THE MELDOLA MEDAL LECTURE*

Chemical Aspects of Glycoproteins, Proteoglycans, and Carbohydrate–Protein Complexes of Human Tissues

By John F. Kennedy
DEPARTMENT OF CHEMISTRY, UNIVERSITY OF BIRMINGHAM, EDGBASTON, BIRMINGHAM B15 2TT

1 Introduction

Most people, at an early age, learn either from elementary instruction in general science, or from their parents, or from the cornflake packet on the breakfast table, that carbohydrates and proteins are very important commodities in the processes and maintenance of life. Although for the majority the descriptions of these commodities go no further, few can be left entirely unmoved by some degree of fascination, even if only fleetingly and to a shallow depth, for the complexity of the automatic chemical reactions which go on day and night in our bodies. Some molecules, including carbohydrates and amino-acids, that are involved in these reactions which provide energy, form tissue and bone, and convey messages, are very simple in structure. For example, the small molecule D-glucose is an important constituent of blood and is involved in the energy cycle. However, most of the material which goes to make up our frame is composed of polymeric structures. A high proportion of these structures has proved to contain carbohydrate and protein and therefore comes under the general classifications of glycoproteins, proteoglycans, or carbohydrate–protein complexes.

2 General Considerations

A. Definitions of Glycoproteins, Proteoglycans, and Carbohydrate–Protein Complexes.—In more detail, glycoproteins contain a protein [poly(amino-acid)] chain which may consist of, for example, some three hundred amino-acid units which can be any of the twenty or so naturally occurring L-α-amino-acids (Table 1). This protein chain is essentially the backbone of the molecule and the carbohydrate part of the molecule takes the form of oligosaccharide chains which are pendant and covalently bound to the protein chain (Figure 1). The oligosaccharide chains are usually hetero-oligosaccharide chains which are frequently branched and consist of neutral monosaccharides (D-galactose, D-glucose, D-mannose, or L-fucose), basic monosaccharides (2-amino-2-deoxy-D-galactose or 2-amino-2-deoxy-D-glucose), and acidic monosaccharide (neuraminic acid) (Table 2). The basic carbohydrate units are *N*-acetylated and the neuraminic acid

* First delivered on 27 September 1972 at the Autumn Meeting of the Chemical Society held at the University of Nottingham.

Table 1 *Structures of α-amino-acids*‡

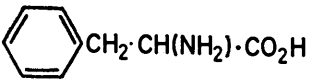
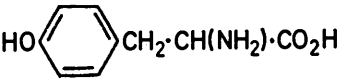
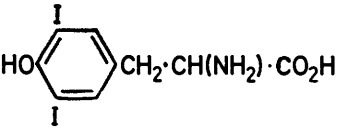
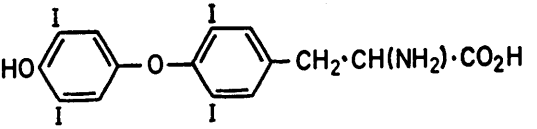
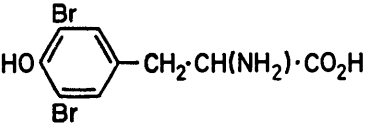
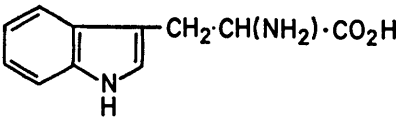
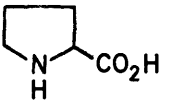
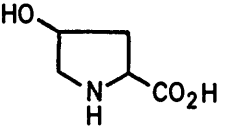
Name	*Formula*
Neutral amino-acids (one amino-group and one carboxy-group)	
1. Glycine (*g*)	$CH_2(NH_2)\cdot CO_2H$
2. Alanine (*g*)	$CH_3\cdot CH(NH_2)\cdot CO_2H$
3. Valine (*g*, *e*)	$(CH_3)_2CH\cdot CH(NH_2)\cdot CO_2H$
4. Leucine (*g*, *e*)	$(CH_3)_2CH\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$
5. Isoleucine (*g*, *e*)	$(C_2H_5)(CH_3)CH\cdot CH(NH_2)\cdot CO_2H$
6. Norleucine (*l*)	$CH_3\cdot(CH_2)_3\cdot CH(NH_2)\cdot CO_2H$
7. Phenylalanine (*g*, *e*)	
8. Tyrosine (*g*)	
9. Serine (*g*)	$HOCH_2\cdot CH(NH_2)\cdot CO_2H$
10. Cysteine (*g*)	$HS\cdot CH_2CH\cdot(NH_2)\cdot CO_2H$
11. Cystine (*g*)	$(—S\cdot CH_2\cdot CH(NH_2)\cdot CO_2H)_2$
12. Threonine (*g*, *e*)	$CH_3\cdot CHOH\cdot CH(NH_2)\cdot CO_2H$
13. Methionine (*g*, *e*)	$CH_3\cdot S\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot CO_2H$
14. Di-iodotyrosine or iodogorgic acid (*l*)	
15. Thyroxine (*l*)	
16. Dibromotyrosine (*l*)	
17. Tryptophan (*g*, *e*)	

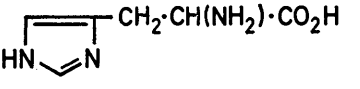
Table 1 (*continued*)

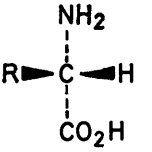
Name	*Formula*
18. Proline (*g*)	
19. Hydroxyproline (*l*)	

Acidic amino-acids (one amino-group and two carboxy-groups)

20. Aspartic acid (*g*)	$HO_2C{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
21. Glutamic acid (*g*)	$HO_2C{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
22. γ-Hydroxyglutamic acid (*l*)*	$HO_2C{\cdot}CHOH{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$

Basic amino-acids (two amino-groups and one carboxy-group)

23. Ornithine†	$NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
24. Arginine (*g*)	
25. Lysine (*g*, *e*)	$NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
26. δ-Hydroxylysine	$NH_2{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO_2H$
27. Histidine (*g*)	

‡General stereochemistry for L-amino-acids 

* Occurrence in proteins uncertain.
† Ornithine is probably not present in proteins, but is formed by the hydrolysis of arginine.
g general occurrence; *l* less common occurrence; *e* essential in man.

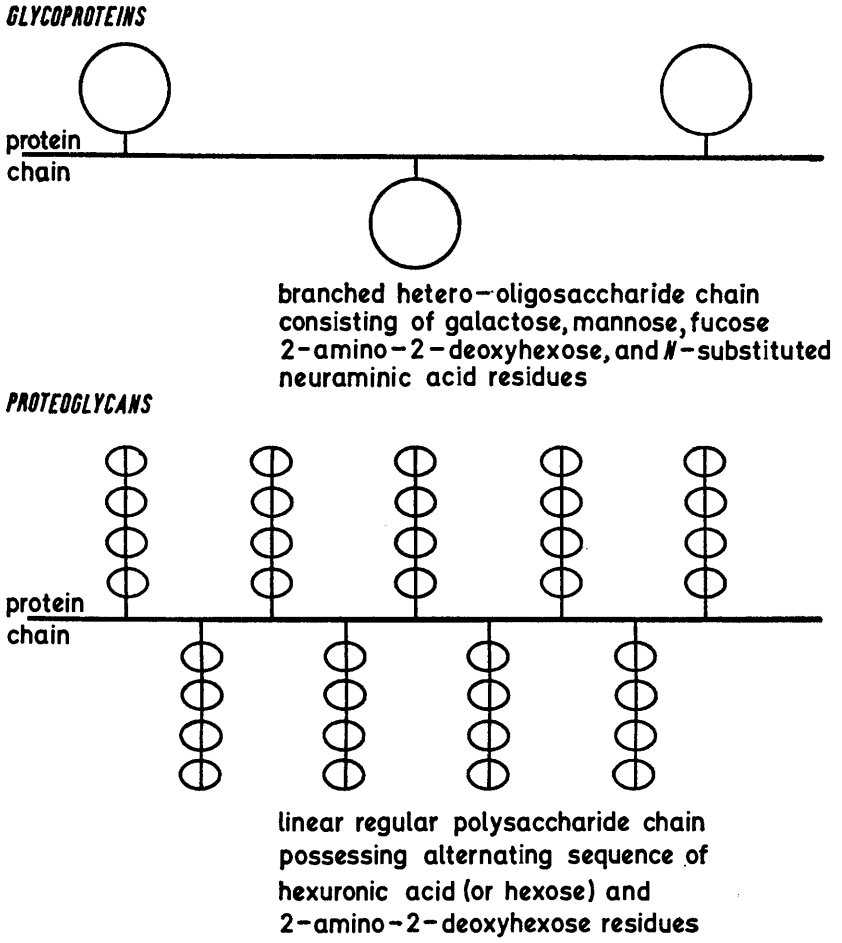
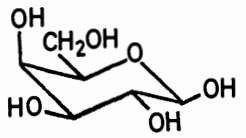


Figure 1 *General representation of glycoproteins and proteoglycans*

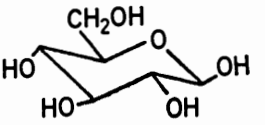
units are *N*-acetylated or *N*-glycolylated and in some cases are also *O*-acetylated. The oligosaccharide chains are therefore overall moderately acidic.

Whilst proteoglycans are also based on a protein backbone, the carbohydrate takes the form of polysaccharide chains which are pendant and covalently bound to the protein chain (Figure 1). These polysaccharide chains are linear and regular, possessing alternating monosaccharide sequences which generally involve acidic monosaccharide (D-glucuronic acid or L-iduronic acid) and basic monosaccharide (2-amino-2-deoxy-D-galactose or 2-amino-2-deoxy-D-glucose). The basic units are *N*-acetylated or *N*-sulphated and are frequently *O*-sulphated. These polysaccharide chains are of course strongly acidic, and were therefore

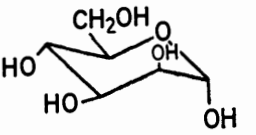
Table 2 *Structures of monosaccharides*



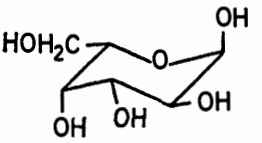
β-D-galactopyranose



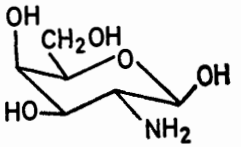
β-D-glucopyranose



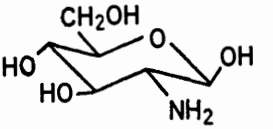
α-D-mannopyranose



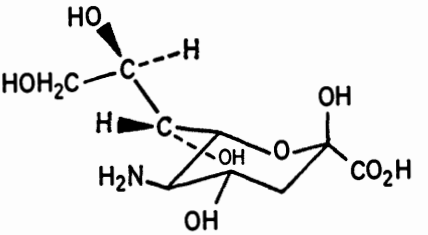
α-L-fucopyranose



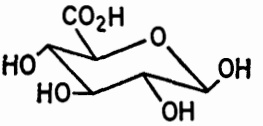
2-amino-2-deoxy-β-D-galactopyranose



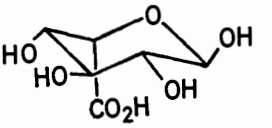
2-amino-2-deoxy-β-D-glucopyranose



5-amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulose
(neuraminic acid)



β-D-glucopyranuronic acid



α-L-idopyranuronic acid

called 'acidic mucopolysaccharides' but are now known as 'glycosaminoglycans' in the interests of systematization.

The number of polysaccharide chains attached to a unit length or unit molecular weight of protein backbone in a proteoglycan is much greater than the number of oligosaccharide chains attached per unit of protein backbone in a glycoprotein.

Thus carbohydrate predominates in a proteoglycan whereas protein predominates in a glycoprotein.

The term carbohydrate–protein complex is applied to situations where glycoproteins, proteoglycans, polysaccharides, oligosaccharides, proteins, peptides, or lipid-type molecules are linked with one another by non-covalent bonds to give complexes which contain both carbohydrate and protein. The nature of the intramolecular linkages is usually ionic.

B. Historical Development of the Concepts of Glycoproteins, Proteoglycans, and Carbohydrate–Protein Complexes.—Historically, proteins such as albumin and gelatin were known before glycoproteins. Probably the first paper on a glycoprotein appeared in 1805 when it was observed[1] that mucus differed in its physicochemical properties from proteins and therefore appeared to be a new type of macromolecular proteinaceous compound. It was not until 1865 that the first chemical evidence for the presence of carbohydrate in mucins was reported, it being observed[2] that elemental analysis of purified mucin gave values for carbon, and more so for nitrogen, that were significantly lower than the corresponding values for proteins, and that acidic hydrolysis of mucin yielded a compound which appeared to be glucose. The presence of basic carbohydrate in glycoproteinaceous materials was particularly recognized in the period 1894–1905, the compound isolated being found to be identical with the hydrolysis product (chitosamine) of chitin, a linear poly(2-acetamido-2-deoxy-D-glucose) which occurs in crab shells.

Nearly coincident with the work on mucinous secretions *etc.*, another type of protein-linked carbohydrate was discovered in the ground substance of connective tissue, it being reported that a carbohydrate termed chondroitin sulphuric acid could be produced by the hydrolysis of hyaline cartilage.[3] This carbohydrate was ultimately purified[4] and was conceived[5] in 1891 to be a polymer which contained glucuronic acid, sulphuric acid, and chitosamine – a polysaccharide which is now known to be a glycosaminoglycan.

Gradually, the picture of carbohydrate firmly bonded to protein emerged. It appeared that all glycoproteins associated with mucous secretions contained 2-amino-2-deoxyhexose and thus the term mucopolysaccharide was introduced[6] in 1938 to describe 2-amino-2-deoxyhexose-containing polymeric materials of animal origin. As more and more information on the occurrence of macromolecular carbohydrate-containing proteinaceous materials was obtained, so it became apparent that not only mucins but all these materials contained 2-amino-2-deoxyhexose.

