-2-furaldehyde(5HF) together with formaldehyde, acetaldehyde and propionaldehyde (Rice and Fishbein 1956). The 5-HMF can undergo further breakdown in acid to levulinic acid and formic acid. The mechanism for the conversion of hexose to 5-HMF has been recently reviewed (Anet 1964). The first and probably rate-limiting step involves enolization at C_1 and C_2 after

protonation of the carbonyl oxygen. Elimination of the oxygen function at C_3 gives a 3-deoxyaldos-2-ene which has been isolated as the 3-deoxyglycosulose. A similar elimination at C_4 results in a 3,4-dideoxyglycosulos-3-ene which by cyclisation and elimination of water gives 5-HMF. It is possible that these carbonyl-containing intermediates also react with the reagent in the colorimetric procedures, since the ultraviolet absorption spectra of acid-treated sugars although similar to that of the 2-furaldehydes are not quantitatively related to the colour yield obtained when a reagent such as carbazole is present (Holtzman et al 1947).

The application of this type of method to the estimation of hexose in glycoproteins has not always given unequivocal results. Several of such methods applied to protein-bound carbohydrate gave different hexose contents although consistent values were obtained for pure carbohydrates (e.g. Sudhof et al 1955). Different hexoses give different colour yields so that the molar ratio of the hexoses in glycoprotein must be first determined before true standards can be prepared (Spik and Montreuil 1964, Neuberger and Marshall 1966a). Other sugars may interfere such as sialic acid in the anthrone reaction (Spiro 1960). Amino acids can also interfere in the reaction. For instance, tryptophan was found to decrease the colour in two colorimetric methods applied to the estimation of mannose in glycoproteins (Sørensen 1936, Hörmann and Gollwitzer 1962, Marshall 1964). The interference was eliminated either by oxidation with performic acid or by reading the absorbance at a wavelength where tryptophan did not interfere (Hörmann and Gollwitzer 1962, 1963). Use of a concentrated reagent showed very little tryptophan effect for mannose

estimation in egg albumin (Marshall 1964). However the general question of correction for non-specific blank values which are often high, has not been satisfactorily answered. Even if a mixture of the individual amino acids which occur in the glycoprotein in question is taken as control, the value obtained may not be identical with that given by these amino acids in peptide linkage (Marshall 1964).

In the methods which depend on the estimation of sugars after release from glycoproteins, the conditions of acid hydrolysis required to effect complete liberation of a sugar from its glycosidic linkage also brings about appreciable destruction of the released sugar, particularly in the presence of some amino acids such as cysteine (François et al 1962) and tryptophan as shown by the experiments recorded above. The destruction of mannose after heating in 2 N HCl for 3 hr. at 100°, as measured by isotope dilution after isolation of the sugar by paper chromatography, was found to be about 4%, a value less than that to be expected from the 23% destruction of mannose in 5 hr. reported by Francois et al (1962). The greater destruction observed by these authors may have been caused by the presence of oxygen as the solutions in the present experiments were heated in an evacuated sealed tube. Oxygen is known to cause increased destruction of hexosamines in hot acid (Walborg and Ward 1963). The orcinol-H_2SO_4 colorimetric method has been used to assess the destruction of various sugars in 1 N and 2 N HCl at 100° (Montreuil and Scheppler 1959), but as in the first 2 hr. of heating the colour yields were greater than those of the unheated sugars, the values obtained probably reflect not only the stability of the sugars but also the stability of the chromogenic acid-degradation products. The

values obtained by Haab and Anastassiadis (1961), who used the anthrone-H_2SO_4 method of estimation, are also unlikely to be an accurate measure of the destruction of the actual sugar. These authors heated the sugars in 4 N HCl and their values show increased rate of destruction with time of heating. If this situation occurs for the sugars it would suggest that the acid-degradation products cause increased destruction of the sugar.

A considerable loss of mannose occurred in the presence of tryptophan in equimolar amounts. The loss of mannose was much less in the presence of an amount of lysozyme which contained a similar amount of tryptophan and also some cystine. This finding is not unexpected because, as Marshall (1964) has suggested for colorimetric estimations, tryptophan alone is not a valid control for tryptophan occurring in a glycoprotein. The peptide-linked tryptophan in a glycoprotein may have a different reactivity so that in the free state and the presence of other amino acids such as cyst(e)ine and serine are known to cause destruction of tryptophan (see Hill 1965). Addition of two molar equivalents of ribose to the solution containing tryptophan reduced the destruction of mannose by about half presumably because it competes with the mannose in degradative reactions.

The optimum conditions of hydrolysis for the release of a particular sugar from a glycoprotein will be such that a compromise is obtained between the extent of cleavage of the appropriate glycosidic bonds and the amount of destruction of the released sugar. As many authors have pointed out, the best conditions should be established experimentally for each particular glycoprotein and for each sugar being

determined (e.g. Montreuil et al 1965). The usual temperature employed for hydrolysis is 100° and the acids commonly used are HCl or H_2SO_4 in concentrations varying from 0.5 to 2 N and for various times. In order to minimise incompleteness of recovery due to formation of hexosaminides linked to neutral sugars, the most concentrated acid compatible with recovery would appear to be the best choice. With 2 N HCl, the optimum time for the release of galactose, mannose and fucose from α_1-acid glycoprotein was 2 hr. (Montreuil and Scheppler 1959) but to what extent the released sugars were destroyed is not known.

The method of isotope dilution was employed to obviate this problem of destruction of released sugar. As the sugar is released from the glycoprotein it mixes with and causes dilution of the radioactive sugar until at complete release the specific activity of the free sugar reaches a constant value which is unaffected by subsequent destruction of the sugar. Destruction of free sugar prior to this time will result in a lower final specific activity and therefore a higher calculated sugar content of the glycoprotein. The effect of the destruction occurring at any time will be inversely proportional to the time of hydrolysis related to the release of sugar, i.e. an appreciable rate of destruction of the radioactive sugar at the beginning of the hydrolysis will have a much greater effect than similar destruction occurring later on. It is to be expected that the rate of destruction of free sugar in the presence of protein during hydrolysis will vary with the degree of fragmentation of the protein since the individual amino acids are likely to have different reactivities, both chemically and kinetically, in the bound and free states.

Neither the rate of destruction nor the variation in the rate is easy to assess except by the type of kinetic experiment suggested earlier. François et al (1962) assessed the relative rates of release to destruction of 12 to 1 for mannose in egg albumin, from the increase in reducing power on hydrolysis and from the rate of the decrease in recovery of mannose phenylhydrazone after complete hydrolysis of the glycoprotein had occurred. Such a high ratio of the rates was considered to be compatible with only a small error in the final result.

The conditions of hydrolysis for the radioisotope dilution experiments reported here were 2 N HCl for 2 hr with egg albumin and rabbit γ-globulin and for 3 hr. with the blood-group substances. Maximum release of mannose from egg albumin as determined by radioisotope dilution had been found to occur after 1 hr. in 2 N HCl (François et al. 1962). Hydrolysis of rabbit γ-globulin with 1 N HCl (experiments 2 and 3, Table 8) did not give lower values. Complete hydrolysis of methyl α-D-glucopyranoside was obtained with 2 N HCl for 2 hr. However for maximum release of amino sugars from several glycoproteins investigated 4 N HCl was required and it may well be that similar conditions are necessary for complete hydrolysis of neutral sugars from some glycoproteins.

