THE CHEMICAL COMPOSITION OF GRASSES WITH PARTICULAR REFERENCE TO THE STRUCTURAL CARBOHYDRATES

A thesis presented to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science

by

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June, 1957

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ACKNOWLEDGEMENTS

I wish to thank the Council and Director of the Hannah Dairy Research Institute for allowing this work to be done and for granting the necessary facilities. I also thank Dr R. Waite for suggesting the work and for his constant advice and guidance. The experiments described in Part II of this thesis have involved much routine laboratory work and in this I have had assistance from other members of the Technical Chemistry Department of the Hannah Dairy Research Institute. In particular I would express my thanks to Miss N.D. McInnes who did much of the routine work.

CONTENTS

SYNOPSIS

The methods of chemical analysis of dried grass have usually included the determination of the ash, ether extract, crude protein and crude fibre. The remainder of the material has been calculated as the nitrogen-free extractives. It was decided to make a more detailed analysis of grasses by the examination of successive extracts, and methods have been developed which allow almost all of the dry matter to be accounted for in terms of the major constituents.

The grass samples were examined by the successive use of various neutral extractants and the amount of material removed by each reagent was determined. The weighed, extracted material together with the fibrous residue accounted for 99-101% of the grass dry matter. When the individual extracts and the fibrous residue were further fractionated, the sum of the individual constituent groups amounted to 93-99% of the grass dry matter. Of this total 3-5% was not identified. Unidentified compounds were found in particular in the extracts obtained by the use of water and ammonium oxalate solution.

After extraction of the readily soluble components from the grass, the structural constituents were

examined in the residue. The protein and lignin were largely removed from the residue by treatment with pepsin and the preparation of chlorite holocellulose. During the examination of the holocellulose, evidence was found to suggest that chloriting had rendered some of the residual lignin alcohol-soluble. The polysaccharides in the holocellulose were studied by successive extraction with water, alkali and acid. The sugars in the hydrolysed extracts were separated by paper chromatography and estimated by periodate oxidation.

These methods of analysis of the grass samples were applied to four perennial grasses and one annual grass, each cut at four stages of growth. The amount of the neutral extracts were determined but not examined in detail since this part of the work was concerned mainly with the changes in the composition and quantity of the fibrous residue. There were only slight differences between the perennial grasses in the amounts of polysaccharides and lignin at corresponding stages of growth. The annual rye-grass contained relatively more lignin, and cellulose represented a greater proportion of the total polysaccharides than in the perennial grasses. These differences may result from the more rapid growth habit of the annual grass.

In all the grasses, cellulose represented approximately half the cell wall polysaccharides. The hemicelluloses contained mainly xylan with smaller amounts of araban, glucosan, galactan and uronic anhydride. The quantity of xylan increased markedly with increasing age whereas the amount of the other constituents changed only slightly. Differences in the composition of the structural material of the leaf and stem portions of the grasses were quite small.

GENERAL INTRODUCTION

Grass is a crop which is well suited to the temperate climate of Great Britain since it neither needs nor experiences extremes of temperature and the rainfall received is fairly evenly distributed throughout the year. The exceptional value of grass in relatively modern agricultural economy has for long been apparent (1, 2) and has been emphasised more recently because of the international economic situation which has developed since 1940. Even yet, however, the assets to be gained from the efficient production and utilisation of grass are often not fully realised.

A plentiful supply of grass with a high digestibility can be obtained from pastures on a good soil which has been suitably sown if the pasture is skilfully managed. This management must include the sufficient and timely application of the appropriate fertilisers and must entail the controlled removal of the resulting growth, either by grazing or by mechanical cutting. Such farming methods are not without substantial costs but by their adoption the need to purchase winter feed can be very much reduced, and so far as weather conditions permit the period of full grazing in the fields may be extended. Even less promising areas such as hill

pastures can be made more profitable by good grassland husbandry.

Need for chemical analyses

It is necessary if full advantage is to be taken of improved farming methods to have a knowledge of the probable composition of the grass produced and of the natural changes in composition which occur as a result of season or stage of growth. The changes which take place in the composition of the grass, either as a result of varying applications of fertilisers or by the employment of different methods of removing the crop, must be known and appreciated.