The occurrence of neuraminic acid in glycoproteins was not recognized until

[1] J. Bostock, *J. Nat. Phil. Chem. Arts*, 2nd Ser., 1805, **11**, 244.
[2] E. Eichwald, *Ann. Chem. Pharm.*, 1965, **134**, 177.
[3] G. Fischer and C. Boedeker, *Ann. Chem. Pharm.*, 1861, **117**, 111.
[4] C. T. Mörner, *Skand. Arch. Physiol.*, 1889, **1**, 210.
[5] O. Schmiedeberg, *Arch. Exp. Pathol. Pharmakol.*, 1891, **28**, 354.
[6] K. Meyer, *Cold Spring Harbor Symposium on Quantitative Biology*, 1938, **6**, 91.

a much later date (1949[7]) on account of the complexity of the monosaccharide structure and the fact that it had not been previously detected in simpler form, as had neutral and basic monosaccharides before their recognition in glycoproteins.

C. Occurrence and General Function of Glycoproteins, Proteoglycans, and Carbohydrate–Protein Complexes.—The course of events has been such that these types of macromolecule have been found extensively in mammalian tissues and fluids, and the literature reporting their occurrence, isolation, purification, and structure is vast (see refs. 8 and 9 for reviews). Thus there is now a general picture of the occurrence in humans of glycoproteins in connective tissues (including bone) as matrix formers; in specialized organs as hormones; in blood cells (erythrocytes and leukocytes) as blood group active substances and in these and other cells as protective coatings; in serum as immunoglobulins and antibodies which give the subject immunity against infection; in serum, milk, urine, saliva, and other secretions and in body fluids as enzymes involved in biosynthesis and metabolism, *etc.* Proteoglycans occur in connective tissues as matrix builders, in joint fluids as lubricants and shock resistors, and in the eye as humour. Carbohydrate–protein complexes occur as cross-linking agents for/of matrix-forming molecules and possibly as glycoprotein–lipid complexes in tissues. Whilst less is known of such non-covalent complexes, there is no doubt that there is much to be discovered in terms of complexes formed by the known glycoproteins and proteoglycans as they perform their various functions.

Overall, whilst many glycoproteins and proteoglycans have been documented as contained in the human body, it may be predicted that more will be found and that molecules presently regarded as proteins will be found to contain carbohydrate as more attention is given to systematic carbohydrate analysis. Clearly, much less is known of the precise function of the macromolecules and of the chemical processes which they perform and undergo.

D. Isolation, Purification, and Compositional and Structural Analysis of Glycoproteins, Proteoglycans, and Carbohydrate–Protein Complexes.—In spite of the fact that the general picture of the occurrence of such macromolecules represents a large number of publications, it is important to realize that a number of problems remain unsolved. Thus many glycoproteins *etc.* have yet to be isolated in a pure form. Even a definition of purity is no longer straightforward since microheterogeneity is now well recognized, *i.e.* a particular glycoprotein may occur in forms which differ from one another by one carbohydrate or amino-acid unit, and it is also recognized that a particular proteoglycan may occur in forms which differ slightly in their glycosaminoglycan composition. Ion-exchange fractionation and gel filtration, which distinguish molecules according to their charge and molecular size, respectively, are used most extensively to effect

[7] A. Gottschalk and P. E. Lind, *Nature*, 1949, **164**, 232.
[8] A. Gottschalk, 'Glycoproteins', Elsevier, Amsterdam, 2nd edn., 1972.
[9] J. S. Brimacombe and J. Webber, 'Mucopolysaccharides', Elsevier, Amsterdam, 1964.

purification. Other techniques include ultracentrifugation, electrophoresis, isoelectric focusing, and affinity chromatography/immunoadsorption, which separate molecules according to their shape/size, charge, pK_a value, and ability to complex with a specific compound, respectively.

Following purification, the next chemical step is a component analysis performed after hydrolysing the macromolecules into their component carbohydrate and amino-acid units. Complete and accurate amino-acid analysis is now an established technique but it is unfortunate that many workers tend to assume that glycoproteins contain L-fucose, D-galactose, D-glucose, D-mannose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, and neuraminic acid as the monosaccharide units, determining neutral sugars and basic sugars only collectively by the classical colorimetric techniques and assuming that the basic sugars are in fact *N*-acetylated. However, with the problems of microheterogeneity it is essential to determine qualitatively and quantitatively each specific type of carbohydrate unit individually and ultimately to determine its D or L configuration. This necessarily involves the use of more advanced techniques such as quantitative gas-phase chromatography, mass spectrometry, ion-exclusion chromatography, and ion-exchange chromatography. Both automated amino-acid and carbohydrate analysers based on ion-exchange chromatography are now available and this should alleviate some of the difficulties of component analysis.

Determination of the primary structure follows component analysis and requires even more skill and attention. Whilst it is true that amino-acid sequences can be determined by automated and semi-automated techniques and peptide mapping, it is not a foregone conclusion that a sequence of a few hundred amino-acid units can be determined without difficulty. Structurally, the carbohydrate portion of the macromolecule is even more elusive since in addition to determining, as in the case of amino-acids, the sequence of the various units, it is necessary to determine which of the hydroxy-groups of the carbohydrate are involved in the linkage to the adjacent units and also the stereochemistry of carbon-1 of the carbohydrate unit. Such parameters give rise to at least eight possibilities for the way in which a neutral hexose unit such as D-galactose is linked within a carbohydrate chain. In the structural determination of both carbohydrate and protein moieties a number of techniques are now employed, and these depend essentially on selective cleavage unit by unit along the chain or on breakdown of the macromolecule into small peptides, oligosaccharides, or glycopeptides which present simpler cases for sequential analysis. As will be seen later, the linkage points and types in the carbohydrate moieties are determined by further specialized techniques.

Finally, from the chemical viewpoint, there remain to be determined the secondary and tertiary structures. The secondary structure involves the complexity that many glycoproteins consist of subunits, *i.e.* they are oligomers of polymeric species. These subunits are frequently different from one another and each has a specific part to play in the overall properties and structure of the molecule. The tertiary structure involves the spatial three-dimensional structure

of the complete molecule. It must not be imagined that the backbone of the macromolecules under consideration is straight – generally it is not and may involve random orientation, coil/helix, ball, and rod type shapes, and indeed may be partially random and partially ordered. Information regarding the particular structures involved in a macromolecule is derived from *X*-ray diffraction analysis, sedimentation analysis, circular dichroism and optical rotatory dispersion spectroscopy, and determination of solution properties.

Complete primary structures have been determined for very few carbohydrate-containing macromolecules and complete information on any tertiary structure has yet to be achieved. This situation holds at present not only on account of the complexity of the structures to be identified but also in the case of human materials on the fact that many of the macromolecules are available only in milligram or even microgram amounts. Thus, as will be seen later, in any such work, the chemist is frequently forced to devise new sensitive analytical methods or to scale down drastically existing methods.

Coupled with structural analysis are investigations of structure–biological activity relationships whether the activity manifested by the macromolecule be enzymic, hormonal, or immunological. Some idea of the involvement of a certain unit type in a structure may be obtained by applying to the macromolecule a reaction which is specific for that unit in bound form and testing for the effect of the derivatization on the activity. Further confirmation of the involvement of the unit may be obtained by reversion of the activity effect on de-derivatization. In other words, monitoring of the activity of the molecule during the reaction sequences is used as a test for the occurrence of a chemical reaction and this technique has a great advantage in that it can be carried out on a microgram or nanogram scale since many of the active macromolecules are active at such levels.

E. Present Work.—Since macromolecules containing carbohydrate and protein are so prolific in the human, it is impossible to research all types and therefore specialization is necessary. Thus our work has involved the investigation of human hormonal glycoproteins in the fertility field and of proteoglycans as constituents of dermal tissue and as excess products of the body in disease. These two classes of molecule will be described in more detail. However, in the case of humans, the chemical and structural investigation is not only academically orientated and is not an end in itself, but is directed at giving a better understanding of health and disease and so to lead the way to chemically based diagnoses and monitoring of treatment. To achieve such aims emphasis must be laid on chemically based analyses as well as structural determination of the macromolecules. In this respect our work has extended to the development of microscale techniques and to the preparation and application of new and improved water-insoluble agents/reactors. These agents are frequently water-insoluble derivatives of enzymes and glycoproteins which can be used for molecular separations, specific purifications, structural studies, and assays of biologically active macromolecules. Chief advantages of these water-insoluble reactors are that they

have greater stability than their soluble counterparts, are easily separated from soluble material, can be re-used, and are amenable to packing into cartridges *etc.* for simplified automated analyses, including clinical chemical analyses. These types of reagent will be described in more detail later.

3 Hormonal Glycoproteins

A. Nomenclature of Hormonal Glycoproteins.—The group of glycoproteins which possess hormonal activity comprises follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, human menopausal gonadotrophin, pregnant mare serum gonadotrophin, and thyroid-stimulating hormone. With the exception of thyroid-stimulating hormone, these hormonal glycoproteins are also known as gonadotrophins on account of their gonad-stimulating ability and their involvement in the fertility cycle. Some aspects of earlier work on the subject of hormonal glycoproteins have been reviewed.[10]

B. Composition of Hormonal Glycoproteins.—These hormones, as already implied, are all macromolecular and are composed of the naturally occurring amino-acids together with the usual carbohydrate components of glycoproteins. Methods and problems in the determination of carbohydrate compositions and structures of glycoprotein hormones have been reviewed.[11] Typical compositions are illustrated by those[12] of human pituitary follicle-stimulating hormone[13] and human chorionic gonadotrophin (Table 3). Generally, these hormones all contain these components in moderately different proportions. It should be noted that the precise composition of each hormone from a number of species has in many cases yet to be determined since complete purity in isolation has yet to be achieved. Furthermore, it now emerges that some of the carbohydrate and amino-acid units, particularly in certain terminal positions, may be 'optional extras', thus giving rise to the problem of microheterogeneity within any hormone preparation. Microheterogeneity may, of course, be the result of incomplete biosynthesis or of metabolism.

It will be realized that many other, totally unrelated glycoproteins have similar compositions, but that it is the precise quantitative composition and more especially the sequence of units along the macromolecular chain (primary structure), the combination of any subunits (secondary structure), the spatial arrangement of the chain (tertiary structure), and the size of the molecule which determine the hormonal character of the molecule.

C. Occurrence, Function, and Use of Hormonal Glycoproteins.—Follicle-stimulating and luteinizing hormones occur in the pituitary gland, and their

[10] J. F. Kennedy, *Endocrinologica Experimentalis*, 1973, **7**, 5.
[11] J. F. Kennedy, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones,' ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 2, Excerpta Medica, Amsterdam, 1972, p. 360.
[12] J. F. Kennedy and M. F. Chaplin, unpublished results.
[13] W. R. Butt, S. S. Lynch, and J. F. Kennedy, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones', ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 2, Excerpta Medica, Amsterdam, 1972, p. 355.

Table 3 *Compositions of human pituitary follicle-stimulating hormone and human chorionic gonadotrophin*

Amino-acid or carbohydrate unit	*Number of residues*		*Percentage by weight*	
	Follicle-stimulating hormone (per mol. wt. of 35 000)	*Chorionic gonadotrophin (per mol. wt. of* 41 000)	*Follicle-stimulating hormone*	*Chorionic gonadotrophin*
L-Aspartic acid*	15.2	17.5	5.00	5.16
L-Threonine	17.3	14.2	4.98	3.68
L-Serine	13.8	15.3	3.43	3.40
L-Glutamic acid	25.0	17.2	9.20	5.65
L-Proline	12.2	29.2	3.37	7.24
Glycine	11.4	15.6	1.85	2.27
L-Alanine	11.4	14.3	2.31	2.60
L-Valine	12.5	13.8	3.54	3.50
L-Methionine	3.2	3.1	1.18	1.02
Half L-cystine	15.2	11.1	4.45	2.86
L-Isoleucine	7.3	5.4	2.24	1.58
L-Leucine	10.5	14.3	3.39	4.13
L-Tyrosine	12.4	5.3	5.81	2.19
L-Phenylalanine	7.6	6.5	3.22	2.43
L-Lysine	12.4	10.1	4.56	3.32
L-Histidine	7.0	4.6	2.73	1.60
L-Arginine	8.2	13.3	3.64	5.33
D-Galactose	13.4	16.6	6.20	6.81
D-Mannose	19.4	19.4	9.01	11.99
L-Fucose	2.0	1.5	0.84	0.56
2-Acetamido-2-deoxy-D-glucose	14.5	15.5	8.45	7.99
2-Acetamido-2-deoxy-D-galactose	1.6	5.0	0.93	2.61
N-Acetylneuraminic acid (acid hydrolysis)	10.6	14.8	8.81	11.10
N-Acetylneuraminic acid (enzymic hydrolysis)	11.1	16.2	9.25	12.10

* With the exceptions of D-galactose and L-fucose, the D and L configuration are assumed by analogy with other glycoproteins.

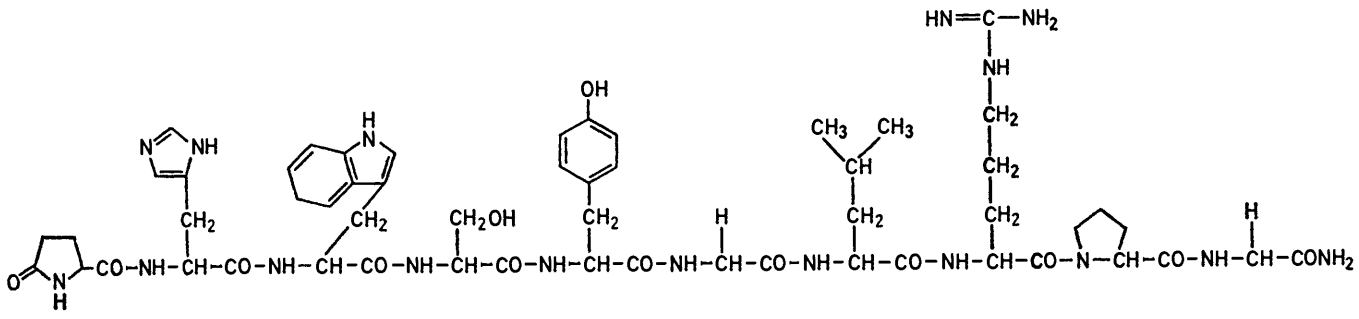
release from this gland is dictated by a releasing hormone/factor produced by the hypothalamus, this hormone being a decapeptide (1).[14] Thyroid-stimulating hormone is similarly released from the pituitary by a tripeptide (2)[15,16] releasing-hormone of hypothalamic origin. Chemico-biological relationships of the two releasing hormones have been investigated.[17,18] Follicle-stimulating and luteinizing hormones act upon the ovary to stimulate follicle growth/rupture according to the stage of the cycle[19] and are ultimately excreted in the urine in macromolecular active forms. The combined follicle-stimulating and luteinizing hormone content of post-menopausal urine is termed human menopausal gonadotrophin. Thyroid-stimulating hormone acts upon the thyroid gland to stimulate production and release of thyroid hormones. Human chorionic gonadotrophin is produced by placental trophoblasts, particularly in the first trimester. No releasing factor for this gonadotrophin has as yet been identified.