In this work the release of mannose from egg albumin in 2 N HCl with time was determined by isotope dilution. The appearance of mannose did not follow first order kinetics which is to be expected for the following reasons. (a) For release of most of the mannose residues in the heterosaccharide, two glycosidic linkages need to be split. (b) All the glycosidic bonds in oligosaccharides formed during the hydrolysis will have a rate of cleavage which differs from that of the same bonds in the

intact heterosaccharide. (c) All the glycosidic linkages involving mannose in the heterosaccharide will not be cleaved at the same rate. If the carbohydrate of egg albumin is heterogeneous, as has been suggested (Cunningham et al. 1963, 1965, Bhoyroo and Marshall 1965), the kinetics of release will be even more complex. The time required for release of half of the mannose was about 7 min. which is a little less than the 10 min. calculated previously as a maximum value (François et al 1962). By comparison the half time for the hydrolysis of methyl α-D-mannopyranoside is 2.8 min. under the same conditions.

The release of glucose from methyl α-D-glucopyranoside in 2 N HCl at 100° has a half-time of 7 min. similar to that of the release of mannose from egg albumin. The estimation of glucose in the glucoside by isotope dilution gave about the theoretical value, but when the hydrolysis was carried out in the presence of egg albumin, with a protein to free sugar ratio of 160 to 1, an overestimate of 18% was obtained. With performic acid-oxidized egg albumin at a lower protein to sugar ratio (37 to 1), the value obtained was 12% higher than that calculated. These results suggest that preferential destruction of radioactive sugar has occurred during hydrolysis. If the value for the mannose content (2.01%) found for egg albumin is corrected by 18%, a value of 1.7% is obtained. For performic acid-oxidized egg albumin the application of a 12% correction to the mannose value obtained (1.85%) gives a mannose content of 1.65%. These values are much lower than the 1.96% found by the orcinol-sulphuric acid method which in general agrees with other independent methods for the estimation of mannose in egg albumin. However, the corrections which were applied may not be valid for the following reasons:

(a) the corrections were based on the estimation of glucose in single experiments which have a standard error of ± 4%; (b) the destruction of glucose and mannose are unlikely to occur at the same rate; (c) although the time for release of half the mannose from egg albumin is similar to that for equivalent cleavage of the glycoside, it would be expected that the release of mannose early in the hydrolysis would be more rapid, as it is known that one of the mannose residues is in terminal position.

The isotope dilution method applied to the estimation of galactose and mannose in rabbit γ-globulin gave considerable variation between single values particularly for galactose. The variation is greater than that to be expected from cumulative errors arising from the estimations. From the model experiments on the destruction of mannose in 2 N HCl at 100° reported here and previously (François et al 1962) it appears that the presence of cysteine, tryptophan or oxygen have considerable influence on the destruction of mannose (and presumably other hexoses) in 2 N HCl at 100°.

The tryptophan and half-cystine contents of rabbit γ-globulin are relatively high, 14 and 22 moles/10^5 g. respectively (Crumpton and Wilkinson 1963), compared with the hexose content about 7 moles/10^5 g. Interaction between these amino acids and reducing sugars during hydrolysis is likely to be less with a lower concentration of glycoprotein during hydrolysis, as in experiment 4 where the volume was 5 times greater than in previous experiments. The effect of the presence of oxygen on the destructive influence of the two amino acid s is not known. Oxygen may depress this influence by oxidation of the amino acids, or it may enhance

their effect on sugar destruction by formation of intermediates which react more readily with the sugars. In these experiments the glycoprotein in acid solution was flushed with nitrogen and heated in stoppered tubes. Variable amounts of oxygen may have been present during the course of hydrolysis, so that variable degrees of sugar destruction may have occurred. High protein to added-sugar ratios and high glycoprotein concentration during hydrolysis are likely to enhance interaction between sugars and amino acid residues and consequently destruction of sugars.

Performic acid oxidation of the glycoprotein prior to hydrolysis should remove the destructive influence of tryptophan and cyst(e)ine. When the oxidation is carried out at 0° for 4 hr., it does not appear to affect the hexose content of glycoproteins such as fetuin (Spiro 1962), egg albumin (Hörmann and Gollwitzer 1962) and fibrin and fibrinogen (Hörmann and Gollwitzer 1963), and of two glycosides tested (see above). Under these conditions tryptophan is destroyed but some of the cyst(e)ine residues may survive. For lysozyme a higher temperature (50°) was required for complete conversion of the cystine to cysteic acid (Edman 1960). This appears also to be the case for egg albumin. The values obtained for galactose and mannose in performic acid-oxidized rabbit γ-globulin fall within the variation obtained with preceding experiments.

The best estimate for the galactose and mannose content of rabbit γ-globulin from these experiments is 2.1 residues of galactose and 4.2 residues of mannose per 10^5 g. The total of 6.3 residues per

10^5 g. is less than the 7.7 mole/10^5 g. hexose (as mannose) found with an orcinol-H_2SO_4 method on the intact glycoprotein even allowing for a small amount of fucose.

The epithelial-type glycoproteins, which are predominantly carbohydrate and lack those amino acids most likely to interact with free sugars in acid, probably have much less destructive effect on reducing sugars. As much larger amounts of radioactive sugar can be added and the fucose will be quickly released early in the hydrolysis, the relative destruction of added sugar should be low. In the estimations reported here greater precautions were taken to exclude oxygen and the concentration of glycoprotein in the hydrolysis mixture was usually much less than 1 mg. per ml., Dilute solutions were recommended for the hydrolysis of proteins for amino acid analysis when large amounts of carbohydrate are present (Dustin et al 1951). A value of about 1 mg. per ml. was recommended by Gottschalk (1963). The values obtained for fucose by the colorimetric method are similar to those obtained by the isotope dilution method except for sample W where the colorimetric method is about 13% higher. The higher value obtained for fucose in blood-group substance Z in the experiment using paper chromatography may have been due to the fact that the glycoprotein sample taken for hydrolysis was not heated whereas the previous samples were dried for 3 to 4 hr. at 78°. Variation in hydrolysis conditions did not seem to have much effect on the value found. The sample Z has a relatively low fucose content but in contrast to other blood-group substances contains a large amount, about 18% of sialic acid and so conforms to the reciprocal trend between fucose and sialic acid contents of glycoproteins observed by Dische (1963).

The values obtained for galactose are probably within the cumulative errors of the method employed, except for the values found for sample Y. There does not appear to be as great a variation in the galactose content of blood-group substances as in the fucose and sialic acid contents.

The reproducibility of the radioisotope dilution method depends on the precision of the estimation of two specific activities. Any error in either specific activity will be magnified in the final result. In this work radioactive samples were counted at infinite thinness which is probably more subject to errors than some other methods, as the samples need to be dried in uniform films in a symmetrical position on the planchets For example, liquid scintillation counting when compared to counting at infinite thinness gave less variation between aliquots taken from the one sample.

The removal of acid from solutions of hexose by some anion-exchange resins was investigated. The hydroxyl form of a weak anion exchange resin did not give a quantitative recovery. Hexose was almost quantitatively recovered from an acid solution containing a mixture of amino acids, by removal of the acid with Dowex 1-bicarbonate, followed by passage of the resulting solution through Dowex 50 - H^+ form. Montreuil et al. (1965) stress that hydrolysates should be kept in acid solution until amino acids have been removed, and they pass the solution through Dowex 50 H^+ form before removing the acid with an anion-exchange resin. However to prevent the elution of amino compounds from the cation-exchange resin by the acid present, a relatively large amount of resin is required.

The chromatographic separation of a mixture of rhamnose, fucose, mannose and glucose on columns of Celite 545 packed in the moist state was described by Hall (1962), confirmed here by obtaining effective separation of a mixture of fucose, mannose and galactose or a mixture of mannose and glucose. On a slightly longer column all four sugars could be completely separated in this system. These columns also gave complete separation of the methyl α- and β- anomers of N-acetylglucosaminide and of the various methyl mannosides. The method was also used for purification of monosaccharides and it would be useful for separating mixtures of sugars from glycoprotein hydrolysates for identification, as the mobile phase can easily be evaporated to dryness leaving uncontaminated sugar.