Grasses contain carbohydrates, proteins, fats, salts and water, all of which are nutritionally valuable, but in addition they contain lignin which is almost completely indigestible and which reduces the digestibility of the other constituents. For many years attempts have been made to predict the feeding value of grass and other forages for farm animals. Agriculturists have desired a sure method by which the energy value, the protein equivalent and the digestibility of a feed can be calculated from a knowledge of the chemical composition of the material. It is not the intention in the present work to examine the success or failure of such methods

except in so far as they have furthered our understanding of the composition of grass. To the biochemist, however, the chemical composition of grass and the variations in composition which are observed, are links towards piecing together the whole picture of plant metabolism - a complicated system which is at last beginning to reveal some of its secrets as a result of the use of new analytical techniques.

Early analyses

The first advances in the knowledge of plant composition were made as a result of pure chemical studies as for example when sucrose was isolated from sugar beet by Marggraf in 1747 (3). Many other substances have been discovered in plants in the course of work not directed to the overall analysis of the plant material.

Proteins were not shown to be present in plants until 1838 (4). Before then Thaer had introduced a system by which fodder was graded in terms of hay values (5, 6) but his scheme was unsuccessful, in particular by lack of consideration of the protein content and of the wide variations in the amount of this fraction which occur in hay. The significance of the proteins was soon realised however, for in 1844 Boussingoult (7) extended Thaer's methods to include the determination of the nitrogen content.

The Weende system of analysis

The work which has made the most lasting impact on the analysis of fodder crops was a system of analysis introduced by Henneberg and Stohmann at Weende in 1860 (8). This method involved the determination of the protein, ether extract, ash and fibre in the dried material. These fractions together did not account for the whole of any sample, and the remaining materials required to account for 100% of the dried material were simply called the nitrogen-free extractives.

A similar analytical scheme was proposed by Voelcker (9, 10) who determined the fibrous material in forages in two stages. The first, which was called "crude woody fibre", was the material insoluble in water, and the other termed "cellular and woody fibre" was a product similar to fibre as prepared by Henneberg's method.

The variability in the actual composition of each of the determined fractions of the Weende analysis was soon realised and Henneberg (11) suggested the terms crude protein and crude fibre.

Typical examples of the range of values that may be expected for grasses when analysed by the Weende system are given in Table 1.

Table 1. Probable range of composition of grass by Weende analysis (as % dry matter)

Ash	6 - 12
Crude protein	2 - 30
Ether extract	2 - 8
Crude fibre	20 - 35
Nitrogen-free extractives (by difference)	30 - 50

The Weende system of analysis has been accepted as the standard method of analysis of fodder materials. It is still used although its shortcomings, in particular the magnitude and unknown nature of the nitrogen-free extractives, have long been recognised, and a more satisfactory alternative has been the desire of agricultural chemists for many years. As yet however, no other method of forage analysis has shown sufficient promise.

Criticism of the Weende scheme of fodder analysis had begun in 1897 when Tollens (12) indicated the vagueness of the nature of the nitrogen-free extractives fraction showing that it contained sugars, polysaccharides, pectin, lignin (a phenolic material of large molecular weight), acids and many lesser constituents such as

tannins, resins, colouring matter and sugar alcohols. A group containing such a variety of compounds can only be acceptable in an analytical method if the constituents maintain a fairly constant distribution in the group. This however does not happen and large changes take place in the amounts of sugars and lignin although the total amount of the nitrogen-free extractives, as a percentage of the grass dry matter may vary within quite small limits. Thus the largest fraction of the forage, the nitrogen-free extractives appears to be fairly constant in quantity whereas marked variations are apparent in the fractions actually determined, i.e. in the ether extract, crude protein, ash and crude fibre.