Human gonadotrophins are used in clinical treatment of infertility where cases have proved resistant to the synthetic low molecular weight drug clomiphene. Great success in such treatment has been achieved by Dr. A. C. Crooke and his group in Birmingham, administration of a combination of follicle-stimulating and luteinizing hormones being followed by human chorionic gonadotrophin. In this respect, apart from general interest in the structure of these glycoprotein hormones, determination of the complete structure is important with a view to establishing structure–activity relationships and identifying the active site(s). Hopefully, this will permit an understanding of the way in which these molecules function and provide information for the design of simulators which can be synthesized.

D. Activities of Hormonal Glycoproteins.—Each hormone not only has a biological activity, *i.e.* its biological function in the species to which it is endogenous, but also an immunological activity, *i.e.* its ability to act as an antigen in an antigen–antibody system. The biological activity is usually tested *in vivo*, the effect of the injected hormone upon certain organs of the test animal being determined. More recently an *in vitro* test has been developed for follicle-stimulating hormone whereby the biological activity can be determined by direct application of the test material to the ovary.[20]

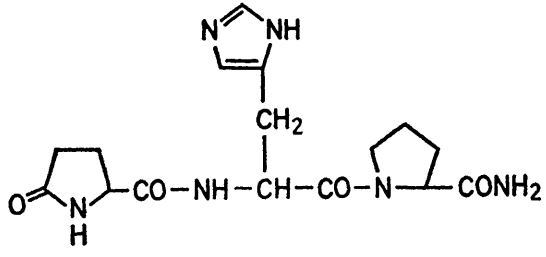
Immunological activity is usually determined in a competitive binding technique where unknown antigen (hormone) competes with a standard amount of

[14] H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Comm.*, 1971, **43**, 1334.
[15] C. Y. Bowers, A. V. Schally, F. Enzmann, J. Boler, and K. Folkers, *Endocrinology*, 1970, **86**, 1143.
[16] R. M. G. Nair, J. F. Barrett, C. Y. Bowers, and A. V. Schally, *Biochemistry*, 1970, **9**, 1103.
[17] J. F. Kennedy, C. J. Gray, S. Ramanvongse, L. Albrighton, and W. F. White, *Life Sciences*, 1973, 12, Pt. I, p. 533.
[18] J. F. Kennedy, C. J. Gray, S. A. Barker, L. Albrighton, C. Y. Bowers, A. V. Schally, and W. F. White, *Life Sciences*, 1971, 10, Pt. II, p. 569.
[19] V. Petrow, *Chem. in Britain*, 1970, **6**, 167.
[20] M. Ryle, M. F. Chaplin, C. J. Gray, and J. F. Kennedy, in 'Gonadotrophins and Ovarian Development', ed. W. R. Butt, A. C. Crooke, and M. Ryle, Livingstone, Edinburgh, 1970, p. 98.



pyroGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Glyamide

(1)



pyroGlu–His–Proamide

(2)

radioactively labelled but otherwise identical hormone for binding to a fixed and substoicheiometric amount of antibody which has been raised specifically to the hormone in question. In order to facilitate separation of bound antigen from unbound antigen, the antibody is frequently immobilized by attachment to a water-insoluble matrix, *e.g.* bentonite,[21] cellulose,[22] or Sephadex.[23] Bioassays require a number of days to elapse between administration and measurement and are rather imprecise, whereas the radioimmunoassay is easily completed in a few hours and is much more accurate.

The methods for determination of the specific activity of hormonal glycoproteins are not without criticism; the greater problem is not the determination of activity on a small scale, but the measurement of mass on a small scale. In view of the instability of the hormones and the small quantities being prepared, dry weight determinations are inaccurate. Significant variations exist[24] between results based upon alternative methods such as u.v. absorption and colorimetric determination of the protein, and such variations must account for some of the differences in potencies reported by various laboratories for the most highly purified materials. Whilst spectrophotometric methods give tolerable approximations, determination of mass by a full carbohydrate and amino-acid compositional analysis is the only absolute method.

E. Isolation and Purification of Hormonal Glycoproteins.—The closely similar chemical and physical natures of the hormones to one another and to other macromolecules, including unrelated glycoproteins endogenous to the media of origin, give rise to difficulties in the isolation and purification of the particular hormone under investigation. A complex series of purification steps is often necessary in which it is important to take advantage of properties peculiar to the hormone molecule, *e.g.* acidic and basic groups provided by certain amino-acid units and acidic groups provided by *N*-acetylneuraminic acid units. Thus the purification methods used by many workers include ion-exchange chromatography and electrophoresis in addition to molecular weight discrimination by gel filtration. It is essential to monitor the purification process by determination of activity as well as protein profiles since no purely chemical technique is hormone-specific. Owing to the sensitive natures of the hormones, where loss of a few residues can abolish all activity, care must be taken to avoid microbial contamination and conditions which deviate markedly from the physiological. Clearly, the final stages of purification prove more and more difficult as the impurities become predominantly more similar to the hormone. A typical purification is one which has been applied to human follicle-stimulating hormone[13,24,25] and which involves a number of steps of chromatography on calcium phosphate, DEAE-cellulose, and Bio-Gel P-150. By such techniques a purification of 5000 fold has been achieved.[13]

[21] W. R. Butt and S. S. Lynch, *Clinica Chim. Acta*, 1968, **22**, 79.
[22] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **30**, 11.
[23] L. Wide, S. J. Nillius, C. Gemzell, and P. Roos, *Acta Endocrinol.*, 1973, **73**, suppl. 174.
[24] S. A. Barker, C. J. Gray, J. F. Kennedy, and W. R. Butt, *J. Endocrinol.*, 1969, **45**, 275.
[25] A. S. Hartree, *Biochem. J.*, 1966, **100**, 754.

Two other approaches to the purification of hormonal glycoproteins have now been pursued. Isoelectric focusing has been applied to the purification of follicle-stimulating and luteinizing hormone from the human and other species.[26-28] However, this technique may suffer from the fact that other materials in the mixture may have pI values analogous to that of the hormone. More important, the technique does not overcome the problem of microheterogeneity since differences of, for example, one or two *N*-acetylneuraminic acid units between molecules will cause them to migrate to slightly different pI values under the electrostatic field in the static pH gradient column. This theory is supported by the fact that human luteinizing hormone was resolved into multiple components in the range pH 7—10 whereas each component showed comparable high biological and immunological activity.[26] Treatment of the components with neuraminidase decreased their number. Immunoadsorption has been applied to the purification of ovine luteinizing hormone.[29] Since antibodies highly specific for the glycoprotein hormones can be produced, this technique is theoretically the most important and advanced, permitting almost complete purification in one step. However, problems preventing the complete success of this type of purification include that of raising the specific antibody. This really requires the administration of high purity material to the animal. A further problem is that of achieving a successful elution of the hormonal glycoprotein specifically bound to the immobilized antibody. Many of the reagents used for such elutions in other antibody–antigen systems are known to cause disruption of the hormonal glycoproteins into subunits.

The stabilities of the most highly purified preparations of certain hormonal glycoproteins have presented some problems, *e.g.* follicle-stimulating hormone preparations are often found to be stable in frozen solution but lose activity dramatically on concentration and freeze drying. Indeed some of the highest potencies described have been for preparations which were obtained in solution.[30,31] The easy loss of activity is undoubtedly due to alterations to the macromolecular structure and again emphasizes the sensitivity of these hormones and the precision with which hormonal activity is related to the molecular structure. Some studies have been carried out on the stability of human pituitary follicle-stimulating hormone to various parameters,[13,24] and a preparation of highest activity which is both biologically and immunologically stable to freeze drying and concentration has now been obtained.[13]

F. Primary and Secondary Structures of the Hormonal Glycoproteins.—The determination of the primary structures of the hormonal glycoproteins is very much in its infancy on account of the difficulties in obtaining quantities of pure

[26] L. E. Reichert, *Endocrinology*, 1971, **88**, 1029.
[27] D. Graesslin, H. C. Weise, and G. Bettendorf, in 'Gonadotropins', ed. B. B. Saxena, C. G. Beling, and H. M. Candy, Wiley–Interscience, New York, 1971, p. 159.
[28] P. Rathnam and B. B. Saxena, *J. Biol. Chem.*, 1970, **245**, 3725.
[29] D. Gospodarowicz, *J. Biol. Chem.*, 1972, **247**, 6491.
[30] P. Roos, *Acta Endocrinol.*, 1968, **59**, suppl. 131.
[31] W. D. Peckam and A. F. Parlow, *Endocrinology*, 1969, **84**, 933.

material. The determination has been approached in three ways – determinations carried out on the intact molecules, determinations carried out on the subunits of the molecules, and *via* structure–activity relationships (see later).

Human pituitary follicle-stimulating hormone has been studied by the direct approach. Treatment with the enzyme neuraminidase released all the *N*-acetylneuraminic acid units from the intact molecule and hence these units must occupy terminal non-reducing positions in the oligosaccharide moieties.[32] The desialylized but not the intact molecule was susceptible to galactose oxidase, indicating that the galactose units have the D-configuration and do not occupy terminal non-reducing positions but are adjacent to the *N*-acetylneuraminic acid units. Some fucose was released by L-fucosidase, but generally the hormone is disappointingly resistant to the action of glycosidases. However, the number of possibilities of linkage positions of the various monosaccharide units in the hormone has been narrowed by periodate oxidation studies in which adjacent carbohydrate hydroxy-groups not involved in glycosidic linkages are oxidized and reduced and the products, liberated by acid hydrolysis, are identified quantitatively.[33] The identification of linkage type in the carbohydrate moieties of follicle-stimulating hormone was finalized by methylation analysis in which unoccupied hydroxy-groups are converted into methyl ether groups whereas occupied hydroxy-groups are exposed in the subsequent acid hydrolysis.[34] Identification of the hydrolysis products by gas–liquid chromatography and mass spectrometry demonstrated that the L-fucose units occupy terminal non-reducing positions, the D-galactose units are linked in the 1- and 2-positions, the mannose units exist in three forms (some as terminal non-reducing residues, some as 1,6-linked residues, and some as 1,3,4-linked branch points), and the 2-acetamido-2-deoxyglucose units are 1,6-linked. The methylation analysis also showed that these four types of unit exist in the pyranose forms and that the 2-amino-2-deoxyglucose units are *N*-acetylated.

The application of glycosidases to human chorionic gonadotrophin has proved successful, revealing the sequences *N*-acetylneuraminosyl-β-D-galactosyl-2-acetamido-2-deoxy-β-D-glucosyl-α-D-mannose and α-L-fucosyl-β-D-galactosyl-2-acetamido-2-deoxy-β-D-glucosyl-α-D-mannose.[35,36] From the data, tentative structures involving branch points at D-mannose units were proposed. Preliminary studies also indicated that 2-acetamido-1-[(*N*-β-L-aspartyl)amino]-2-deoxy-β-D-glucopyranosylamine and 2-acetamido-2-deoxy-β-D-glucopyranosyl-serine type linkages are involved at the carbohydrate–protein junctions.

Turning to the structural approach *via* the subunits, the phenomenon of subunit formation by the gonadotrophins and thyroid-stimulating hormone

[32] M. F. Chaplin, C. J. Gray, and J. F. Kennedy, in 'Gonadotropins and Ovarian Development', ed. W. R. Butt, A. C. Crooke, and M. Ryle, Livingstone, Edinburgh, 1970, p. 77.
[33] J. F. Kennedy and W. R. Butt, *Biochem. J.*, 1969, **115**, 225.
[34] J. F. Kennedy and M. F. Chaplin, *Biochem. J.*, 1972, **130**, 417.
[35] O. P. Bahl, *J. Biol. Chem.*, 1969, **244**, 575.
[36] O. P. Bahl, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones', ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 1, Excerpta Medica, Amsterdam, 1971, p. 99.

has been reviewed.[37] First evidence that the pituitary glycoprotein hormones could exist in the form of subunits came from work on ovine luteinizing hormone, which was dissociated by hydrochloric acid at pH 1.3.[38,39] Many reports on the use of various dissociative reagents such as dichloroacetic acid, urea, and guanidine have followed. Human luteinizing hormone has been shown to be dissociated at a pH of 1.3,[40] and by dichloroacetic acid,[41] urea, or guanidine.[42,43]

Evidence for the dissociation of human pituitary follicle-stimulating hormone was originally presented in terms of quantized molecular weights according to the ionic strength of the solvent used for gel filtration.[44] Treatment of the hormone with sodium dodecyl sulphate gave two fragments of different molecular weights,[45] and treatment with acid at pH 1.0 at 20 °C also gave rise to fragments.[46] The hormone has also been segregated into its subunits by the action of urea[47] and propionic acid.[48] Human chorionic gonadotrophin has been separated into non-identical subunits by preparative gel electrophoresis[49] and urea treatment.[50,51] Work on thyroid-stimulating hormone has been confined to that of bovine origin, but again two subunits have been separated and identified. From these studies and those on such hormones from other species[37,52] it has emerged that all the glycoprotein hormones can be dissociated into dissimilar subunits (α and β).