For the estimation of hexosamine in glycoproteins by the radioisotope dilution method, hydrolysis of the glycoprotein was carried out in 6 N HCl at 100° for 6 hr. Under these conditions the possibility of forming acid-stable hexosaminides should be less than when hydrolysis is performed in 4 N HCl which results in a maximum yield of hexosamine from several glycoproteins (see above). The greater destruction of amino sugar which occurs in 6 N HCl should not affect the results obtained with the isotope dilution method.

For isolation and estimation of the hexosamines from hydrolysates the naphthylisothiocyanate method of Scott was used. Amino acids and 2-aminosugars in the glycoprotein hydrolysate were converted to naphthylthiocarbamyl derivatives and to imidazolidinethione compounds respectively. The derivatives of the acidic and neutral amino acids were extracted with a liquid anion-exchange resin or dimethyldodecylamine in benzene. The

hexosamine derivatives were resolved and separated from the basic amino acids by paper electrophoresis in tungstate buffer, eluted from the paper and estimated by measurement of the absorption at 240 mμ or 222 mμ. The advantages of this method are that it is more rapid than that usually employed which involves separation on cation-exchange resins, several samples can be estimated simultaneously and it is more sensitive. The molar extinction coefficients at 240 mμ and 222 mμ are about 74,000 and 18,000 respectively compared to about 16,000 for the Cessi method used here.

The naphthylisothiocyanate derivative of glucosamine was prepared and appeared to separate on the basis of solubility in methanol into two compounds which differed in melting point and optical rotation but which gave similar ultraviolet absorption spectra and similar molar extinction coefficients. It is likely that they represent isomers in which the hydroxyl group at C 1 of the original glucosamine is situated on opposite sides of the imidazolidine ring. (However see Addendum, p. 163)

The conditions employed for the release of hexosamines from glycoproteins, 6 N HCl at 100° for 6 hr., is likely to give nearly quantitative release of hexosamine as recovery of glucosamine from a mixture of glucosamine hydrochloride and methyl β-N-acetyl-D-glucosaminide was 97.5%. Some of this loss will be due to destruction. Boas (1953) found 20% loss of glucosamine in 15 hr. under the same conditions but he took no precautions to exclude oxygen. However, if any of the amino groups of the hexosamines in glycoproteins are free or substituted with a very acid-labile acyl group, their glycosidic linkages will not be completely split under these conditions; e.g. some glycoproteins such as ovomucoid (Marshall and Neuberger 1960) and a glycoprotein from urine (Hakamori et al 1961) contain

formyl groups which may be N-substituents of the amino sugars.

Hydrolysis of glycoproteins was carried out in evacuated sealed tubes to avoid destruction of hexosamine by oxygen as was reported by Walborg and Ward (1963). When the method was applied to hydrolysates of blood-group substances, electrophoresis gave well-defined spots in the areas corresponding to the glucosamine and galactosamine derivatives with other spots near the origin. With hydrolysates of γ-globulin long trails were obtained which had to be resubmitted to electrophoresis to obtain spots free from interfering substances. These substances may be products of interaction between amino acids and neutral sugars since model experiments showed that these compounds do give spots near the origin of the electrophoretic strip. Another possibility is that they may arise from peptides due to incomplete hydrolysis of the protein in the short time of hydrolysis.

The estimates of total hexosamine in the blood-group substances are similar to those found by Morgan using the Elson-Morgan method. However the ratios of glucosamine to galactosamine differ, the reason for this discrepancy is not apparent. The glucosamine values in the method used here are probably less subject to interference than the galactosamine values, as on electrophoresis the glucosamine derivative migrates more rapidly on the paper whereas the less mobile galactosamine derivative runs closer to interfering substances near the origin. If the galactosamine area contains other naphthyl compounds, a high value would be expected.

The estimation of glucosamine in rabbit γ-globulin by isotope dilution gave a value (5.7 mole/10^5 g.) which is a little lower than that obtained by the Cessi method after hydrolysis for 4 or 6 hr. in 4 N HCl.

The values correspond to about 8 to 8.3 moles respectively of hexosamine per 140,000 g of rabbit γ-globulin. These values agree with the 8.2 mole reported by Fleischman et al. (1963).

The method of radioisotope dilution offers a sound basis for the estimation of neutral sugars in glycoproteins. In comparison with colorimetric methods on the intact glycoprotein it is specific for the individual sugar. It has the advantage over estimations, which are carried out on sugars after hydrolytic release from glycoproteins that more drastic conditions of hydrolysis can be employed, thus removing the need to establish optimum conditions of hydrolysis, and decreasing the possibility of trapping sugars in hexosaminide linkages. In addition the yields of sugar from hydrolytic and separation processes do not have to be quantitative.

The problem which may arise from significant destruction of added free radioactive sugars to a glycoprotein may be minimised by taking precautions: (i) the glycoprotein concentration during hydrolysis should be low, such as 1 mg. per ml. (Gottschalk 1963); (ii) exclusion of oxygen seems to be important. An effective way is to carry out the hydrolysis in an evacuated sealed tube; (iii) protection of the added sugar may be partly effected by addition of a sugar such as a pentose which does not occur in the glycoprotein; (iv) reactive amino acids such as tryptophan and cyst(e)ine in the glycoprotein may be oxidized by prior treatment with performic acid.

A suggested method to overcome the problem of preferential destruction of added radioactive sugar is to add the labelled sugar in the form of

a glycoside which more closely approaches the form of the sugar in the glycoprotein. In this case the conditions, particularly during the early part of the hydrolysis will be less biased towards the destruction of the added radioactive sugar. It would be preferable for the added glycoside to have a rate of cleavage similar to that of the sugar bound in the glycoprotein but even if the rates differ somewhat the situation should be improved. The alkyl glycopyranosides, which are readily prepared, appear to be fairly stable. For example, the rate of cleavage of methyl α-D-glucopyranoside in 2 N HCl at 100° has a similar rate of hydrolysis to that of mannose in egg albumin. The methyl α-D-mannopyranoside is hydrolysed at about twice the rate.

The radioisotope dilution method is likely to be particularly valuable in certain cases:

e.g. (a) the estimation of the remaining hexose in periodate-oxidized glycoproteins where the aldehyde groups from oxidized sugars interfere in colorimetric methods. Fletcher et al. (1963) have applied it to the estimation of mannose in oxidized egg albumin.

(b) the estimation of hexosamine in glycosaminoglycuronans where considerable destruction of hexosamine may occur during hydrolysis (Ogston 1963).

(c) the estimation of sialic acid in glycoproteins.

SUMMARY

1. Present knowledge on the general structure of glycoproteins has been reviewed. The basis for a subdivision of glycoproteins into serum-type and epithelial-type glycoproteins was proposed. The characteristic features of the subdivision are (a) the serum-type glycoproteins have a relatively high polypeptide content per carbohydrate prosthetic group, i.e. greater than about 4,000 whereas the same parameter for the epithelial-type glycoproteins is less than 1000.

(b) The carbohydrate to protein linkage in the serum-type glycoproteins, which have been investigated, is the N-glycosyl-amide structure involving an asparagine residue whereas the linkage which appears to predominate in the epithelial-type glycoproteins is the O-glycosyl bond involving the hydroxyl group of a serine or a threonine residue.