Criticism of the Weende scheme of analysis was extended to the crude fibre determination, a very active critic being Norman (13) who showed that crude fibre was not what it was claimed to be. It was originally presumed to be a measure of the structural components of the feeding-stuff and as such to be indigestible (8) but by the method of isolation we now know that it contained only part of the intended material. The method of fibre estimation [see review by Dougall (14)] involved consecutive treatments with dilute acid and alkali, which removed part of the structural carbohydrates and

part of the lignin (13). Also the residual fibre is by no means indigestible (15, 16, 17, 18, 19). Because successive extractions bring into solution more material than is intended, the nitrogen-free extractives fraction includes part of the lignin and structural carbohydrate contents. This again is contrary to the original intention that the nitrogen-free extractives should represent only materials which are completely digestible.

The various constituents concerned in the Weende system of forage analysis will now be discussed since they must obviously find some place in any system of analysis. The fractions suggested by Henneberg will be considered in the light of present knowledge and it will be shown just how serious chemically were the false conclusions which resulted from such a fractionation of grass. When discussing the nitrogen-free extractives the soluble carbohydrates will be dealt with rather more fully than the other constituents.

Ether-soluble materials

This fraction which may amount to 8% of the grass dry matter contains the lipids and waxes and most of the pigments. In comparison with the studies that have been made on other lipids there has been very little work done on these materials in grass, probably because

of the difficulty of separating them from the pigments: however, the isolation of pure fractions is now possible by chromatographic methods. The most important investigations of these lipids were those by Smith and Chibnall (20) and more recently Shorland (21). Preparations from cocksfoot and ryegrass (20) and from a mixed pasture (21) were examined and separated into glycerides, waxes, phosphatides and non-saponifiable fractions. The major fatty acids in the glycerides were the unsaturated C_{18} acids, linoleic and linolenic acids along with substantial amounts of oleic acid and the saturated acid, palmitic acid. In the waxes obtained from other samples of cocksfoot only one alcohol was found in any significant amount, n-hexacosonol (22). Although occurring in only a very small amount, the unsaponifiable fraction of the ether extract is a vital portion of the grass. It contains the class of compounds known as sterols, including ergosterol which is a precursor of Vitamin D (23). It also contains the grass pigments.

The pigments in the ether extract contain the group of substances known as the carotenoids, the parent substance of which, carotene, has a number of isomers: of these the β form can be converted by animals to

Vitamin A (24). The carotenoids contribute to the yellow colouring of plants and various other members of the group share with β-carotene the ability to be converted to Vitamin A, to varying extents (25). Another compound present in grass pigments is α-tocopherol which is particularly important because of its Vitamin E activity. The occurrence of this compound was studied by Brown (26) who showed that its amount was greatest in young leaves (20-30 mg./100 g. grass dry matter). The principal pigments of grass are however the chlorophylls which are a group of heterocyclic compounds containing magnesium and of which the fundamental structure is a condensed pyrrole nucleus. All these grass pigments and sterols are susceptible to oxidation or pH changes, making their extraction and examination a matter of some difficulty.

Crude protein

The protein content of forages is usually determined by estimation of the total nitrogen in the material. From this is calculated the value for "crude protein", a designation necessary because the total nitrogen is derived from other compounds as well as proteins. The other nitrogenous substances include amino acids, peptides, amides, ammonium salts and compounds having

nitrogen as part of a heterocyclic structure. If the true protein content is required, the non-protein nitrogen is estimated and deducted from the amount of total nitrogen. For many purposes the crude protein estimate is satisfactory, as the non-protein nitrogen frequently amounts to only 10% of the total nitrogen.

It has been well established that the crude protein content of the dry matter of grasses is at its maximum in very young tillers and decreases rapidly and steadily with progressive growth. Spring grass, for example, which may have 25% of the dry weight as crude protein, if allowed to grow unchecked will eventually yield by mid-summer a product in which the crude protein is only 5% of the dry matter. Most of this decline will have occurred in the first few weeks of rapid growth.