Recent studies of the subunits of the hormonal glycoproteins have been reviewed[52] and the complete amino-acid sequences have been determined in a number of instances for subunits from non-human sources. Of the human hormones, most work has been done on chorionic gonadotrophin, the α- and β-subunits (HCG-α, HCG-β) possessing 92 and 139 amino-acid residues, respectively, [(3), (4)] the points of attachment of carbohydrate being indicated.[53] The sequence of the *N*-terminal 35 residues of the α-subunit of human luteinizing

[37] W. R. Butt and J. F. Kennedy, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones', ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 1, Excerpta Medica, Amsterdam, 1971, p. 115.
[38] C. H. Li and B. Starman, *Nature*, 1964, **202**, 291.
[39] D. N. Ward and M. S. Arnott, *Analyt. Biochem.*, 1965, **12**, 296.
[40] A. S. Hartree, in 'Protein and Polypeptide Hormones', ed. M. Margoulies, International Congress Series 161, Part 3, Excerpta Medica, Amsterdam, 1969, p. 799.
[41] L. E. Reichert, A. R. Midgley, G. D. Niswender, and D. N. Ward, *Endocrinology*, 1970, **87**, 534.
[42] R. J. Ryan, N. Jiang, and S. Hanlon, *Recent Prog. Hormone Res.*, 1970, **26**, 105.
[43] L. E. Reichert and D. N. Ward, *Fed. Proc.*, 1969, **28**, 505.
[44] C. J. Gray, *Nature*, 1967, **216**, 112.
[45] J. F. Kennedy, W. R. Butt, W. Robinson, and M. Ryle, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones', ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 2, Excerpta Medica, Amsterdam, 1972, p. 348.
[46] J. F. Kennedy and M. F. Chaplin, *J. Endocrinol.*, 1973, **57**, 501.
[47] B. B. Saxena and P. Rathnam, *J. Biol. Chem.*, 1971, **246**, 3549.
[48] L. E. Reichert, *Endocrinology*, 1972, **90**, 1119.
[49] F. J. Morgan and R. E. Canfield, *Endocrinology*, 1971, **88**, 1045.
[50] N. Swaminathan and O. P. Bahl, *Biochem. Biophys. Res. Comm.*, 1970, **40**, 422.
[51] S. Donini, V. Olivieri, G. Ricci, and P. Donini, *Acta Endocrinol.*, 1973, **73**, 133.
[52] S. M. Amir, *Acta Endocrinol.*, 1972, **70**, 21.
[53] O. P. Bahl. R. B. Carlsen, R. Bellisario, and N. Swaminathan, *Biochem. Biophys. Res. Comm.*, 1972, **48**, 416.

H-Ala-Pro-Asx-Val-Glx-Asx-Cys-Pro-Glx-Cys-Thr-Leu-Glx-Glx-Asx-Pro-Phe-Phe-Ser-Glx-Pro-Gly-Ala-Pro-Ile-Leu-Glx-Cys-
CHO
|
Met-Gly-Cys-Cys-Phe-Ser-Arg-Ala-Tyr-Pro-Thr-Pro-Leu-Arg-Ser-Lys-Lys-Thr-Met-Leu-Val-Glx-Lys-Asn-Val-Thr-Ser-Glx-
CHO
|
Ser-Thr-Cys-Cys-Val-Ala-Lys-Ser-Tyr-Asx-Arg-Val-Thr-Val-Met-Gly-Gly-Phe-Lys-Val-Glx-Asn-His-Thr-Ala-Cys-His-Cys-
Ser-Thr-Cys-Tyr-Tyr-His-Lys-Ser-OH

(3)

CHO CHO
| |
H-Ser-Lys-Glx-Pro-Leu-Arg-Pro-Arg-Cys-Arg-Pro-Ile-Asn-Ala-Thr-Leu-Ala-Val-Glx-Lys-Glx-Gly-Cys-Pro-Val-Cys-Ile-Asn-
Val-Thr-Thr-Ile-Cys-Ala-Gly-Tyr-Cys-Pro-Thr-Met-Thr-Arg-Val-Leu-Glx-Gly-Val-Leu-Pro-Ala-Leu-Pro-Glx-Leu-Val-Cys-
Asx-Tyr-Arg-Asx-Val-Arg-Phe-Glx-Ser-Ile-Arg-Leu-Pro-Gly-Cys-Pro-Arg-Gly-Val-Asx-Pro-Val-Val-Ser-Tyr-Ala-Val-Ala-
Leu-Cys-Arg-Ser-Thr-Thr-Asx-Cys-Gly-Gly-Pro-Lys-Asx-His-Pro-Leu-Thr-Cys-Asx-Asx-Pro-Arg-Phe-Glx-Asx-Ser-Ser-Ser-
CHO CHO CHO
| | |
Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asx-Thr-Pro-Ile-Leu-Pro-Glx-Ser-Leu-Pro-OH

(4)

H-Val-Gln-Asp-Cys-Pro-Glu-Cys-Thr-Leu-Gln-Glu-Asn-Pro-Phe-Phe-Ser-Gln-Pro-Gly-Ala-Pro-Ile-Leu-Gln-Cys-Met-Gly-
Cys-Cys-Phe-Ser-Arg-Ala-Tyr-Pro

(5)

hormone (HLH-α) has been reported[54] (5) and is identical with the *N*-terminal residues of HCG-α except that the latter has three additional residues at the *N*-terminus. The amino-acid sequence of HCG-α also shows extensive analogies with the α-subunits of ovine luteinizing hormone (OLH-α) and of bovine thyroid-stimulating hormone (BTSH-α) although the carbohydrate compositions are quite different.

The amino-acid sequences of the β-subunits of these two hormones from non-human species are, however, quite distinct from one another and from that of HCG-β. No sequence data are as yet available for the subunits of human follicle-stimulating hormone but the α-subunit of human follicle-stimulating hormone (HFSH-α) has been shown to possess an amino-acid composition analogous to HLH-α and HCG-α.[55] Although the picture is far from complete, it appears at this stage that the β-subunits are individually unique, whereas the α-subunits have some common amino-acid but not carbohydrate sequences. Tertiary structures have yet to be studied seriously but physicochemical studies of human chorionic gonadotrophin indicate a spherical structure for the hormone.[56]

G. Primary Structure–Activity Relationships of Hormonal Glycoproteins.—The involvement of specific units within the primary structures of the molecules may be determined by combined chemical and biological techniques as already described. Earlier results for such studies on the gonadotrophins have been summarized.[11,57] In general it has been found that removal of the *N*-acetylneuraminic acid units from follicle-stimulating hormone and human chorionic gonadotrophin destroy the biological (*in vivo*) but not the immunological activities. In contrast, removal of the same units from luteinizing hormone has no effect upon either activity of the hormone. Further investigation of the desialylization of human pituitary follicle-stimulating hormone showed that whereas the modified molecule is inactive *in vivo* it is biologically active *in vitro*, *i.e.* when presented direct to the target organ of the hormone, the ovary, the molecule still exhibits follicle-stimulating ability.[20] This was interpreted as indicating the necessity of the *N*-acetylneuraminic acid units only for transport of the hormone from the site of production to the site of action. Such a phenomenon has subsequently been shown to be the general case for a number of glycoproteins. Reduced, periodate-oxidized human pituitary follicle-stimulating hormone was found to be immunologically active but biologically (*in vivo*) inactive, suggesting that although the carbohydrate is essential for biological activity, it is not a requirement for immunological activity, which appears to be a function of the protein portion of the molecule.[33] Modification of human follicle-stimulating hormone by its reaction with chloramine-T, diazosulphanilic

[54] T. Inagami, K. Murakami, D. Puett, A. S. Hartree, and A. Nureddin, *Biochem. J.*, 1972, **126**, 441.
[55] P. Rathnam and B. B. Saxena, in 'Gonadotropins', ed. B. B. Saxena, C. G. Beling, and H. M. Candy, Wiley–Interscience, New York, 1971, p. 120.
[56] K. F. Mori and T. R. Hollands, *J. Biol. Chem.*, 1971, **246**, 7223.
[57] W. R. Butt, *Acta Endocrinol.*, 1969, **64**, suppl. 142, p. 13.

acid, maleic anhydride, citraconic anhydride, *N*-acetylimidazole, t-butyl azidoformate, and proteases, and by photo-oxidation has shown that lysyl, histidyl, methionyl, cysteinyl, and some tyrosyl residues are essential for manifestation of biological (*in vivo*) activities whereas arginyl, tryptophanyl, and phenylalanyl residues are not so involved.[32,58,59] Biological properties of human chorionic gonadotrophin after removal of the sialic acid and D-galactose residues have been investigated in detail.[60]

H. Secondary Structure–Activity Relationships of Hormonal Glycoproteins.—The subunits already described for the hormonal glycoproteins have been extensively examined for their biological and immunological activities with respect to those of the parent intact molecules and such phenomena have been reviewed and summarized.[10,37,52] The α- and β-subunits of human luteinizing hormone were found to possess some residual immunological activity and small amounts of biological activity.[41,61] Recombination of the subunits was achieved by incubation together on a 1:1 basis whereupon both immunological and biological activities were restored. Analogous results have been obtained for follicle-stimulating hormone[47,48] and for human chorionic gonadotrophin.[49,50] From these studies and those on gonadotrophins and thyroid-stimulating hormone from non-human species it is now clear that the loss of immunological and biological activity on subunit formation, which is reversible, is a general property of the hormones. However, the small amount of residual biological activity in the separate subunits may be an artefact, owing to the presence of traces of the complementary subunit; this view is supported by the fact that the residual biological activity of HCG-β has been largely eliminated by further purification.[62] It has also been found that the subunits of a particular hormone from various species are interchangeable so far as activity is concerned. More important, however, is the fact that hybrid gonadotrophins have been produced, *e.g.* the combination of HCG-α with BTSH-β gives a molecule with thyroid-stimulating hormone activity.[63] Also, the α-subunits of human luteinizing and follicle-stimulating hormones and chorionic gonadotrophin can be substituted for one another in combination with the β-subunit of each hormone.[64] A pattern is now emerging in which the type of hormonal activity of the hybrid gonadotrophin is designated by the activity in which the β-subunit used was originally involved.

[58] W. R. Butt, S. S. Lynch, M. F. Chaplin, C. J. Gray, and J. F. Kennedy, in 'Gonadotrophins and Ovarian Development', ed. W. R. Butt, A. C. Crooke, and M. Ryle, Livingstone, Edinburgh, 1970, p. 171.

[59] J. F. Kennedy, S. Ramanvongse, W. R. Butt, W. Robinson, M. Ryle, and A. Shirley, in 'Structure–Activity Relationships of Protein and Polypeptide Hormones', ed. M. Margoulies and F. C. Greenwood, International Congress Series 241, Part 2, Excerpta Medica, Amsterdam, 1972, p. 351.

[60] T. Tsuruhara, M. L. Dufau, J. Hickman, and K. J. Catt, *Endocrinology*, 1972, **91**, 296.

[61] A. Nureddin, A. S. Hartree, and P. Johnson, in 'Gonadotropins', ed. B. B. Saxena, C. G. Beling, and H. M. Candy, Wiley–Interscience, New York, 1971, p. 167.

[62] P. Donini and S. Donini, personal communication, 1973.

[63] J. G. Pierce, O. P. Bahl, J. S. Cornell, and N. Swaminathan, *J. Biol. Chem.*, 1971, **246**, 2321.

[64] P. Rathnam and B. B. Saxena, *J. Biol. Chem.*, 1971, **246**, 7087.

This phenomenon has yet to be tested for all the human hormonal glycoproteins but nevertheless it seems quite certain that the β-subunits are hormone-specific whereas the α-subunits are interchangeable. Such a finding is in keeping with the similarities of the amino-acid sequences of the α-subunits but the unique characters of the β-subunits.

Apart from such work, it is also important to try and produce fragments of the hormones, which retain activity, thus permitting a localization of the active site(s) of the molecules. Some success has been achieved in this respect, fragments produced from human follicle-stimulating hormone retaining immunological activity and biological activity (*in vitro*).[45,46]

Conclusions and future objectives are included in the final section of this article, page 392.

4 Proteoglycans

A. Nomenclature of Proteoglycans.—On account of the apparent regularity of the polysaccharide chains in proteoglycans and the earlier belief that the protein present in preparations of the polysaccharide parts represented impurity, greater attention has been given to the polysaccharides themselves. Thus the polysaccharides, glycosaminoglycans (earlier named acidic mucopolysaccharides), have been classified and named according to their monosaccharide contents and apparent repeating structures. In comparison with glycoproteins, the proteoglycans are apparently few in type and present only eight glycosaminoglycan components: hyaluronic acid, chondroitin, chondroitin 4-sulphate (chondroitin sulphate A), chondroitin 6-sulphate (chondroitin sulphate C), dermatan sulphate (chondroitin sulphate B, β-heparin), heparin, heparan sulphate (heparitin sulphate), and keratan sulphate (keratosulphate). Earlier[9] and more recent[65] aspects of the subject of the glycosaminoglycans have been reviewed.

B. Composition of Proteoglycans.—The proteoglycans, as already implied, are all macromolecular and are composed of the naturally occurring amino-acids, hexuronic acid (or in one case hexose), 2-amino-2-deoxyhexose, acetate, and sulphate (in some cases) together with the following types of unit which may be present in small amounts: hexose, pentose, 6-deoxyhexose, sialic acid. The determination of amino-acid and 2-amino-2-deoxyhexose presents no problem, methodology being well established and a sensitive fluorimetric method[66,67] being available for identification of the latter in microgram amounts. Similarly, determination of the hexoses, although present in small amounts, is easily performed and the gas-phase chromatographic behaviour of the pentoses as volatile derivatives has been studied in detail.[68] Although determination of the uronic acid content by the modified carbazole procedure is a well-established

[65] J. F. Kennedy, *Biochem. Soc. Trans.*, 1973, **1**, 807.
[66] H. Cho Tun, J. F. Kennedy, M. Stacey, and R. R. Woodbury, *Carbohydrate Res.*, 1969, **11**, 225.
[67] J. F. Kennedy, in 'Automation in Analytical Chemistry', Technicon, Basingstoke, 1973, p. 528.
[68] J. F. Kennedy, *Chromatographia*, 1970, **3**, 316.

technique, identification of the uronic acid is not so easy. These units may be partially identified either enzymically (see later) or by conversion into the corresponding hexose and identification as such.[69] The gas-phase chromatographic and mass spectrometric behaviour of the uronic acids as volatile derivatives has also been studied as an aid to identification.[70] The determination of sulphate has also presented a problem for microscale work where the sulphate content is below the lower limit of the techniques, which depend on the formation of insoluble sulphates. A flame-photometric method based on determination of barium sulphate in the range in which it is soluble has been reported[71] and it is to be hoped that an ion-selective electrode which is being developed for sulphate[72] could also be used in this field. Determination of the amino-acid composition is similar to that for any proteinaceous molecule, but as will be evident from the trend throughout this section, much less attention has been given to the amino-acid compositions of the proteoglycans.