(c) The only sugar so far found to be involved in the carbohydrate-protein linkage for the serum-type glycoproteins is N-acetylglucosamine whereas N-acetylgalactosamine appears to play a similar role for the epithelial-type glycoproteins.

A feature which appears to distinguish further the glycoproteins and glycosaminoglycans is that in the latter, xylose is the sugar involved in the linkage of carbohydrate to protein.

2. The Celite chromatography of neutral sugars as described by Hall was found to give excellent separation of galactose, mannose, and fucose from glycoprotein hydrolysates. The method was also found to be useful for the separation of anomeric glycosides.

3. A method of estimation of small amounts of galactose, mannose and

fucose in glycoproteins by radioisotope dilution was worked out. ^{14}C-Labelled sugars were added to the glycoprotein before hydrolysis in 2 N HCl at 100° for 2 or 3 hr. After removal of charged substances from the hydrolysate the remaining sugars were separated on columns of Celite and assayed for specific activity.

4. Estimation of the galactose and mannose content of rabbit γ-globulin by the radioisotope dilution method gave values of 2.2 ± 0.2 and 4.3 ± 0.2 mole per 10^5 g. respectively. After performic acid-oxidation of the glycoprotein the values obtained were 2.0 and 4.3 mole per 10^5 g. respectively.

5. From model experiments, in which glucose was estimated in methyl α-D-glucopyranoside by radioisotope dilution in the presence of egg albumin, it appeared that the radioisotope dilution procedure may give high results under some circumstances, a situation which was improved by prior performic acid-oxidation of the glycoprotein.

6. The half-time for the release of mannose from egg albumin in 2 N HCl at 100° was found to be 7 min.

7. The content of fucose and galactose in two samples of blood-group B specific substance and one sample of Le^a specific substance was estimated by the radioisotope dilution method.

8. The influence of various substances on the destruction of mannose in 2 N HCl for 3 hr. in an evacuated sealed tube was determined by radioisotope dilution. Tryptophan, present in equimolar amount to mannose, increased the destruction of mannose from 4 to 15%. If ribose was also added the loss of mannose was decreased by one half. The presence of oxygen appears to enhance mannose destruction as much greater losses of mannose in 2 N HCl have been reported previously.

9. A radioisotope dilution method was applied to the estimation of glucosamine in rabbit γ-globulin and of galactosamine and glucosamine in three samples of blood-group specific substances, after isolation of the naphthylisothiocyanate derivatives of the hexosamines by electrophoresis in tungstate buffer. The values obtained for total hexosamine were generally in agreement with those found by other methods.

10. The reaction between D-glucosamine and naphthylisocyanate resulted in two main products which were partly characterized.

Addendum

The substances A and B which were obtained by the reaction of naphthylisothiocyanate with glucosamine were submitted to electrophoresis in tungstate buffer at pH 7. Substance B migrated rapidly towards the anode but substance A did not move. Therefore substance A can not possess the tetrahydroxybutyl group and is probably the product of an intramolecular cyclisation involving the coupling of C_1 of the original glucosamine moiety with one of the carbon atoms of the tetrahydroxybutyl group, and accompanied by the loss of a molecule of water (J.E. Scott, personal communication).

REFERENCES

Adams, J.B. (1965a) Biochem. J. 94, 368.

Adams, J.B. (1965b) Biochem. J. 97, 345.

Aminoff, D. (1961) Biochem. J. 81, 384.

Aminoff, D. and Morgan, W.T.J. (1951) Biochem. J. 48, 74.

Aminoff, D. and Morgan W.T.J. and Watkins, W.M. (1952) Biochem. J. 51, 379.

Anderson, A.J. (1962) Biochem. J. 82, 372.

Anderson, B., Hoffman, P. and Meyer, K. (1963) Biochim. Biophys. Acta 74,309

Anderson, B., Hoffman,P. and Meyer, K. (1965) J. Biol. Chem. 240, 156.

Anderson, B., Seno, N., Sampson, P., Riley, J.G., Hoffman, P. and Meyer, K. (1964) J. Biol. Chem. 239, PC 2716.

Anet, E.F.L.J. (1964) Adv. Carb. Chem. 19, 181.

Annison, E.F. and Morgan, W.T.J. (1952a) Biochem. J. 50, 460.

Annison, E.F. and Morgan, W.T.J. (1952b) Biochem. J. 52, 247.

Annison, E.F., James, A.T. and Morgan, W.T.J. (1951) Biochem. J. 48, 477.

Armour, C., Bunton, C.A., Patai, S., Selman, L.H. and Vernon, C.A. (1961) J. Chem. Soc. 412.

Ashwell, G. (1957) Meth. Enzymol. 3, 73.

Avigad, G., Amaral, D., Asensio, C. and Horecker, B.L. (1962) J. Biol. Chem. 237, 2736.

Balazs, E.A. and Jeanloz, R.W. (1965). In E.A. Balazs and R.W. Jeanloz (Eds) "The Amino Sugars", IIA, (Academic Press, New York) p. xiv.

Ballou, C.E. (1954). Adv. Carb. Chem. 9, 59.

Bamford, C., Capon, B. and Overend, W.G. (1962) J. Chem. Soc. 5138.

Banks, B.E.C., Meinwald, Y., Rhind-Tuft, A.J., Sheft, I. and Vernon, C.A. (1961) J. Chem. Soc. 3240.

Barker, S.A., Pardoe, G.I., Stacey, M. and Hopton, J.W. (1962) Nature 197,231

Barker, S.A., Pardoe, G.I., Stacey, M. and Hopton, J.W. (1963a) Protides of the Biological Fluids, XI, 284.

Barker, S.A., Cruickshank, C.N.D. and Morris, J.H. (1963b) Biochim. Biophys. Acta, 74, 239.

Bentley, R. (1963) in P.D. Boyer, H. Lardy and K. Myrbäck (Eds.) The Enzymes (Academic Press, New York and London), p. 567.

Berenson, G.S. and Fishkin, A.F. (1962) Arch. Biochem. Biophys. 97, 18.

Best, J.S., Bhavanandan, V.P. and Gottschalk, A. (1965) Biochim. Biophys. Acta, 101, 141.

Bettelheim-Jevons, F.R. (1958) Adv. Prot. Chem. 13, 35.

Bhavanandan, V.P., Buddecke, E., Carubelli, R. and Gottschalk, A. (1964) Biochem. Biophys. Res. Commun. 16, 353.

Bhoyroo, V.D. and Marshall, R.D. (1965) Biochem. J. 18P, 97.

Bierry, H. (1929) Compt. Rend. Soc. Biol. 101, 544.

Blass, J., Macheboeuf, M. and Nunez, G. (1950). Bull. Soc. Chim. Biol. 32, 130

Blix, G. (1936) Z. Physiol. Chem. 240, 43.

Blix, G. (1951) Glycoproteide. In Physiologische Chemie, B. Flaschenträger and E. Lehnarty, Eds. Springer, Berlin, Vol. I, p. 751.

Blix, G. (1963) Ann. N.Y. Acad. Sci. 106, 164.

Blix, G., Svennerholm, L. and Werner, I. (1952) Acta Chem. Scand. 6, 358.
Blix, G., Lindberg, F., Odin, L. and Werner, I. (1956) Acta Soc. Med. Upsalien. 61, 1.
Blumenfeld, O.O., Paz, M.A., Gallop, P.M. and Seifter, S. (1963). J. Biol. Chem. 238, 3835.

Boas, N.F. (1953) J. Biol. Chem. 204, 553.

Bogdanov, V.P., Kaverzneva, E.D. and Andreyeva, A.P. (1964). Biochim. Biophys. Acta, 83, 69.

Bourrillon, R. and Michon, J. (1959) Bull. Soc. Chim. Biol. 41, 267.