Inorganic constituents

The inorganic constituents are usually grouped as a single quantity referred to as the ash obtained as the residue after controlled incineration of the grass. The ash is derived from mineral salts, some of which play an obvious and vital role in the metabolism of both plants and animals. It is at present impossible to say what direct associations exist between the various cations and anions in grass tissue, though as is

mentioned elsewhere, pectin seems possibly to be linked with calcium and magnesium ions and part of the magnesium is a constituent of chlorophyll. Silica, which may represent half the isolated ash, has been found as insoluble deposits in cocksfoot (27), although it must presumably have entered the plant as a soluble silicate through the roots in the same manner as other minerals. Soil contamination is often the reason for much of the silica in grass samples. In the usual estimation of silica in grass both the soluble and insoluble forms are isolated together, so any distinction is obscured.

The metal ions present in plants occur in widely differing amounts, thus each of the more abundant elements calcium, sodium and potassium are present as 0.5 - 4.0% of the dry matter, but the equally important trace elements such as cobalt and copper are present as only a few parts per million. A proportion of the cations will act in the grass in a buffering capacity for the plant acids. Magnesium is a necessary constituent of chlorophyll and several of the trace elements present are essential for the functioning of the many enzyme systems of the plant.

Another element found in large quantities in the ash of grass is phosphorus which is present as phosphate

phosphate and amounts to about 1% of the dry matter of the grass. In many instances this is used, not so much as a component of the enzyme systems but rather as one of the reactants. As will be shown later, the chemical transformations which take place in plants are nearly always dependent upon transfer of phosphate groups, particularly amongst the carbohydrates.

Nitrogen-free extractives

The errors introduced into the analysis of forage crops in general, and into the analysis of grasses in particular, by the use of the quantity referred to as the nitrogen-free extractives has been mentioned in a previous section (p. 8). The actual constituents of the nitrogen-free extractives will now be discussed. There are, of course, polysaccharides and lignin, which were intended by early analysts to be regarded as part of the crude fibre, but these will be discussed later when considering the grass fibre. The other main components of the nitrogen-free extractives are the soluble carbohydrates and organic acids.

Soluble carbohydrates

For many years it was not realised what a large proportion of the dry matter of some grasses the soluble carbohydrates represent at certain stages of growth.

In many cases the previous management of the grass had been, in the light of present knowledge, such as to provide samples with the lowest possible content of soluble carbohydrates. Thus in samples from nine different grasses cut in June, the total sugars found by Buston (28) were less than 10% in all but two instances. Some of the earliest results, which showed the enormous variations possible in this group were produced by Norman (29) who showed that the soluble sugars might amount to over 30% of the grass dry matter. It is such results which emphasise the undesirability of measuring the nitrogen-free extractives simply by difference.

The principal sugars obtained by a water extraction of grass are the monosaccharides glucose and fructose, the disaccharide sucrose and the polysaccharide fructosan, which is also variously given names such as graminin and phlein (30) depending on the species from which it has been isolated. There are also a number of carbohydrates intermediate in molecular size between sucrose and fructosan, loosely called oligosaccharides (31). The fructosan appears to be a straight chain polymer in which the fructofuranose units have 2:6 linkages and in which a glucose residue is an end group (32). It is likely that fructosan is a temporary

reserve carbohydrate and as such is an energy store.

The free hexoses and sucrose are early products of photosynthesis and, as will be discussed later, are essential metabolites of grasses, so it is not surprising that the amounts of these sugars remain fairly constant. The hexoses usually represent 1 - 4% of the dry matter. The sucrose content may rise to as much as 10% of the dry matter when the plant is growing actively but more usually it lies between 2 and 6%.

If fructosan is a reserve material in grasses the quantity of it may be governed by the energy requirements of the plant. Thus, when the grasses are growing rapidly it might be expected that very little surplus energy (in the form of sugars) would be available to allow the production of fructosan. This relationship is revealed by the differences in fructosan content between grasses analysed at various stages of growth (33, 34).

Grasses which have to establish their whole plant from seed at the beginning of a growing season, have high energy needs and consequently comparatively low fructosan contents, for instance the maximum value in a perennial rye-grass in its first year was 15% of the dry matter compared with 24% in its second year (34).