C. Occurrence, Function, and Use of Proteoglycans.—The glycosaminoglycans of the proteoglycans occur generally in mammalian species. Of the glycosaminoglycans, hyaluronic acid was reported first, although 'unwittingly', since it was extracted, albeit in a crude form, from human umbilical cords as Wharton's jelly in 1656.[73] In terms of the modern approach it was discovered in bovine vitreous humour in 1934,[74] and in the human the glycosaminoglycan is now known to be an important component of bone, fibroblasts, skin, aorta, and connective tissues, and also of two body fluids – synovial fluid and vitreous humour; it is also produced by a few Streptococcal bacteria. Chondroitin 4-sulphate was first isolated in 1861 from cartilage[3] although the workers were somewhat uncertain of themselves; chondroitin 6-sulphate was reported in 1936.[75] These two isomers usually occur together, *e.g.* in bone, cornea, aorta, umbilical cord, and connective tissue from humans and other species. Polysulphated variants of these two glycosaminoglycans occur predominantly in squid and shark cartilage. Chondroitin itself has a more limited occurrence, being found in cornea.[76] Dermatan sulphate[77] is widely spread in cornea, sclera, skin, lung, aorta, and connective tissues, and has also been detected in a bacterium.[78] Heparin,[79] heparan sulphate,[80] and keratan sulphate[76] are found extensively in connective tissues *etc.* and the latter at least is also a component of cartilage. Polysulphated varieties of keratan sulphate have been found in shark cartilage. The reports of the occurrence of

[69] R. L. Taylor and H. E. Conrad, *Biochemistry*, 1972, **11**, 1383.
[70] J. F. Kennedy and S. M. Robertson, unpublished results.
[71] S. A. Barker, J. F. Kennedy, P. J. Somers, and M. Stacey, *Carbohydrate Res.*, 1968, **7**, 361.
[72] G. G. Guilbault, personal communication, 1973.
[73] T. Wharton, 'Adenographia: Sive Clandularum Totius Corporis Descriptio', Londini, 1656.
[74] K. Meyer and J. W. Palmer, *J. Biol. Chem.*, 1934, **107**, 629.
[75] K. Meyer and J. W. Palmer, *J. Biol. Chem.*, 1936, **114**, 689.
[76] K. Meyer, A. Linker, E. A. Davidson, and B. Weissman, *J. Biol. Chem.*, 1953, **205**, 611.
[77] K. Meyer and E. Chaffee, *J. Biol. Chem.*, 1941, **138**, 491.
[78] G. K. Darby, A. S. Jones, J. F. Kennedy, and R. T. Walker, *J. Bacteriol.*, 1970, **103**, 159.
[79] J. McLean, *Amer. J. Physiol.*, 1916, **41**, 250.
[80] J. E. Jorpes and S. Gardell, *J. Biol. Chem.*, 1948, **176**, 267.

glycosaminoglycans now run into hundreds, and, as already indicated, these molecules are essentially parts of the proteoglycans. The corresponding proteoglycans have more recently been isolated intact from the same sources.

The general function of the proteoglycans as essential components in the block building of the macromolecular framework of connective and other tissues from these molecules and collagen has already been cited. Hyaluronic acid, on account of the viscosity it imparts to its solutions, appears to act as a lubricant for joints and also possesses physicochemical characteristics which permit the solution to be fluid except under compression (shock) when it acts more as a shock-absorbing gel. On account of their macromolecular and charged natures, the proteoglycans may operate to perform filtration and exclusion of diffusable molecules. Beyond these, the true role of the proteoglycans in greater detail is uncertain since they do not appear to manifest biological, hormonal, or enzymic properties.

Clinically, the glycosaminoglycans and proteoglycans are of little use in terms of therapy of non-production since most diseases known to involve them specifically produce them in excess. However, heparin alone has potent blood anticoagulent properties and is therefore used extensively for the prevention of clotting.

D. Isolation and Purification of Proteoglycans.—The quantitative isolation of the glycosaminoglycans frequently presents a problem since the parent proteoglycans are often strongly associated with insoluble collagen fibres *etc.* Various techniques have been used for such cases including enzymic degradation with proteases and collagenases, and alkaline extraction. Some tissues are relatively easily solubilized but studies on human skin, chemically one of the most inaccessible tissues, have shown that extensive disruption of the fibres is essential to permit complete attack of de-bonding *etc.* reagents.[81] A typical process to obtain a mixed glycosaminoglycan fraction involves the following: drying of tissue, solvent extraction of lipid, extensive homogenization, proteolytic digestion, brief alkaline treatment, denaturation of residual protein, and dialysis, the product being purified as described subsequently.

Although isolation of the proteoglycans intact may be considered an easier task, de-bonding agents are still somewhat necessary. However, initial emphasis was again laid on disruption, and the extraction of tissue with water alone, with high speed homogenization, yielded a proteoglycan fraction.[82] It was subsequently found that the use of salts greatly enhanced the yield of proteoglycan, up to 90% of the total being extracted when guanidinium chloride was used.[83] This is attributable to the fact that such an extractant causes dissociation of proteoglycan aggregates, and the process is therefore a dissociative extraction. Dissociative extraction is to be preferred since the macromolecules are more likely to remain intact. Ethylenediaminetetra-acetic acid–sodium chloride solution has also proved useful for the extraction of proteoglycans, and many other agents have

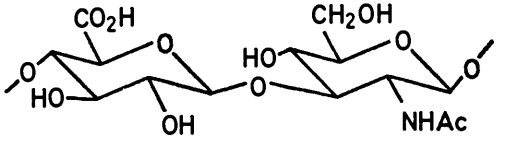
[81] S. A. Barker, J. F. Kennedy, and P. J. Somers, *Carbohydrate Res.*, 1969, **10**, 57.
[82] J. Shatton and M. Schubert, *J. Biol. Chem.*, 1954, **211**, 565.
[83] S. W. Sajdera and V. C. Hascall, *J. Biol. Chem.*, 1969, **244**, 77.

been tested. The use of cathepsins holds potential. Generally, it has been proposed that the extractability of a proteoglycan is inversely parallel to the protein content and is also dependent upon the age of the tissue.[84]

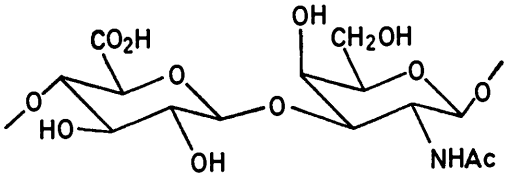
Having obtained an initial crude extract, it is necessary to achieve a fractionation into the various molecular types present. In the case of glycosaminoglycans, one might expect this to be relatively easy on account of their individual charge characteristics. However, such simple theory is overshadowed by molecular weight heterogeneity within a fraction and the presence of small amounts of residual peptide. Numerous techniques, including selective precipitation, thin-layer chromatography, and isoelectric focusing, have been employed. No one method separates completely all glycosaminoglycans but ion-exchange chromatography (see ref. 81), electrophoresis (see ref. 85), and the cetylpyridinium chloride–cellulose column technique[86] are probably the best, the latter method being based on the fact that glycosaminoglycans can be partially separated by precipitation as their cetylpyridinium salts.[87] Gel filtration may prove useful at various stages in a purification procedure, but recent studies have shown that the glycosaminoglycans can become associated with some gel-filtration matrices.[88] However, none of these techniques should be regarded as doing more than providing a means of separating the glycosaminoglycans, structural analysis being necessary for complete identification (see later). In the case of proteoglycans, a combination of selective precipitation and high speed centrifugation has been used extensively and separates the proteoglycans into heavy (PPH) and light (PPL) fractions, *e.g.* ref. 89. Fractionations of proteoglycans and derived subfractions may also be achieved by equilibrium density-gradient ultracentrifugation, gel filtration, and ion-exchange chromatography.

E. Primary Structures of the Glycosaminoglycan Components of Proteoglycans.—The glycosaminoglycans may be distinguished by their compositions and primary structures, since repeating disaccharide structures have been discovered as general features of all of them. Thus the repeating unit of hyaluronic acid is (6), the molecule containing no sulphate. Chondroitin, the only other non-sulphated glycosaminoglycan, is an isomer of hyaluronic acid, the 2-amino-2-deoxyhexose component of the disaccharide repeating unit (7) having the D-*galacto*- rather than the D-*gluco*-configuration. As implied, chondroitin 4-sulphate and chondroitin 6-sulphate are sulphated variants of chondroitin, the sulphate ester groups being situated on the 2-amino-2-deoxy-D-galactose units [(8) and (9), respectively]. Dermatan sulphate (10) is an isomer of chondroitin 4-sulphate in which the uronic acid has the L-*ido*- rather than the D-*gluco*-configuration. As with other, perhaps better known, polysaccharides, irregularities have been observed in

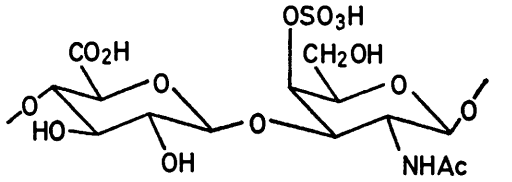
[84] A. A. Hallén, in 'Chemistry and Molecular Biology of the Intercellular Matrix', ed. E. A. Balazs, Academic Press, London, 1970, vol. 2, p. 903.
[85] P. W. Lewis, J. F. Kennedy, and N. D. Raine, *Biochem. Soc. Trans.*, 1973, **1**, 844.
[86] J. Svejcar and W. Van B. Robertson, *Analyt. Biochem.*, 1967, **18**, 333.
[87] J. E. Scott, *Methods Biochem. Analysis*, 1960, **8**, 145.
[88] J. F. Kennedy, *J. Chromatog.*, 1972, **69**, 325.
[89] R. M. Mason, *Biochem. J.*, 1970, **119**, 599.


CO2H
O
CH2OH
HO
O
O
HO
O
OH
NHAc

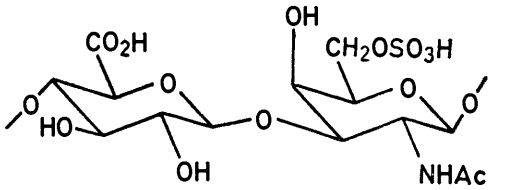
(6)


OH
CO2H
CH2OH
O
O
O
HO
OH
NHAc

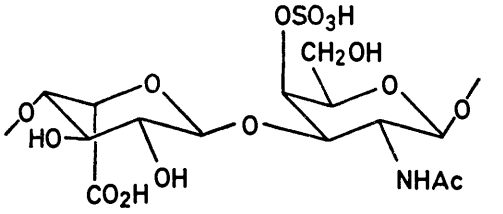
(7)


OSO3H
CO2H
CH2OH
O
O
HO
O
OH
NHAc

(8)


OH
CO2H
CH2OSO3H
O
O
HO
O
OH
NHAc

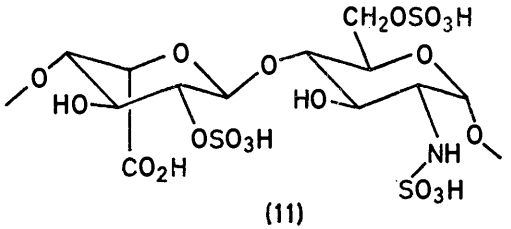
(9)


OSO3H
CH2OH
O
O
HO
O
CO2H
OH
NHAc

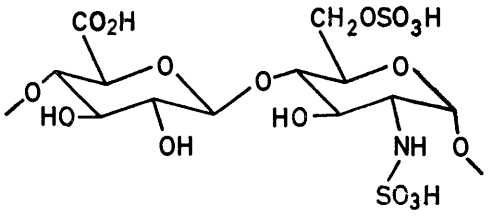
(10)

these primary structures. For example, using hyaluronidase, an enzyme which specifically cleaves 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-D-glucuronic acid bonds in glycosaminoglycan structures, it has been found that the uronic acid units of dermatan sulphate may occasionally have the *D-gluco-* rather than the L-*ido*-configuration.[90,91] Degradation into disaccharide units with other specific enzymes has shown that the repeating disaccharide structures of the chondroitin sulphates may occasionally be di- or non-sulphated[92] whereas the L-iduronic acid units of dermatan sulphate may bear a sulphate group in addition to the normal sulphate group on the 2-amino-2-deoxy-D-galactose unit.[93] Chondroitin 4- and 6-sulphate-type and dermatan sulphate-type structures also exist as copolymers, the heterogeneous molecules containing equimolar proportions of the standard disaccharide repeating units for these three glycosaminoglycans.[94] Dermatan and 'dermatan 6-sulphate' have not yet been reported as occurring naturally, although dermatan sulphate may be desulphated synthetically.

The structures of the repeating units of heparin, heparan sulphate, and keratan sulphate are, thus far, less clearly defined. It has, however, been established that the repeating structures for heparin and heparan sulphate possess a general disaccharide repeating sequence of →4)-hexopyranuronosyl-(1→4)-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→ and keratan sulphate of →3)-hexopyranosyl-(1→4)-(2-amino-2-deoxy-D-glucopyranosyl)-(1→. Structures (11)



(11)



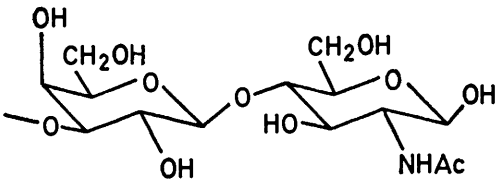
(12)

[90] L.-Å. Fransson and L. Rodén, *J. Biol. Chem.*, 1967, **242**, 4161.
[91] L.-Å. Fransson and L. Rodén, *J. Biol. Chem.*, 1967, **242**, 4170.
[92] K. Murata, T. Harada, T. Fujiwara, and T. Furuhashi, *Biochim. Biophys. Acta*, 1971, **230**, 583.
[93] S. Suzuki, H. Saito, T. Yamagata, K. Anno, N. Seno, Y. Kawai, and T. Furuhashi, *J. Biol. Chem.*, 1968, **243**, 1543.
[94] L.-Å. Fransson and B. Havsmark, *J. Biol. Chem.*, 1970, **245**, 4770.

and (12) have been found to contribute[95,96] to the overall structure of heparin but the structure appears to be much more complex, if not random, in terms of the location and abundance of the sulphate groups and also the configuration of the uronic acid units. Until quite recently, heparin was considered to contain D-glucuronic acid exclusively as the uronic acid. However, L-iduronic acid has been isolated from pure heparin *via* mild acid hydrolysis,[97] additional experiments establishing that it was neither derived from contaminant dermatan sulphate nor through epimerization at C-5 of D-glucuronic acid. Sulphation of the 2-amino-2-deoxy-D-glucose residues of heparin occurs mainly at C-6,[98] whereas the uronic acid residues are predominantly non-sulphated. Non-sulphated and 3,6-di-sulphated 2-amino-2-deoxy-D-glucose residues also occur in small proportions.[99]

The 2-amino-2-deoxy-D-glucose units of heparan sulphate are *N*-sulphated and less frequently *O*-sulphated,[100,101] and nitrous acid degradation[102] and enzymic hydrolysis[103] studies have led to the suggestion of some repeating monosaccharide sequences. Structure (13) contributes to the overall structure of keratan sulphate, the 2-amino-2-deoxy-D-glucose residues sometimes bearing 6-*O*-sulphate groups in addition to the hexose units.[104,105] Occasionally the hexose units may be non-sulphated.[106] Although this glycosaminoglycan is generally assumed to contain D-galactose as its constituent hexose, it is frequently reported to contain mannose, fucose, and sialic acid.[107]

Apart from the glycosaminoglycans, the primary structures of the proteoglycans have been given little attention, apart from determining the amino-acids involved in the glycopeptide linkage regions (see later).