Bourrillon, R., Cornillot, P. and Got, R. (1962) Clin. Chim. Acta, 7, 506

Bourrillon, R., Michon, J and Got, R. (1959) Bull. Soc. Chim. Biol. 41, 493.

Boyer, P.D. (1954) J. Am. Chem. Soc. 76, 4331.

Bragg, P.D. and Hough, L. (1961) Biochem. J. 78, 11.

Bray, H.G., Henry, M. and Stacey, M. (1946) Biochem. J., 40, 124.

Brimacombe, J.S. and Webber, J.M. (1964) "Mucopolysaccharides" (Elsevier Publishing Co., Amsterdam).

Broda, E. (1960) Radioactive Isotopes in Biochemistry (Elsevier Publishing Co., Amsterdam) p. 111.

Brossmer, R. (1962) Meth. Carb. Chem. 1, 216.

Brown, H.C. and Borkowski, M. (1952). J. Am. Chem. Soc. 74, 1894.

Bruno, G.A. and Christian, J.E. (1961) Anal. Chem. 33, 1216.

Bunton, C.A., Lewis, T.A., Llewellyn, D.R. and Vernon, C.A. (1955). J. Chem. Soc. 4419.

Calvin, M. Heidelberger, C., Reid, J.C., Tolbert, B.M. and Yankwich, P.F. (1949) Isotopic Carbon (John Wiley & Sons, New York, U.S.A.) p.27.

Canfield, R.E. (1963) J. Biol. Chem. 238, 2691.

Capon, B. and Overend, W.G. (1960) Adv. Carb. Chem. 15, 33.

Carsten, M.E. and Pierce, J.G. (1963) J. Biol. Chem. 238, 1724

Carubelli, R., Bhavanandan, V.P. and Gottschalk, A. (1965) Biochim. Biophys. Acta 101, 67.

Cessi, C. (1952) Boll. Soc. Ital. Biol. Sper. 28, 858.

Cessi, C. and Piliego, F. (1960) Biochem. J. 77, 508.

Cessi, C. and Serafini-Cessi, F. (1963) 88, 132. Biochem. J.

Chatterjee, A.K. and Montgomery, R. (1962) Arch. Biochem. Biophys. 99, 426.

Cheftal, R.I., Cloarec, L., Moretti, J., Rafelson, M. and Jayle, M.F. (1960) Bull. Soc. Chim. Biol. 42, 993.

Cheftal, R.I., Cloarec, L., Moretti, J. and Jayle, M. (1965) Bull. Soc. Chim. Biol. 47, 385.

Clamp, J.R. and Hough, L. (1963) Chem. Ind. 82.

Clamp, J.R. and Putnam, F.W. (1964) J. Biol. Chem. 239, 3233.

Comb, D.G. and Roseman, S. (1960) J. Biol. Chem. 235, 2529.

Committee on Protein Nomenclature, J. Biol. Chem., 4, xlviii (1908).

Cook, G.M.W. and Eylar, E.H. (1965) Biochim. Biophys. Acta 101, 57.

Cornforth, J.W. and Firth, M.E. (1958) J. Chem. Soc. 1091.

Côté, R.H. and Morgan, W.T.J. (1956) Nature 178, 1171.

Crumpton, M.J. (1959) Biochem. J., 72, 479.

Crumpton, M.J. and Wilkinson, J.M. (1963) Biochem. J. 88, 128

Cunningham, L.W., Ford, J.D. and Rainey, J.M. (1965) Biochim. Biophys. Acta 101, 233.

Cunningham, L.W., Clouse, R.W. and Ford, J.D. (1963) Biochim. Biophys. Acta, 78, 379.

Cynkin, M.A. and Haschenmeyer, R.H. (1964) Red. Proc. 23, 273.

Dische, Z. (1928) Biochem. Z. 201, 74

Dische, Z. (1955) Meth. Biochem. Anal. 2.

Dische, Z. (1962) Meth. Carb. Chem. 1, 477.

Dische, Z. (1963) Ann. N.Y. Acad. Sci. 106, 259.

Dische, Z. and Borenfreund, E. (1950) J. Biol. Chem. 184, 517

Dische, Z. and Shettles, L.B. (1948) J. Biol. Chem. 175, 595

Donna, R.A., Orr, A.C. and Drake, N. (1957) Anal. Chem. 29, 464.

Dorfman, A., Roseman, S., Moses, F.E., Ludowieg, J. and Mayeda M. (1955) J. Biol. Chem. 212, 583.

Dubois, M. Gilles, K., Hamilton, J.K., Rebers, P.A. and Smith, F. (1951) Nature, 168, 167.

Dubois, M., Gilles, K., Hamilton, J.K.,Rebers, P.A. and Smith F. (1956) Anal. Chem. 28, 350.

Duff, R.B. and Eastwood, D.J. (1950) Nature 165, 848.

Dustin, J.M., Czajkowska, C., Moore, S. and Bigwood, E.J. (1951) Anal. Chim. Acta 9, 256.

Edman, P. (1960) Ann. N.Y. Acad. Sci. 88, 602.

Edward, J.T. (1955). Chem. and Ind. 1102.

Eichwald, E. (1865) Ann. Chem. Pharm. 134, 177.

Elson, L.A. and Morgan, W.T.J. (1933) Biochem. J. 27, 1824.

Eylar, E.H. (1962) Biochem. Biophys. Res. Commun. 8, 195.

Eylar, E.H. and Jeanloz, R.W. (1962a) J. Biol. Chem. 237, 622.

Eylar, E.H. and Jeanloz, R.W. (1962b). J. Biol. Chem. 237, 1021.

Fairbridge, R.A., Willis, K.J. and Booth, R.G. (1951) Biochem. J. 49, 423.

Fischer, E. and Leuchs, H. (1903) Ber. 36, 24.

Fischer, F.G. and Nebel, H.J. (1955) Z. physiol. Chem. 302, 10.

Fishkin, A.F. and Berenson, G.S. (1961) Arch. Biochem. Biophys. 95, 130

Fleischman, J.B., Porter, R.R. and Press, E.M. (1963) Biochem J. 88, 220.

Fletcher, A.P., Marshall, R.D. and Neuberger, A. (1963a) Biochim. Biophys. Acta 71, 505.

Fletcher, A.P., Marks, G.S., Marshall, R.D. and Neuberger, A. (1963b) Biochem. J. 87, 265.

Flood, W.E., Hirst, E.L. and Jones, J.K.N. (1947) Nature 160, 86.

Folkes, B.F., Grant, R.A. and Jones J.K.N. (1950) J. Chem. Soc. 2136.

Foster, A.B. and Horton, D. (1959) Adv. Carb. Chem. 14, 213.

Foster, A.B., Horton, D. and Stacey, M. (1957) J. Chem. Soc. 81.

Foster, A.B., Overend, W.G., Stacey, M. and Vaughan, G. (1954) J. Chem. Soc. 3367.

François, C., Marshall, R.D. and Neuberger, A. (1962) Biochem. J. 83, 335.

Fränkel, S. and Jellinek, C. (1927) Biochem. Z. 185, 392.

Gardell, S. (1953) Acta Chem. Scand. 7, 207.

Gibbons, M.N. (1955) Analyst 80, 268.

Gibbons, R.A. (1959) Biochem. J. 73, 209.

Gibbons, R.A. and Morgan, W.T.J. (1954) Biochem. J. 57, 283.

Gibbons, R.A. and Roberts, G.P. (1963) Ann. N.Y. Acad. Sci. 239, 3312.

Gibbons, R.A., Morgan, W.T.J. and Gibbons, M.N. (1955) Biochem. J. 60, 428.