The rate of growth of grasses and hence their fructosan contents is influenced by several factors. In temperate climates different species may have widely differing fructosan contents, established plants of timothy and cocksfoot grown together have been reported (34) with maximum values of 25 and 8% of the dry matter respectively. This difference was probably related to the greater rate of growth of the cocksfoot. The application of fertilisers usually increases the rate of growth and as a result it usually depresses the fructosan content (35).

Over-riding all these considerations of variations in energy requirements due to rate of growth are the changes which take place in grasses during a growing season (33, 34). With an established grass such as timothy there is a period in early spring when growth is slow and during which the fructosan content may increase to as much as 18% of the dry matter. Rapid growth then ensues, with stem elongation and head emergence, after which the fructosan content may be only 12%. After this phase the rate of growth of the grass is very much reduced and the fructosan content may increase again to as much as 25% of the dry matter. As the growing period ends the leaves of the grass tend to wither and their

photosynthetic ability decreases so that although the grass does not then have large energy requirements the fructosan content of the whole plant decreases.

Perennial rye-grasses differ from the above pattern in that they appear to be able to photosynthesise at a sufficient rate to allow accumulation of fructosan even when growing rapidly (33, 34). Consequently they usually have only one maximum in the fructosan content, during the whole growing season and this may be about 24% of the dry matter.

Also present in the soluble carbohydrates of grasses is a second polysaccharide or mixture of polysaccharides of unknown origin. At least two instances have been reported when such a fraction has been recognised. McIlroy (36) isolated from a mixed rye-grass-clover pasture, a fraction which he considered to be a sulphuric acid ester of a glucogalactan in view of the large amount of non-removable ash. The published report of this leaves doubt as to the correctness of his conclusions. Harwood (37) obtained from a hot water extract of rye-grass a product which yielded glucose, galactose, arabinose and xylose on hydrolysis. He also isolated free D-mannitol from this extract. These polysaccharides were obtained only in very small amounts (e.g. 1% of the grass) so that it would not be

unreasonable to assume that they represented degradation products of the structural carbohydrates.

Starch

Starch is not normally found in grasses except in the heads and then presumably only in the seeds (the starch representing less than 2% of the whole grass dry matter). Treatment to cause swelling of the granules is required before the starch is rendered soluble and hot aqueous solutions of acid or alkali and hot water are often used, though 72% perchloric acid at 25°C has also been found suitable (38). Clegg reported starch in whole young grass (39) but has stated that the small values estimated might be accounted for by interferring substances other than starch (40).

Organic acids

Closely associated with the sugars in the present theories of plant metabolism are the organic acids. Though a certain fraction of these compounds will be estimated in the ash (being present as salts) the majority will be included in the nitrogen-free extractives. There are very few results available relating to the acids in grass but much work has been done on other plants especially by Bennett-Clark (41) and the American school led by Pucher and Vickery (42, 43).

It appears that two metabolic systems may be characterised, the one termed the Crassulacean type results in an accumulation of malic acid during periods of darkness whereas in the other type malic acid concentration decreases during darkness (44). It is possible that grasses may have the Crassulacean type of metabolism (45) but in the more general examples from this type of system (46) the gain in malic acid is usually accompanied by a loss of starch and a gain in citric acid. Grass leaves and stem contain no starch though a decrease in sucrose (including oligosaccharides) is found in grass during the night (33). The gain in malic acid reported for grass was associated however with a loss of citric acid and this does not agree with the changes normally experienced in plants having the Crassulacean type of metabolism.

The acids isolated from plants have included all those necessary for the various stages of the Krebs tricarboxylic acid cycle (47a, b) but as yet it cannot be said with certainty that the Krebs cycle operates in grasses. The isolation in grasses of malonic acid (45), which is an inhibitor of succinic acid dehydrogenase, would suggest that at the best only a modification of the tricarboxylic acid cycle is possible.

The published results of the analysis of organic acids in grasses (45, 48) indicate that the total acid concentration in grasses increases with the growth of the plant, and that this is due mostly to the increase in malic acid which more than balances the decrease in citric acid: these two acids together account for about half the total organic acids. The gross changes in the concentration of the acids are inversely related to the crude protein changes. When grass that was cut regularly and allowed to regrow was examined the later samples were found to have a higher crude protein and lower acid concentration than the earlier samples (49). The total amounts of the acids in the grasses which have been examined are 2-5% of the dry matter.