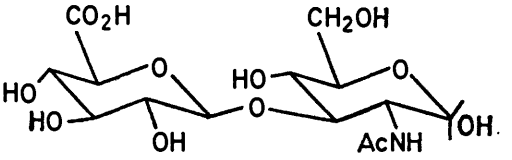


(13)

[95] A. S. Perlin, D. M. Mackie, and C. P. Dietrich, *Carbohydrate Res.*, 1971, **18**, 185.
[96] T. Helting and V. Lindahl, *J. Biol. Chem.*, 1971, **246**, 5442.
[97] M. L. Wolfrom, S. Honda, and P. Y. Wang, *Carbohydrate Res.*, 1969, **10**, 259.
[98] M. L. Wolfrom, P. Y. Wang, and S. Honda, *Carbohydrate Res.*, 1969, **11**, 179.
[99] I. Danishefsky, H. Steiner, A. Bella, and A. Friedlander, *J. Biol. Chem.*, 1969, **244**, 1741.
[100] J. A. Cifonelli and J. King, *Biochim. Biophys. Acta*, 1970, **215**, 273.
[101] C. P. Dietrich, H. B. Nader, L. R. G. Britto, and M. E. Silva, *Biochim. Biophys. Acta*, 1971, **237**, 430.
[102] J. A. Cifonelli, *Carbohydrate Res.*, 1968, **8**, 233.
[103] A. Linker and P. Hovingh, *Biochim. Biophys. Acta*, 1968, **165**, 89.
[104] V. P. Bhavanandan and K. Meyer, *J. Biol. Chem.*, 1967, **242**, 4352.
[105] V. P. Bhavanandan and K. Meyer, *J. Biol. Chem.*, 1968, **243**, 1052.
[106] J. Hirano and K. Meyer, *Biochem. Biophys. Res. Comm.*, 1971, **44**, 1371.
[107] N. Toda and N. Seno, *Biochim. Biophys. Acta*, 1970, **208**, 227.

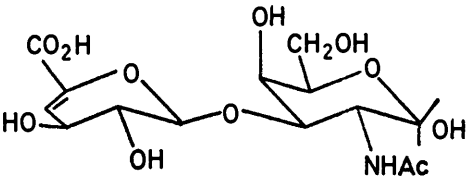
F. Identification of the Glycosaminoglycans.—The basic disaccharide repeating structures of the glycosaminoglycans were originally determined by classical carbohydrate chemistry.[9] The relatively large scales employed were possible since the tissue sources were non-human. Subsequently, in investigating new sources of glycosaminoglycans, workers have relied either on compositional data obtained *via* acid hydrolysis or on comparison of chromatographic data for the unknowns with that of 'standard preparations'. With the possible exception of the simplest glycosaminoglycans, such methods are imprecise for at least two reasons. Compositional data may be misleading owing to the difficulty in determining individual uronic acids[108] and owing to the variation in the degree of sulphation which may occur. Comparative chromatography may lead to a false identification since the 'standards' employed may have special characteristics related to the source from which they were prepared, and may in fact differ markedly in composition and molecular weight, and therefore physicochemical properties, from the unknown molecule under investigation. The only completely satisfactory method of individual measurement and identification is one based on structural recognition, and one which is applicable on a microgram scale to cope with the fact that often only milligram amounts of human tissue are available.

New methods for the microscale chemical identifications of hyaluronic acid, chondroitin, chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate have therefore been developed.[109] Hyaluronic acid is degraded with hyaluronidase, the products being further degraded with β-D-glucuronidase and β-D-acetamidodeoxyglucosidase to a characteristic disaccharide (14). Chondroitin, chondroitin 4- and 6-sulphates, and dermatan sulphate are distinguished with hyaluronidase and treated with chondroitin sulphate lyases and chondrosulphatases from *Proteus vulgaris* to give a sulphate-free unsaturated disaccharide (15), which can be identified *via* specific periodate oxidation to β-formylpyruvic acid. The positions of linkages in the disaccharides (14) and (15) may be established by specific photometric analysis. Component analyses are combined with these specific methods, and new more-sensitive techniques were developed for the determination of sulphate[71] (by flame photometry) and quantitative identification of the 2-amino-2-deoxyhexoses[66,67] (by fluorimetry). These combined techniques, which are specific for the identification and characterization of the



(14)

[108] J. F. Kennedy, *Biochem. Soc. Trans.*, 1974, **2**, in the press.
[109] S. A. Barker, J. F. Kennedy, and P. J. Somers, *Carbohydrate Res.*, 1968, **8**, 482.

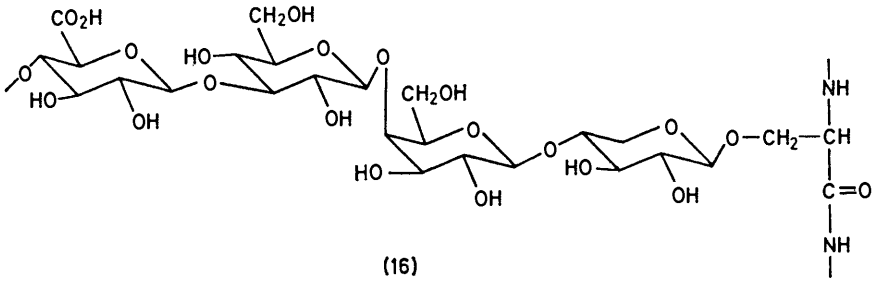


(15)

individual glycosaminoglycans, require only 150 micrograms total of each glycosaminoglycan. The chondroitin sulphate lyases and the chondrosulphatases have also been applied to chondroitin sulphates and dermatan sulphate by others[110,111] to differentiate between them, the products being identified by chromatographic comparison with standards.

Application of the microscale techniques permitted the identification of three types of hyaluronic acid, chondroitin 4- and 6-sulphates, and dermatan sulphate in human skin.[81] However, it occurred to us that such tissues might contain only nanogram amounts of proteoglycans, which would escape identification by the microgram techniques. Methods were therefore developed to label the glycosaminoglycans by culture of the living tissue in the presence of radioactive precursors,[112,113] the glycosaminoglycans being subsequently separated and being monitored for radioactivity.[114] Furthermore, by careful selection of the labelled precursor used in the culture medium (*e.g.* D-glucuronic acid, D-galactose, sulphate), it has been found that some specificity of labelling of certain glycosaminoglycans can be achieved. Such studies permitted the additional identification of heparin and keratan sulphate in human skin and differences in the glycosaminoglycan distribution between the dermal and epidermal layers.[112]

G. Primary Structures of the Glycopeptide Linkages of Proteoglycans.—A common glycopeptide linkage sequence (16) has been found for chondroitin

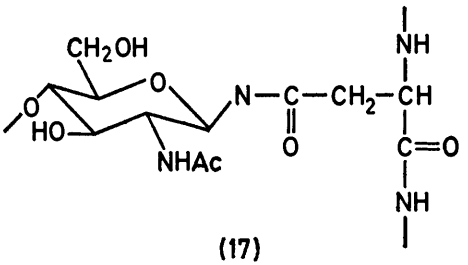


(16)

[110] T. Yamagata, H. Saito, O. Habuchi, and S. Suzuki, *J. Biol. Chem.*, 1968, **243**, 1523.
[111] H. Saito, T. Yamagata, and S. Suzuki, *J. Biol. Chem.*, 1968, **243**, 1536.
[112] S. A. Barker, J. F. Kennedy, and C. N. D. Cruickshank, *Carbohydrate Res.*, 1969, **10**, 65.
[113] J. F. Kennedy, *J. Labelled Compounds*, 1970, **VI**, 201.
[114] J. F. Kennedy, *Experientia*, 1969, **25**, 1120.

4-sulphate and heparin,[115,116] chondroitin 6-sulphate,[117] and dermatan sulphate[118,119] and, as will be seen, this involves neutral sugar residues which are quite different from the normal monosaccharide units of the glycosaminoglycans themselves. The linkage between the D-xylose residue and serine is of the *O*-glycosyl type to the hydroxy-group of the amino-acid. In the case of keratan sulphate the linkage is quite different (17),[120] does not involve atypical carbohydrate units, and is of the glycosylamine type to the side-chain of asparagine. The overall picture for the involvement of such glycopeptide linkages in proteoglycans is gradually emerging and appears complex since the proteoglycan protein backbone may bear more than one type of glycosaminoglycan. Chondroitin 4-sulphate and keratan sulphate frequently occur together in this way.[121,122]

Although hyaluronic acid isolated by non-degradative methods is known to contain protein, it is not certain that the overall protein-containing molecule is analogous to the other proteoglycans. Also in contrast, the glycopeptide linkage of hyaluronic acid has not been elucidated, although it has been suggested that this involves neutral carbohydrate residues.



(17)

H. Secondary and Tertiary Structures of Glycosaminoglycans and Proteoglycans.—The proteoglycans are generally considered to lie in the tissue matrix with their backbones parallel to collagen fibres, with the pendant polysaccharide chains forming non-covalent bonds with the collagen, so forming a three-dimensional network. Many model systems have been devised to simulate and elucidate in greater detail the tertiary structures of the proteoglycans in the natural state, but at the moment no clear solution to the problem is available. However, evidence has been presented[123] for the existence of specialised crosslinks between the proteoglycans themselves and of subunit secondary structures, whilst some information is forthcoming from ultracentrifuge data. *X*-Ray crystallographic studies of the simpler glycosaminoglycuronans have, however, reached a more

[115] U. Lindahl, *Biochim. Biophys. Acta*, 1966, **130**, 268.
[116] U. Lindahl and L. Rodén, *J. Biol. Chem.*, 1966, **241**, 2113.
[117] T. Helting and L. Rodén, *Biochim. Biophys. Acta*, 1968, **170**, 301.
[118] A. Bella and I. Danishefsky, *J. Biol. Chem.*, 1968. **243**, 2660.
[119] E. L. Stern, J. A. Cifonelli, L.-Å. Fransson, B. Lindahl, L. Rodén, S. Schiller, and M. L. Spach, *Arkiv Kemi*, 1969, **30**, 583.
[120] H. W. Stuhlsatz, R. Kisters, A. Wollmer, and H. Greiling, *Z. physiol. Chem.*, 1971, **352**, 289.
[121] K. D. Brandt and H. Muir, *Biochem. J.*, 1971, **123**, 747.
[122] H. Lyons and J. A. Singer, *J. Biol. Chem.*, 1971, **246**, 277.
[123] M. B. Matthews, *Biochem. J.*, 1971, **125**, 37.

advanced state[124,125] and it is now possible to attempt rationalizations of the tertiary structures of the molecules in the crystalline state in terms of some of the functions of the molecules.

I. The Involvement of Proteoglycans in Diseases.—On account of their fundamental function in tissue structure, it is not surprising that the proteoglycans are involved chemically in conditions which cause/give rise to changes in tissue. The glycosaminoglycans have been examined in disease, but on account of the problem of availability of tissue from living subjects many investigations have relied only on histological methods. It has not been until the advent of the micro-techniques that differences could be examined in detail and on a quantitative and structural basis. Also, using the previously cited radioactive incorporation techniques,[112] it has been possible to demonstrate glycosaminoglycan disorders in various tissue conditions and to monitor correction of the disorders during treatment.[126–128] A further approach has been the application of the radioactive incorporation method to the testing of drugs, *etc*, for their action and side-effects on the structure, biosynthesis, and metabolism of tissue as judged by glycosaminoglycan biosynthesis and metabolism.[129] In view of the versatility of the radioactive incorporation technique, which can be carried out on a biopsy specimen of five milligrams, it holds potential for further development in the understanding of tissue in health and disease.

A group of diseases[130] (mucopolysaccharidoses) in which various glycosaminoglycans with peptide attached are excreted in excess (glycosaminoglycanuria) has been given more attention on account of the large and easily collected amounts of glycosaminoglycans produced. However, clinical distinction between the various mucopolysaccharidoses is somewhat difficult, and therefore a chemically based diagnosis is required. Many qualitative and quantitative glycosaminoglycan tests have been reported, but research in this area has more recently been aimed at reproducible qualitative tests and methods of analysis for screening infants[85] and identification on the basis of chemical structure.[131]

A further approach to an understanding of the involvement of proteoglycans in disease and their faulty metabolism lies in simulation of the abnormal processes involved. In association with certain arthritic conditions in which hyaluronic acid chains become degraded, the free-radical degradation of the glycosaminoglycuronan with ferrous ions has been investigated.[132]

(Conclusions and future objectives are included in the final section of this article, page 392.)

[124] E. D. T. Atkins, C. F. Phelps, and J. K. Sheehan, *Biochem. J.*, 1972, **128**, 1255.
[125] E. D. T. Atkins and J. K. Sheehan, *Nature New Biol.*, 1972, **235**, 253.
[126] S. A. Barker and J. F. Kennedy, *Life Sciences*, 1969, 8, Pt. II, p. 989.
[127] E. J. Moynahan and J. F. Kennedy, *Proc. Roy. Soc. Med.*, 1966, **59**, 1125.
[128] E. J. Moynahan and J. F. Kennedy, 'XIII Congressus Internationalis Dermatologiae – München, 1967', Springer-Verlag, Berlin, 1968, p. 1543.
[129] S. A. Barker and J. F. Kennedy, *Carbohydrate Res.*, 1969, **11**, 27.
[130] V. A. McKusick, D. Kaplan, D. Wise, W. B. Hanley, S. B. Suddarth, M. E. Sevick, and A. E. Maumanee, *Medicine*, 1965, **44**, 445.
[131] J. F. Kennedy, C. H. Sinette, and J. B. Familusi, *Clinica Chim. Acta*, 1970, **29**, 37.
[132] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1972, **22**, 43.