Good, T.A. and Bessman, S.P. (1964) Anal. Biochem. 9, 253.

Gottschalk, A. (1952) Nature 170, 662.

Gottschalk, A. (1960) The Chemistry and Biology of Sialic Acids. Cambridge University Press.

Gottschalk, A. (1962) Perspectives in Biol. Med. 5, 327.

Gottschalk, A. (1963) Ann. N.Y. Acad. Sci. 106, 168.

Gottschalk, A. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 21.

Gottschalk, A. and Ada, G.L. (1956) Biochem. J. 62, 681.

Gottschalk, A. and Fazekas de St. Groth, S. (1960) 43, 513.

Gottschalk, A. and Graham, E.R.B. (1959) Biochim. Biophys. Acta 34, 380.

Gottschalk, A. and Graham, E.R.B. (1966) In H. Neurath (Ed.) "The Proteins", 2nd Ed., Vol IV (Academic Press, New York) in the press.

Gottschalk, A. and Murphy, W.H. (1961) Biochim. Biophys. Acta 46, 81.

Gottschalk, A. and Neuberger, A. (1966) In A. Gottschalk (Ed.) "Glycoprotei (Elsevier Publishing Co., Amsterdam) p. 594.

Graham, E.R.B. (1961) Australian J. Sci. 24, 140.

Graham, E.R.B. and Gottschalk, A. (1960) Biochim. Biophys. Acta 38, 513.

Graham, E.R.B., Murphy, W.H. and Gottschalk A. (1963) Biochim. Biophys. Acta, 74, 222.

Gregory, J.D., Laurent, T.C. and Roden, L. (1964) J. Biol. Chem. 239, 3312.

Gyorky, G. and Houck, J.C. (1965) Canad. J. Biochem. 43, 1807.

Haab, W. and Anastassiadis, P.A. (1961) Can. J. Biochem. Physiol. 39, 671.

Hagedorn, H.C. and Jensen, B.N. (1923) Biochem. Z. 135, 46.

Hall, R.H. (1962a) J. Biol. Chem. 237, 2283.

Hall, R.H. (1962b) Anal. Biochem. 4, 395.

Hall, A.N., Hollingshead, S. and Ryder, H.N. (1961) J. Chem. Soc. 4290.

Hammarsten, O. (1888) Physiol. Chem. 12, 163.

Harbon, S., Herman, G., Rossignol, B., Jolles, P. and Clauser, H. (1964) Biochem. Biophys. Res. Commun. 17, 57.

Hartree, E.F. (1964) Anal. Biochem. 7, 103.

Hashimoto, Y. and Pigman, W. (1962) Ann. N.Y. Acad. Sci. 93, 541.

Hashimoto, Y., Hashimoto, S. and Pigman, W. (1964) Arch. Biochem. Biophys. 104, 282.

Hashimoto, Y., Tsuiki, S., Nisizawa, K. and Pigman, W. (1963) Ann. N.Y. Acad. Sci. 106, 233.

Heath, H. and Wildy, J. (1956) Biochem. J. 64, 612.

Heidt, L.J. and Purves, C.B. (1944) J. Am. Chem. Soc. 66, 1385.

Heimmens, W.F. (1965) Nature, 207, 973.

Hewitt, L.F. (1934) Biochem. J. 28, 2080.

Hewitt, L.F. (1937) Biochem. J. 31, 360.

Hewitt, L.F. (1938) Biochem. J. 32, 1554.

Hill, R.L. (1965) Adv. Prot. Chem. 20, 37.

Holtzmann, G., McAllister, R.V. and Niemann, C. (1947) J. Biol. Chem.171, 27.

Hörmann, H. and Gollwitzer, R. (1963) Protides of the Biological Fluids.332

Hörmann, H. and Gollwitzer, R. (1962) Ann. Chem. 655, 178.

Horowitz, M.I., Hashimoto, Y. and Pigman, W. (1963) Biochim. Biophys. Acta 83, 209.

Horscroft, R.C. (1963) J. Chem. Soc. 5247.

Hough, L. and Jones, J.K.N. (1962) Meth. Carb. Chem. 1, 6.

Hough, L., Jones, J.K.N. and Wadman, W.H. (1950) J. Chem. Soc. 1702.

Hughes, R.C. and Jeanloz, R.W. (1964a) Biochemistry 3, 1535.

Hughes, R.C. and Jeanloz, R.W. (1964b) Biochemistry 3, 1543.

Imanaga, Y. (1963) J. Biochem. 53, 80.

Inazawa, H. (1951) Tohoku J. Exp. Med.54, 53.

Inone, S. and Egami, F. (1963) J. Biochem. 54, 557.

Ingram, V.M. (1953) J. Chem. Soc. 3717.

Isbell, H.S., Frush, H.L. and Holt, N.B. (1960) J. Nat. Bur. Stand. 64A,363

Jackson, R.H., Coulson, E.J. and Clark, W.R. (1962) Arch. Biochem. Biophys. 97, 373.

Jamieson, G.A. (1965a) J. Biol. Chem. 240, 2019.

Jamieson, G.A. (1965b) J. Biol. Chem. 240, 2914.

Jeanloz, R.W. (1960) Arthritis Rheum. 3, 233.

Johansen, P.G. (1963) Biochem. J. 87, 63.

Johansen, P.G., Marshall, R.D. and Neuberger, A. (1960) Biochem. J. 77, 239.

Johansen, P.G., Marshall, R.D. and Neuberger, A. (1961) Biochem. J. 78, 518.

Johnston, I.R. (1963) Biochem. J. 86, 254.

Jolles, Z.E. and Morgan, W.T.J. (1940) Biochem. J. 34, 1183.

Kamiyama, S. and Schmid, K. (1962) Biochim. Biophys. Acta 58, 80.

Karkas, J.D. and Chargaff, E. (1964) J. Biol. Chem. 239, 949.

Kaverzneva, E.D. and Bogdanov, V.P. (1962) Biokhimiya 27, 273.

Kekwick, R.A. (1940) Biochem. J. 34, 1248.

Kent, P.W. and Marsden, J.C. (1963) Biochem. J. 87, 38P.

Kent, P.W. and Whitehouse, M.W. (1955) Biochemistry of the Amino Sugars, (Butterworths, London).

Kowkabany, G.N. (1954) Adv. Carb. Chem. 9, 303.

Kraan, J.G. and Muir, H. (1957) Biochem. J. 66, 55P.

Kuhn, R. and Haas, H.J. (1955) Angew. Chem. 67, 785.

Kuhn, R. and Kirschenlohr, W. (1954) Ber. 87, 560, 1547.

Kuhn, R. and Kirschenlohr, W. (1956) Ann. 600, 126.

Kuhn, R., Leppelmann, H.J. and Fischer, H. (1959) Ann. 620, 15.

Laidlaw, R.A. and Reide, S.G. (1950) Nature, 166, 476.

Langstein, L. (1900). Z. Physiol. Chem. 31, 49.

Laver, W.G. (1961) Biochim. Biophys. Acta 53, 469.

Lee, Y-C. and Montgomery, R. (1961a) Arch. Biochem. Biophys. 93, 292.

Lee, Y.C., Wu, Y-C. and Montgomery, R. (1964) Biochem. J. 91, 9C.

Leloir, L.F. (1951) Arch. Biochem. Biophys. 33, 186.

Lemieux, R.U. (1962) Meth. in Carb. Chem. 1, 45.

Leskowitz, S. and Kabat, E.A. (1954) J. Am. Chem. Soc. 76, 4887.

Levene, P.A. (1925) Hexosamines and Mucoproteins, Longmans, Green & Co., London.