Pectin

The remaining material which would be included in a value for the nitrogen-free extractives and which occurs in significant quantity is pectin. Pectin is now considered to be a chain of galacturonic acid residues with 1:4 linkages and a proportion of the uronic acid groups are present as the methyl esters. This polyuronide is associated, in a manner as yet not completely understood, with polysaccharide material which yields galactose and arabinose on hydrolysis (50, 51).

The proportion of the various components varies according to the origin of the pectin (52) and although fractions containing only arabinose or galactose have been isolated from various pectins (53, 54) it cannot be said at present how many types of polysaccharides are in the pectic complex. Some physical evidence obtained by electrophoresis suggested a structure with a core of all three carbohydrate units, galactose, arabinose and uronic acid, combined (55).

Pectin from grass has not been examined in detail, though Buston (28) estimated the amount present in a series of grass samples and concluded that the pectin was of a type similar to previous preparations from other plants. More recently its occurrence in timothy was investigated (56). The amount was usually small, 1 - 3% of the dry matter, and was said to decrease with increasing age of the grass.

The overall physiological function of pectin remains unknown. It is thought to be one of the first materials deposited in the cell walls. It is well known that pectin holds together the cells of certain tissues, but not apparently the cellulose micelles (57). Pectin occurs in all plants that have been examined but in particular it is found in young actively growing tissues

and fruits. Magnesium and calcium ions are found in unusually large amounts in areas rich in pectin, such as the intercellular layer, and this results in the pectin being insoluble in water. It has been suggested (58) that the metal ions link the uronic acid groups of the pectin to other carboxyl groups in plants, such as those of hemicelluloses. Pectin has been said (see the discussion by Buston (59)) to be a precursor of lignin, as well as of most other cellular constituents, on account of its decreasing proportion (as a percentage of the dry matter) with increasing age of the plant but the rise in lignin far outstrips any change in pectin content. The three pectic components were thought to be directly interconvertible, oxidation of the galactan giving pectic acid which on decarboxylation would yield the araban.

Now that part of the structural configuration of each has been determined any such simple conversion has been shown to be impossible for stereochemical reasons, (51) since the glycosidic linkages are of the β configuration in the galactan, whilst in the pectic acid and araban they are in the α form. Also the pectic acid and galactan units are considered to be pyranose in

structure and have 1:4 linkages but the araban is furanose with the main chain having 1:5 linkages.

Grass fibre

In the Weende analysis the fraction containing structural material of the plant was termed crude fibre. It has been mentioned previously (p. 9) that this determination is chemically unsatisfactory since parts of some of the constituents which it was intended to include, such as the lignin and polysaccharides, are in fact not included, because, being soluble, they appear in the nitrogen-free extractives. For the purpose of the following discussion the whole of the structural components will be considered, not only that fraction which would be estimated as crude fibre by the Weende system.

The development by Norman and Jenkins of a method for the estimation of cellulose (60) and the examination of the sources of error in the lignin determination (61) allowed a better appreciation of the crude fibre fraction obtained in the Weende system of analysis. The cellulose estimation was an attempt to equal in a more convenient manner the estimation introduced by Cross and Bevan (62, 63) and as such was quite successful. The product was not pure cellulose. Using these methods

Norman (13) was able to show that grass with a high lignin content did not necessarily yield a crude fibre with a large amount of lignin though the product, as expected, was composed mainly of cellulose with most of the lignin. These methods for the determination of cellulose and lignin were the first important departures from the Weende method and they in their turn were variously modified, either as a result of errors that were detected or in attempts to produce a more discrete fraction. The modification of the lignin determination which has been most generally accepted is the use of a dilute acid pretreatment (64). In the method for cellulose, Crampton and Maynard (15) introduced the use of a digestion mixture containing acetic acid and nitric acid. This modification had originally been developed in a slightly different form by Kurscher and Hoffer (65). The method for cellulose by Crampton and Maynard yields values about 10% lower than those obtained by the chlorination methods of Norman and Jenkins. It is claimed (15) that these lower values measure more closely "true cellulose" i.e. polymeric 1-4' α glucosan, whereas the other measures "natural cellulose" or some approximation to it and hence include various amounts of the associated polysaccharides.