5 Water-insoluble Biologically Active Reactors

The chemical attachment of molecules, particularly biologically active macromolecules, to water-insoluble matrices has received considerable attention in the past decade, and the products of such attachments have been put to a number of uses, the most common of which is as a new phase-type of biologically active compound. However, one must not be led by all the extensive literature which has now been published on the chemical production of such compounds to think that the principle of insolubilization is something new. The overall principle of attachment of a biologically active molecule to an insoluble matrix is simple and simulates the natural mode of action and environment of enzymes, antibodies, antigens, *etc.* which are carried on the surfaces or in the interiors of cells, or which are embedded in biological membranes and tissues. Indeed, as is often discovered, 'Creation was there first'. In fact, it may be said that in the human the greater proportion of the biologically active molecules of the body exist at some time in insolubilized form. Apart from all the molecules present in the body known to have biological activity, many if not all of the others can be regarded as insoluble biological reactors of some description, *e.g.* the proteoglycans in their tissue matrix-forming role. However, perhaps less attention has been given to the insoluble forms rather than the soluble forms of such molecules since most chemical techniques, analyses, and manipulations are designed to be carried out in solution, and the chemistry of activity in the solid phase is less well developed.

In natural systems, the insolubilization of biologically active macromolecules such as enzymes and glycoprotein hormones may well be a reversible process, according to whether the macromolecule is originally synthesized in the solid or liquid phase. However, it is quite certain that insolubilized forms of such active macromolecules easily become converted into soluble forms to be transported to a new site at which they perform their function. In this respect the natural insolubilized molecules differ markedly from those produced in the laboratory. This is because synthetically insolubilized biologically active molecules are usually required to perform their biological function without being released into the surrounding solution and thereby contaminating it.

As already indicated, there are many applications of insolubilized biologically active molecules. Insolubilized enzymes are principally used to effect the reaction catalysed by the free enzyme, but in a simplified form since the enzyme (insoluble) can be very easily and simply removed from the substrate and products (soluble) by filtration or centrifugation, whereas use of the soluble enzyme in the conventional fashion requires subsequent laborious separation of the enzyme from the products by, for example, gel filtration and ion-exchange chromatography. Further advantages of insolubilized enzymes are that an enzyme is often stabilized to decomposition in storage and to heat on insolubilization, and that such enzymes can be re-used. On these accounts, insolubilized enzymes also lend themselves to application as reactors in continuous analytical and industrial processes.

Insolubilized antibodies are principally useful for the purification of homo-

logous antigens, usually by a type of column chromatography (immunoadsorption) in which the solution of impure antigen is passed through a bed of insolubilized antibody: the specific antigen is adsorbed by the antibody whilst impurities are washed through the column. Subsequently, the antigen may be desorbed from the column in pure form. Thus the lengthy conventional techniques of various types of column *etc.* chromatography are short-circuited. Immunoadsorption can of course also be applied in the reverse sense, using insolubilized antigen to purify an antibody. Immunoadsorption is a very versatile technique since many macromolecules are antigenic and therefore antibodies can be raised to them; but an important prerequisite is of course that the antigen that is to be insolubilized be obtained in pure form or that the antibody to be insolubilized be obtained in pure and/or highly specific form. Insolubilized antigens and antibodies are also of use in radioimmunoassay techniques, as described earlier for the radioimmunoassay of follicle-stimulating hormone (see page 366).

Analogous to immunoadsorption is the technique of affinity chromatography, a technique which can be applied to the purification of enzymes, *etc.* Here the insolubilized molecule is usually one of low molecular weight but one for which the macromolecule to be purified has a specific affinity. Nucleic acids may also be used in an analogous way for the purification of complementary nucleic acids. Insolubilized molecules may be useful as enzyme substrates in the simplification of enzyme assays. The most recent innovation in the field of insolubilization has been the preparation of insolubilized antibiotics.

When attaching a biologically active molecule to an insoluble support, it is important to avoid a mode of attachment that reacts with or disturbs the active site(s) of the molecule, as otherwise a loss of activity will result on binding. It is also important to avoid overloading the matrix when binding molecules, since overloading leads to overcrowding and hence reduced activity by reason of steric hindrance of approach of the substrate *etc.* molecules to the active sites of the bound molecules. Attention to the way in which the macromolecule can be attached to the insoluble matrix and the choice of matrix is also a matter of importance. A number of matrix types have been used in the field of insolubilization, and polysaccharide derivatives have been used extensively. Methods of insolubilization of enzymes, antigens, antibodies, nucleic acids, antibiotics, and affinants and descriptions of the insolubilized molecules have been reviewed in detail in the sister article to this,[133] to which the reader is referred for such information and for greater details of the principles of insolubilization. Presently, our own approach to the field of insolubilization will be described.

We have been concerned with the preparation of a matrix suitable for insolubilization of a wide range of types of molecule with a view to providing a basis for (i) enzyme insolubilization for the use of enzymes in studies of carbohydrate unit sequences in glycoprotein structures and for simplification of industrial processes, (ii) glycoprotein purification by immunoadsorption, (iii) immunoglobulin purification by immunoadsorption, (iv) antigen and

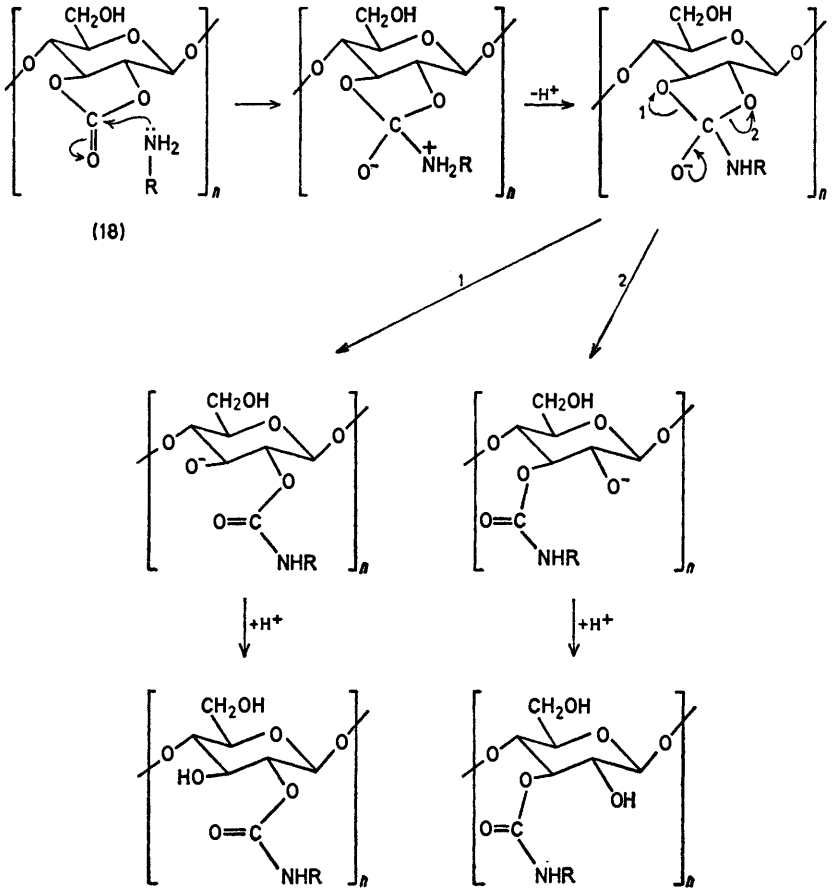
[133] J. F. Kennedy, *Adv. Carbohydrate Chem. Biochem.*, 1973, **29**, in the press.

antibody insolubilization for rapid, specific, and accurate radioimmunoassay techniques for clinical assays, (v) affinity chromatography in relation to carbohydrate structures, and (vi) antibiotic insolubilization, with a view to paving the way to a number of applications.

Of the general modes available – covalent attachment, non-covalent attachment and physical entrapment/inclusion in a fibre or lattice, or cross-linking of the molecule itself to form a matrix – covalent attachment is to be preferred since it is more permanent and the attached molecule is unlikely to be inadvertently released into solution during use. We have therefore followed the lines of a covalent-type insolubilization and have given attention to the derivatization of the insoluble polysaccharide cellulose with ethyl chloroformate to give the reactive cellulose *trans*-2,3-carbonate (18).[134] Using microcrystalline cellulose, the conditions for achieving a maximum degree of substitution with the cyclic group and a minimum degree of substitution with the acyclic carbonate (ethyloxycarbonyl) group which occurs as a side-reaction were defined.[134,135] The strain and electronic arrangement in the ground state of a *trans*-1,2-carbonate ring fused to a six-membered ring in the chair conformation are such that the carbonyl carbon atom of the carbonate ring is susceptible to nucleophilic attack. Thus cellulose *trans*-2,3-carbonate was shown to exhibit suitable reactivity for the covalent coupling of enzymic protein under mild aqueous conditions to give an active insoluble derivative of β-D-glucosidase.[136,137] From a study of the reaction of simple nucleophilic compounds with cellulose *trans*-2,3-carbonate[138] it is envisaged that the coupling reaction involves the nucleophilic attack of a free amino-group in the enzyme *etc.* protein on the strained *trans*-cyclic groups (Scheme 1). As can be seen, the carbonate ring may open in two ways to give products in which the D-glucopyranose ring is substituted at the 2- or 3-position.

The initial successes in forming a stable covalent bond between cellulose *trans*-2,3-carbonate and an enzyme were magnified by the fact that, contrary to expectation, the optimum pH for the covalent coupling reaction is approximately pH 7.8, and not considerably higher, as might be expected for a nucleophilic reaction. This was of course very important in avoiding, during the coupling, damage to biologically active molecules by subjecting them to other than physiological (near neutral) pH values. Thus further studies were carried out to optimize the coupling conditions, and highly active derivatives of several enzymes have now been prepared.[139] Cellulose *trans*-2,3-carbonate has also proved very useful in the insolubilization of human immunoglobulins for purification of antibodies to them,[140] and its properties for this immunoadsorption were found to surpass

[134] S. A. Barker, H. Cho Tun, S. H. Doss, C. J. Gray, and J. F. Kennedy, *Carbohydrate Res.*, 1971, **17**, 471.
[135] S. A. Barker, J. F. Kennedy, and C. J. Gray, B.P. 1289548.
[136] S. A. Barker, S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacey, and T. H. Yeo, *Carbohydrate Res.*, 1971, **20**, 1.
[137] S. A. Barker, J. F. Kennedy, and C. J. Gray, B.P. 1289549.
[138] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **29**, 246.
[139] J. F. Kennedy and A. Zamir, *Carbohydrate Res.*, 1973, **29**, 497.
[140] D. Catty, J. F. Kennedy, R. Drew, and H. Cho Tun, *J. Immunological Methods*, 1973, **2**, 353.



R = rest of molecule being insolubilized

Scheme 1

those of other matrices which could be used for insolubilization. Purification of these antibodies is important for structural investigation and for investigation of antibody specificity and reactivity with respect to health and disease.

Both human pituitary follicle-stimulating hormone and its homologous antibody have been covalently attached to cellulose *trans*-2,3-carbonate.[22] The anti-follicle-stimulating hormone retains its immunological reactivity on insolubilization, and is therefore suitable for use in the solid-phase radioimmunoassay of unknown amounts of the hormone by competitive binding of

radioactively labelled and unlabelled hormone. Acceptable inhibition curves can be obtained, and the low, non-specific adsorption characteristics have advantages over other systems. Follicle-stimulating hormone itself also retains immunological reactivity on insolubilization, and this derivative holds potential for the radioimmunoassay of the hormone as it can be layered immunologically with anti-follicle-stimulating hormone and then the hormone itself. The comparatively recent discoveries of myeloma forms of human immunoglobulin IgE have made possible the development of a sensitive radioimmunoassay for estimating antibody IgE in normal and pathological sera and other body fluids with a view to diagnosis of myeloma conditions. Antibodies, to immunoglobulin IgE, attached to cellulose *trans*-2,3-carbonate have been applied in this respect.[141] Work is presently in hand for the utilization of antigens and antibodies attached to cellulose *trans*-2,3-carbonate for other, automated clinical chemical analyses and diagnoses.

The coupling of a number of antibiotics to cellulose *trans*-2,3-carbonate under a series of coupling conditions has been investigated, and it has been shown that by such couplings active insoluble derivatives of antibiotics can be produced.[142] Such pioneer experiments are particularly exciting since they open the way to the production of antimicrobial surfaces, coatings, dressings, *etc.* for use in clinics and hospitals and for the protection of industrial membranes *etc.* It was also found that the antibiotics became firmly bound to cellulose itself, whereas use of the cellulose *trans*-2,3-carbonate extended the range of antibacterial activity retained. In certain cases slow release of the antibiotic from the matrix occurred when cellulose was used, and this phenomenon holds potential in the development of slow-release antibiotic and drug formulations. On the industrial side, since cellulose is the chemical basis of many packaging materials, it would appear that long-lasting antimicrobial protection may be afforded by a single treatment with antibiotic solution.