Levvy, G.A. and McAllan, A. (1959) Biochem. J. 73, 132.

Lindahl, U. and Roden, L. (1964) Biochem. Biophys. Res. Commun. 17, 254.

Lindahl, U. and Roden, L. (1965) J. Biol. Chem. 240, 2821.

Lindahl, U, Cifonelli, J.A., Lindahl, B. and Roden, L. (1965) J. Biol. Chem. 240, 2817.

Lüderitz, O., Simmons, D.A.R., Westphal, O. and Strominger, J.L. (1964) Anal. Biochem. 9, 263.

McFarren, E.F., Brand, K. and Rutkowsky, H.R. (1951) Anal. Chem. 23, 1146.

Marks, G.S., Marshall, R.D. and Neuberger A. (1963) Biochem. J. 87, 274.

Marshall, R.D. (1963) Nature 199, 998.

Marshall, R.D. Biochim. Biophys. Acta (1964) 93, 182.

Marshall, R.D. and Neuberger, A. (1960) Nature 186, 311.

Marshall, R.D. and Neuberger, A. (1964) Biochemistry, 3, 1596.

Martin, A.J.P. and Synge, R.L.M. (1945) Adv. Prot. Chem. II, 1.

Masamune H. (1955) Proc. Intern. Congr. 3rd. Brussds, (1955) 72.

Masamune, H. and Yosizawa, Z. (1956) Tohoku J. Exp. Med. 64, 53.

Maxfield, M. and Stefanye, D. (1962) J. Biol. Chem. 237, 2522.

Melamed, M.D. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 317.

Mester, L. Moczar, E., Medgyesi, G. and Laki, K. (1963) Compt. Rend. 256, 3210.

Meyer, K. (1945) Advances in Protein Chem. 2, 249.

Meyer, K. (1953a) In"Some Conjugated Proteins" W.H. Cole (Ed.) Rutgers University Press, New Brunswick, New Jersey.

Michon, J. and Bourrillon, R. (1961) Bull. Soc. Chim. Biol. 41, 277.

Moelwyn-Hughes, E.A. (1929) Trans. Faraday Soc. 25, 503.

Moggridge, R.C.G. and Neuberger, A. (1938) J. Chem. Soc. 745.

Montgomery, R. (1961) Biochim. Biophys. Acta 48, 591.

Montgomery, R. and Wu, Y-C. (1963) J. Biol. Chem. 238, 3547.

Montreuil, J. (1949) Bull. Soc. Chim. Biol. 31, 1639.

Montreuil, J. (1957) Bull. Soc. Chim. Biol. 39, Suppl. III, 3.

Montreuil, J. and Scheppler, N. (1959) Bull. Soc. Chim. Biol. 41, 13.

Montreuil, J. Spik, G., Dumaisnil, J. and Monsigny, M. (1965) Bull. Soc. Chim. France, 239.

Moore, S. (1963) J. Biol. Chem. 238, 235.

Moore, S., Cole, R.D., Gundlach, H.G. and Stein, W.H. (1958) Symposium on Proteins, IVth International Congress of Biochemistry, Vienna (Pergamon Press Inc., London) Vol. VIII, p. 52.

Morgan, W.T.J. (1958) Ciba Found. Symp. Chem. Biol. Mucopolysaccharides, 1.

Morgan, W.T.J. (1963) Ann. N.Y. Acad. Sci. 106, 177.

Morgan, W.T.J. and Elson, L.A. (1934) Biochem. J. 38, 988.

Morgan, W.T.J. and King, H.K. (1943) Biochem. J. 37, 640.

Muir, H. (1958) Biochem. J. 69, 195.

Muir, H. (1964) In DA.Hall (Ed.) Connective Tissue Research Vol. 2. (Academic Press, New York and London) p. 101.

Müller, F. (1901) Z. Biol. 42, 468.

Murphy, W.H. and Gottschalk, A. (1961) Biochim. Biophys. Acta 52, 349.

Nath, R.L. and Rydon, H.N. (1954) Biochem. J. 57, 1.

Neimann, M.B. (1958) Internat. J. Appl. Radiation Isotopes 3, 20.

Neish, A.C. (1950) Can. J. Res. B 28, 535.

Neuberger, A. (1938) Biochem. J. 32, 1435.

Neuberger, A. and Marshall, R.D. (1966a) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 190.

Neuberger, A. and Marshall, R.D. (1966b). In A. Gottschalk (Ed.)"Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 299.

Neuberger, A., Gottschalk, A. and Marshall, R.D. (1966a) In A. Gottschalk (Ed.), "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p.273.

Neuberger, A., Gottschalk, A. and Marshall, R.D. (1966b) In A. Gottschalk (Ed.), "Glycoproteins" (Elsevier Publishing Co., Amsterdam).p.158.

Neuberger, A. and Pitt-Rivers, R.V. (1939) J. Chem. Soc. 122.

Nolan, R. and Smith, E.L. (1962) J. Biol. Chem. 237, 446.

Nuenke, R.H. and Cunningham L.W. (1961) J. Biol. Chem. 236, 2452.

Odin, L. (1958) Ciba Found. Symp. Chem. Biol. Mucopolysaccharides, 234.

O'Gorman, J.M. and Lucas, H.J. (1950) J. Am. Chem. Soc. 72, 5489.

Ogston, A.G. (1964) Anal. Biochem. 8, 337.

Opienska-Blouth, J. Chanczinski, M and Berbec, H. (1963) Anal. Biochem.6,69

Orton, K.J.P. and Bradfield, A.E. (1924) J. Chem. Soc. 125, 960.

Overend, W.C., Rees, C.W. and Sequeira, J.S. (1962) J. Chem. Soc. 3429.

Painter, T.J., Watkins, W.M., and Morgan, W.T.J. (1965) Nature 206, 594.

Papkoff, H. (1963) Biochim. Biophys. Acta 78, 384.

Papkoff, H. (1966) In A. Gottschalk (Ed.,) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 532.

Park, J.T. and Johnson, M.J. (1949) J. Biol. Chem, 181, 149.

Partridge, S.M. (1949) Nature, 164, 443.

Pearson, C.H. (1963) Biochem. J. 88, 540.

Perlmann, G.E. (1955) Adv. Prot. Chem. 10, 1.

Perry, M.B. (1964) Canad. J. Biochem. 42, 451.

Pigman, W. (1957) The Carbohydrates, p. 60 (Academic Press, New York).

Pigman, W. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p. 439.

Pigman, W. and Gottschalk, A. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam). p. 434.

Pigman W., Rizvi, S. and Holley H. (1961) Biochim. Biophys. Acta 53, 254.

Plummer, T.H. and Hirs, C.H.W. (1964a) J. Biol. Chem. 238, 1396.

Plummer, T.H. and Hirs, C.H.W. (1964b) J. Biol. Chem. 239, 2530.

Pogell, B.M. and Koenig, D.F. (1958) Nature, 182, 127.

Popenoe, EA. and Drew, R.M. (1957) J. Biol. Chem. 228, 673,

Porter, R.R. (1958) Biochem. J. 73, 119.

Press, E.M. and Porter, R.R. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam). p. 395.

Pusztai, A. and Morgan, W.T.J. (1961a) Biochem. J. 78, 135.

Pusztai, A. and Morgan, W.T.J. (1961b) Biochem. J. 80, 107.

Pusztai, A. and Morgan, W.T.J. (1963) Biochem. J. 88, 546.

Pusztai, A. and Morgan, W.T.J. (1964) Biochem. J. 93, 363.

Radhakrishnamurthy, B. Fishkin, A.F. and Berenson, GS. (1965) 101, 129.

Reeves, R.E. (1950) J. Am. Chem. Soc. 72, 1499.

Reichstein, T. and Weiss, E. (1962) Adv. Carb. Chem. 17, 99.

Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217, 959.

Rice, F.A.H. and Fishbein, L. (1956) J. Am. Chem. Soc. 78, 3731.

Rimington, C. (1929) Biochem. J. 23, 430.

Robert, L. and Dische, Z. (1963) Biochem. Biophys. Res. Commun. 10, 209.

Roberts, G.P. and Gibbons, R.A. (1966) Biochem. J. 98, 426.

Rondle, C.J.M. and Morgan, W.T.J. (1955) Biochem. J. 59, xiii.

Roseman, S. and Daffner, 1. (1956) Anal. Chem. 28, 1743.

Roseman, S., Abeles, R.H. and Dorfman, A. (1952) J. Biol. Chem. 36, 232.

Rosevear, J.W. and Smith, E.L. (1961) J. Biol. Chem. 236, 425.

Rothfus, J.A. and Smith, E.L. (1963) J. Biol. Chem. 238, 1402.

Satake, M., Okuyama, T., Ishihara, K. and Schmid, K. Biochem J. (1965) 95, 749. in press.

Schloss, B. (1951) Analyt. Chem. 23, 1321.

Schram, E., Dustin, J.P., Moore, S. and Bigwood, E.S. (1953) Anal. Chim. Acta, 9, 149.

Schram, E., Moore, S. and Bigwood, E.J. (1954) Biochem. J. 57, 33.

Schultze, H.E. (1958) Deut. Med. Wochschr. 83, 1742.

Schultze, H.E. (1963) Protides of the Biological Fluids. H. Peeters (Ed.) Elsevier Publishing Co., Amsterdam) p. 288.

Schwebel A., Isbell, H.S. and Moyer, J.D. (1954) J. Nat. Bur. Stand. 53, 221.

Scott, J.E. (1962) Biochem. J. 82, 43P.

Scott, J.E. (1964) Biochem. J. 92, 57P.

Seeman, J. (1898) Dissertation, University of Marburg. (Quoted in A. Gottschalk. "Sialic Acids" (1960), Cambridge University Press).

Semke, L.K., Thompson, N.S. and Williams, D.G. (1964) J. Org. Chem. 29, 1041.

Sempere, J.M., Gancedo, C. and Asensio, C. (1965) Anal. Biochem. 12, 509.

Shafizedeh, F. (1958) Adv. Carb. Chem. 13, 25.

Sjoquist, J. (1959) Ark. Kemi 14, 423.

Slein, M.W. (1952) Proc. Soc. Exptl. Biol. Med. 80, 646.

Smith, E.L. and Stockell, A. (1954) J. Biol. Chem. 207, 501.

Smith, H. and Zwartouw, H.T. (1956) Biochem. J. 63, 447.

Smithies, O. (1959) Adv. Prot. Chem. 14, 65.

Somogyi, M. (1937) J. Biol. Chem. 117, 771.

Somogyi, M. (1945) J. Biol. Chem. 160, 61.

Sørensen, M. (1934) C.R. Trav. Lab. Carlsberg. 20, No. 3.

Sørensen, M. (1936) C.R. Trav. Lab. Carlsberg, 21, 123.

Sørensen, M. (1938) C.R. Trav. Lab. Carlsberg, 22, 487.

Sowden, J.C. and Schaffer, R. (1952) J. Am. Chem. Soc. 74, 499.

Sowden, J.C. and Spriggs, A.S. (1954) J. Am. Chem. Soc. 76, 3539.

Spik, G. and Montreuil, J. (1964) Bull. Soc. Chim. Biol. 46, 739.

Spiro, R.G. (1960) J. Biol. Chem. 235, 2860.

Spiro, R.G. (1962) J. Biol. Chem. 237, 382.

Spiro, R.G. (1963a) New England J. Med. 269, 566.

Spiro, R.G. (1963b) J. Biol. Chem. 238, 644.

Spiro, R.G. (1964) J. Biol. Chem. 239, 567.

Spiro, R.G. and Spiro, M.J. (1964) Fed. Proc. 22, 538.

Spiro, R.G. and Spiro, M.J. (1965) J. Biol. Chem. 240, 997.

Stacey, M. (1946) Advances in Carb. Chem. 2, 161.

Stacey, M. and Barker, S.A. (1962) "Carbohydrates of Living Tissue" (van Nostrand, London).

Sudhof, H., Kellner, H. and Schultze, N. (1955) Z. Physiol. Chem. 300, 68.

Svennerholm, L. (1956) Neurochem. 1, 42.

Szejtli, J. (1965a) Acta. Chim. Hung. 45, 141.

Szejtli, J. (1965b) Acta. Chim. Hung. 45, 153.

Szejtli, J. (1965c) Acta Chim. Hung. 46, 77.

Szejtli, J. (1965d) Acta Chim. Hung. 46, 369.

Tanaka, M. (1961) Yakugaku Zasshi 81, 1470.

Tanaka, K., Bertolini, M. and Pigman, W. (1964) Biochem. Biophys. Res. Commun. 16, 404.

Timell, T.E. (1964) Canad. J. Chem. 42, 1456.

Timell, T.E. (1965) Canad. J. Chem. 43, 2296.

Timell, T.E., Glandermans, CP.J. and Currie, A.L. (1956) Anal. Chem. 28, 1916

Toennies, G. and Homiller, R. (1942) J. Am. Chem. Soc. 64, 3054.

Tracey, M.V. (1952) Biochem. J. 52, 265.

Tracey, M.V. (1955) Biochim. Biophys. Acta 17, 159 (1955).

Tsuiki, S., Hashimoto, Y. and Pigman, W. (1961) J. Biol. Chem. 236, 2172.

Tsukamoto, H. Yamamoto, A. and Miyashita, C. (1964) Biochem. Biophys. Res. Commun. 15, 151.

Udranszky L.V., (1888) Z. Physiol. Chem. 12, 377.

Vasseur, E. (1948) Acta Chem. Scand. 2, 693.

Vogel, A.I. (1954) Practical Organic Chemistry (Longmans, Green & Co., London).

Walborg, K.F. and Ward, D.N. (1963) Biochim. Biophys. Acta 78, 304.

Warren, L. (1959) J. Biol. Chem. 234, 1971.

Warren, L. (1963) Comp. Biochem. Physiol. 10, 153.

Watkins, W.M. (1966) In A. Gottschalk (Ed.) "Glycoproteins" (Elsevier Publishing Co., Amsterdam) p 462.

Weigel, H. (1963) Adv. Carb. Chem. 18, 61.

Whistler, R.L. and BeMiller, J.N. (1962) Meth. Carb. Chem. 1, 42.

Willstätter, R. and Schudel, G. (1918) Ber., 51, 780.

Wilson, C.M. (1959) Anal. Chem. 31, 1199.

Winzler, R.J. (1955) Meth. Biochem. Anal. 2, 279.

Winzler, R.J. (1958) Ciba Found. Symp. Chem. Biol. Mucopolysaccharides, 93

Yamashina, I. (1956) Acta Chem. Scand. 10, 1666.

Yamashina, I., Ban-i, K. and Makino, M. (1963) Biochem. Biophys. Acta 78, 382.

Yamashina, I., Makino, M., Ban-i, K. and Kojima, T. (1965) J. Biochem. (Japan) 58, 168.

Zanetti, C.U. (1903) Gazz. Chim. Ital. 33, 160.

Zillikin, F., Rose, C.S., Braun, G.A. and Gyorgy, P. (1955) Arch. Biochem. Biophys. 54, 392.