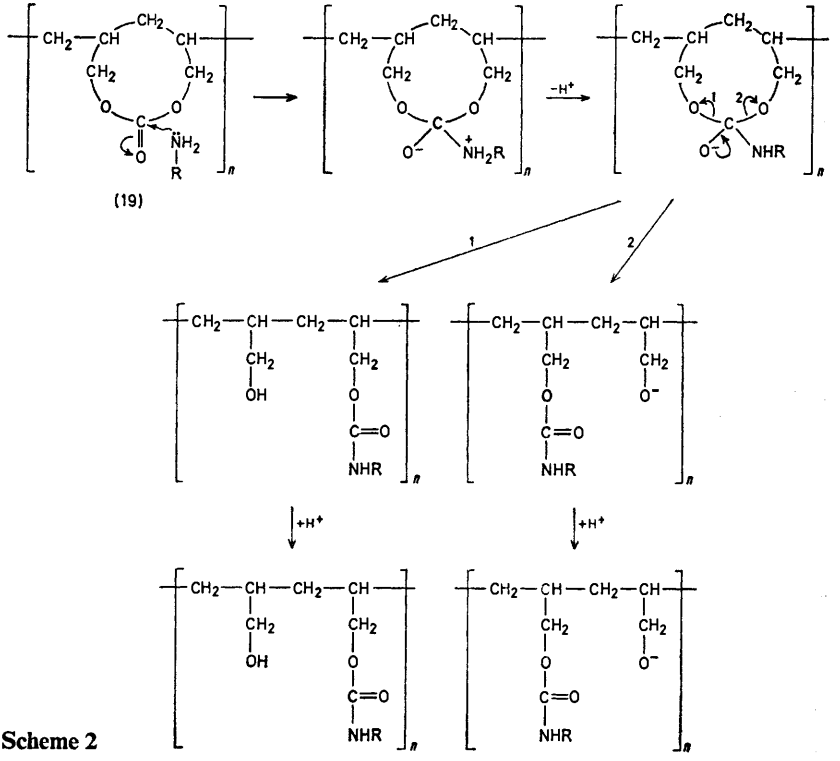
Finally, the use of cellulose *trans*-2,3-carbonate derived from microcrystalline cellulose in the field of insolubilization of affinants typified by the phytohaemagglutinin concanavalin A has been investigated.[143] Thus cellulose *trans*-2,3-carbonate has proved to be a very versatile material for insolubilization.

However, one disadvantage to be expected from the use of a standard powder for insolubilization of macromolecules is that substrate macromolecules cannot rapidly diffuse into the particles. For this reason we have extended the derivatization of cellulose to its *trans*-2,3-carbonate to macroporous cellulose in which there is a gel-type network that is permeable to macromolecules. Derivatization of such macroporous cellulose with retention of macroporosity has been achieved,[144] the degree of substitution with the cyclic carbonate groups being controlled by the addition of water to the reaction medium. Thus it has

[141] P. McLaughlan, D. R. Stanworth, J. F. Kennedy, and H. Cho Tun, *Nature New Biol.*, 1971, **232**, 245.
[142] J. F. Kennedy and H. Cho Tun, *Antimicrobial Agents and Chemotherapy*, 1973, **3**, 575.
[143] N. Ling, J. Bray, and J. F. Kennedy, unpublished observations.
[144] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1973, 2293.

been possible to achieve an insolubilized enzyme with a high retention of the natural activity against a substrate of high molecular weight.[144,145] Cyclic carbonate derivatives of other polysaccharides and cycloamyloses, including inulin cyclic carbonate, which involves some novel carbonate rings, have also been formed[146] and some of these may have application in the attachment of enzymes to charged matrices for displacement of the enzyme pH optimum by microenvironmental effects. Mixed enzyme derivatives of other cellulose derivatives have also been prepared.[147]

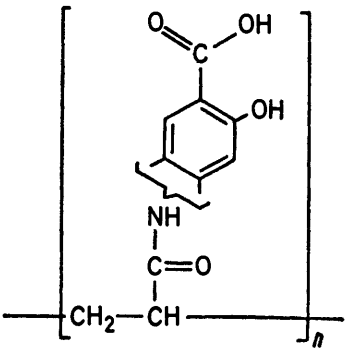
Whereas the use of a polysaccharide-type material for insolubilization is to be considered advantageous since in the solid state residual hydroxy-groups provide a hydrophilic environment for the attached macromolecule, and insolubilization within a macroporous structure gives added protection from exposure, polysaccharides cannot be expected to be universally applicable since they are biodegradable. We have therefore paralleled our approach to insolubilization by devising some non-biodegradable matrices. One of these, poly(allyl cyclic carbonate)[148] (19), is the product of subjecting poly(allyl alcohol) to the ethyl



(19)

Scheme 2

[145] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, in the press.
[146] J. F. Kennedy and H. Cho Tun, *Carbohydrate Res.*, 1973, **26**, 401.
[147] J. F. Kennedy, *Z. klin. Chem. klin. Biochem.*, 1971, **9**, 71.
[148] S. A. Barker, J. F. Kennedy, and A. Rosevear, *J. Chem. Soc.* (*C*), 1971, 2726.



(20)

chloroformate reaction and it reacts with enzymes[148,149] in an analogous way to cellulose *trans*-2,3-carbonate (Scheme 2). Poly(4- and 5-acrylamidosalicylic acids)[150] (20) have proved versatile matrices, being suitable for antibiotic insolubilization,[151] and enzyme insolubilization *via* metal ion chelation.[152] They are also applicable to selective extraction of certain metal ions from solution.[153] Enzyme insolubilization, using alginic acid, chitin, and Celite as matrices,[154] and antibiotic insolubilization, using cellulose as matrix,[155] have also been achieved *via* metal ion chelation, whilst studies with glass have shown that care must be exercised in handling of enzyme solutions in glass since the enzyme may become adsorbed with retention of activity.[156]

(Conclusions and future objectives are included in the following section.)

6 Conclusions and Future Objectives

Whilst it is clear that the present state of chemical knowledge of glycoprotein hormones and proteoglycans is well advanced, it is also certain that there is a considerable amount of work to be done, involving further development of microscale analytical techniques suitable for dealing with minute samples from humans, improved and more direct purifications, primary, secondary, and tertiary structural identification, and determination of chemico-biological relationships and biosynthetic pathways.

In terms of improved and automated component analyses, our interests have required us to devise a number of techniques, principally for carbohydrate components. Carbohydrates are frequently separated and identified by ion-

[149] J. F. Kennedy, S. A. Barker, and A. Rosevear, *J.C.S. Perkin I*, 1972, 2568.
[150] J. F. Kennedy, S. A. Barker, J. Epton, and G. R. Kennedy, *J.C.S. Perkin I*, 1973, 488.
[151] J. F. Kennedy, J. Epton, and G. R. Kennedy, *Antimicrobial Agents and Chemotherapy*, 1973, **3**, 29.
[152] J. F. Kennedy and J. Epton, *Carbohydrate Res.*, 1973, **27**, 11.
[153] J. F. Kennedy, S. A. Barker, A. W. Nicol, and A. Hawkins, *J.C.S. Dalton*, 1973, 1129.
[154] J. F. Kennedy and C. E. Doyle, *Carbohydrate Res.*, 1973, **28**, 89.
[155] J. F. Kennedy and A. Zamir, unpublished observations.
[156] J. F. Kennedy and P. M. Watts, *Carbohydrate Res.*, 1974, **32**, 155.

exchange chromatography (see ref. 108 for review) and by gas-phase chromatography.[157] In connection with ion-exchange chromatography we have been interested in separations that may be carried out in water alone, and have extended the use of an ion-exchange resin as an ion-exclusion matrix to the separation of carbohydrates according to molecular weight,[158] the pores of the resin being utilized as in gel filtration. Other applications of ion-exchange chromatography have been the sensitive identification of 2-amino-2-deoxyhexoses[66,67] and erythritol and threitol.[159] Improved separations and sensitivities demand improved and sensitive automated analyses and such spectrophotometric methods have been reviewed.[108] An even more sensitive general fluorimetric method for carbohydrates, amino-acids, peptides, proteins, glycoproteins, and proteoglycans has been developed.[66,67] Spectrophotometric determinations for phosphate[160] in monitoring columns in glycoprotein *etc.* fractionations and for formic acid[161] in structural analysis by periodate oxidation have also been reported. In connection with gas chromatographic analysis we have been concerned with the separation of pentoses,[68] of methylated monosaccharides[34] in structural analysis by methylation, of hexoses and periodate oxidation products[33] in structural analysis, and of uronic acids,[70] each compound being pre-derivatized to render it volatile. Mass spectrometry of the compounds coupled with gas-phase chromatography has provided additional identification data, and breakdown patterns have been investigated.[34,70]

In view of the increasing need for component analyses of glycoproteins, but the high cost of automated equipment, we have established and set up in our laboratory the University of Birmingham Macromolecular Analysis Centre. The purpose of this Centre is to provide a service of carbohydrate and amino-acid analysis to all members of the University. The Centre uses a Locarte analyser for amino-acid analysis and a Jeol JLC-6AH analyser for carbohydrate analysis: both machines operate on ion-exchange chromatography and are fully automatic. Programming work is in hand to process data and results from the two analysers to customer requirements, the two machines being on line to a Nova 1220 Mini-Computer in the Centre. It is hoped shortly to commission a peptide synthesizer and ultimately to extend the service to peptide sequencing.

So far as better purifications are concerned, it appears at the moment that immunoadsorption and affinity chromatography will be of use here and will reduce the complexity of many purification procedures, including those for hormonal glycoproteins and proteoglycans. In the case of carbohydrate-containing molecules, the lectins or phytohaemagglutinins are also of use since they complex specifically with certain carbohydrate structures. Our assessment[162] of a water-insoluble, active form of one phytohaemagglutinin, concanavalin A, has established that a number of straight-chain and branched-chain polysaccharides

[157] J. R. Clamp, *Biochem. Soc. Trans.*, 1974, **2**, in the press.
[158] S. A. Barker, B. W. Hatt, J. F. Kennedy, and P. J. Somers, *Carbohydrate Res.*, 1969, **9**, 327.
[159] D. B. Lowrie and J. F. Kennedy, *F.E.B.S. Letters*, 1972, **23**, 69.
[160] J. F. Kennedy and D. A. Weetman, *Analyt. Chim. Acta*, 1971, **55**, 448.
[161] J. F. Kennedy, *Methods Carbohydrate Chem.*, 1971, **6**, 93.
[162] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, 1973, 2041.

and several monosaccharides may be separated by elution from a column of insoluble lectin, monosaccharides and weakly interacting polysaccharide fractions being eluted with phosphate buffer and more tightly bound fractions being eluted with borate buffer. The use of borate buffer for this purpose overcomes the problems arising in the common use of competitive carbohydrates for the elution of bound material. This work has also demonstrated that mixtures of carbohydrates which are not separable by complex formation with concanavalin A in solution may be separated by use of the immobilized form; immobilized lectins are now finding use in glycoprotein fractionation.

The importances of all types of structural identification have been mentioned, and for the hormonal glycoproteins it is to be hoped that further information of this type along with chemico-biological studies will further aid identification of biosynthetic, metabolic, and message-transport defects and assist in improved treatment of clinical conditions. For the proteoglycans, more attention needs to be given to the absolute primary structures of the glycosaminoglycans and to the structures of the protein backbone, and indeed might lead to an identification of a spectrum of sequences, thus revealing that the proteoglycans are not such a discrete set of compounds as is imagined at present. Furthermore, the question arises as to whether the distribution of carbohydrate units and sulphate residues along the glycosaminoglycan chains and the sequence of glycosaminoglycan chains along the proteoglycan backbone have any coding function, *e.g.* for deposition of various tissue types which go to make up the various regions and organs of the body. Other useful extensions of work on the proteoglycans would include: exploration of their interactions with other macromolecules and ions to provide connective-tissue models, determination of relationships between primary and tertiary structures and susceptibility to endogeneous enzymes in relation to living processes, and development of automated analyses for chemical diagnosis of clinical conditions.

In the field of insolubilization, there is now sufficient known of how to insolubilize a biologically active molecule with retention of activity, and emphasis must now be laid on application. Thus, for example, insolubilized enzymes must now be developed more earnestly for application in clinical analyses and in clinical machines such as blood purifiers *etc.*, and for the simplification of industrial processes. Insolubilized immunologically active agents must also be applied in clinical chemistry for the monitoring of patient conditions, for treatment, and immunological reaction of rejection of transplants. Insolubilized antibiotics and drugs must be developed for *in situ* sustained activity. Insolubilization of hormonal glycoprotein subunits may aid purification of complementary subunits and provide means of assessing the interactions of the hormones with insoluble target sites. Insolubilization of proteoglycans and glycosaminoglycans could also provide models for studies of tissue processes, and on account of the ionic natures of the glycosaminoglycans such derivatives could well prove useful as new types of matrices for fractionations. In this respect, it is worthwhile remembering that in the body many specific interactions occur between molecules in solution and molecules (receptors) in the solid phase, and thus may ultimately

be extrapolated to laboratory separations. Also on the synthetic side is the possibility of testing modified hormones, and the synthesis of pseudo-hormones and pseudo-proteoglycans by covalent attachment to non-biodegradable soluble molecular chains with a view to increasing their half-life in the human system. One specific application of such would be a joint lubricant for treatment where the natural lubricant, hyaluronic acid, has failed, as in arthritis, a common and painful complaint which demands attention since so little is known of the underlying chemistry. Automated systems must also be developed for testing the effects of drugs upon hormonal glycoprotein and proteoglycan biosynthesis and metabolism and for the discovery of better treatments of clinical conditions.

Since enormous effort is currently being put into research on glycoproteins and proteoglycans, the number of papers published annually on the chemical, chemico-biological, chemico-medical, and biosynthetic aspects runs into thousands. This of course presents a problem for any worker to keep up with the literature. Computerized output of titles of computer-selected relevant papers is now being offered by some organisations, including the Chemical Society, whilst current awareness with detailed reports on each paper is facilitated by an annual review of such papers.[163]

In conclusion, it is hoped that the reader will have been fascinated by the chemical architecture on which the chemical and biological aspects of glycoproteins, proteoglycans, and carbohydrate-protein complexes of human tissues *etc.* are based. Indeed, with such complexity and yet harmony within ourselves, it is true that, as was acknowledged long before chemistry as we know it was established, we are 'fearfully and wonderfully made'.[164] It is also anticipated that the reader will recognize the tremendous scope for future work in this important area and will acknowledge that such chemistry will be supreme in deriving further benefits for the maintenance of health and treatment of disease on a physical plane.

It is indeed a very great honour for me to receive the Meldola Medal and I am very grateful to the Society of the Maccabæans and the Royal Institute of Chemistry for making this award. I am also grateful to the numerous colleagues, relatives, friends and research students who have encouraged me to this success.

[163] J. F. Kennedy, in 'Carbohydrate Chemistry', ed. J. S. Brimacombe, (Specialist Periodical Reports) The Chemical Society, London, 1971, 1972, 1973, vols. 4, 5, and 6, Part II.
[164] King David, *The Psalms*, BC 1040, Ps. 139, v. 14.