Hemicelluloses and cellulose - a definition of terms

The polysaccharides which are associated with cellulose were for a long time estimated only in terms of the total weight of material extracted from cellulosic material under given conditions, or by determination of the furfural produced by reaction with hot strong mineral acid or by measurement of the reducing power of a mixed hydrolysate. They have been the subject of investigation at least since 1890 (66), on account of the ease with which xylose may be obtained from the products of hydrolysis, and in 1891 Schulze (67) suggested the name hemicellulose for these polysaccharides. This name was unfortunate, implying a similarity to cellulose that is unfounded: since then it has become a source of confusion, particularly when used in association with the later developments of terminology such as cellulosans and polyuronides. Similarly the name cellulose has suffered from abuse, its meaning ranging from the pure glucosan to crude polysaccharide mixtures obtained by any of a multitude of procedures. In this thesis the terms hemicelluloses and cellulose will be used as originally intended unless suitably prefixed, i.e. hemicelluloses are taken to include all structural polysaccharides other than cellulose by which is meant

the pure glucosan with α - 1:4 linkages.

Hemicelluloses

Some of the earliest work on grass hemicelluloses was done by Buston (28) who identified, in the acid hydrolysates of various fractions obtained by alkaline extraction of cocksfoot (Dactylis glomerata), the sugars xylose, arabinose, galactose and galacturonic acid. It was realised at that time that the fractions resulting from the alkaline extractions and subsequent precipitations were not necessarily distinct nor did they represent chemical entities, but like so many careful original observations this warning has not always been heeded by later workers, some of whom have given undue emphasis to the products of a particular fractionation technique.

The hemicelluloses have most often been estimated by weighing them after precipitation from suitable extracts in the manner introduced by Preece (68). This was modified by Weihe and Phillips (69) who used delignified tissues, thus removing a major source of contamination of the hemicelluloses. Methods involving furfural formation from pentosans, by reaction with strong acids, have been developed with considerable ingenuity, the

yields of furfural being determined after various treatments of the grass. Values have been obtained for total pentosans, for pentosans in cellulose residues (obtained by various methods) and, by difference, for pentosans due to hemicelluloses.

Uronic acids too are converted to furfural in this estimation and must be allowed for: their determination is not very accurate in the presence of neutral sugars. The other analytical methods employed in place of the direct isolation of the hemicelluloses were reviewed by Phillips (70) who showed how difficult it was to translate results in terms of reducing power or furfural yield into weights of hemicelluloses, since each of the sugars present in a hydrolysate have different reducing values and since some of the sugars in the hemicelluloses yield no furfural.

Recent advances in analyses of fibre

The present possibilities for an improved method for the estimation of hemicelluloses originate from two recent advances in technique. One is the easy preparation of holocellulose in almost quantitative yield and the other is the development of chromatography, in particular paper chromatography. Holocellulose, in theory, is the material representing only the structural carbohydrates

of plant tissue but in practice a small amount of lignin remains as well as some ash and protein. Complete removal of lignin is presumably possible but, beyond a certain limit, its continued removal is accompanied by loss of polysaccharides. Holocellulose was first isolated in 1921, when Schmidt and Graumann made it using free chlorine dioxide gas (71). This was a method involving personal risk and the methods developed subsequently, until the use of prolonged treatment with sodium chlorite and acetic acid for the preparation of wood holocellulose by Jayme (72), were all to some extent objectionable. For soft, porous material like grass, a shorter treatment is more suitable (73), but grass has a far greater protein content than wood so that a preliminary de-proteinising treatment is necessary. This is usually accomplished by the action of an enzyme preparation such as pepsin in dilute acid.

The main advantages in the use of holocellulose for the quantitative examination of the structural carbohydrates of material such as grass are that it allows the extraction of the various fractions under much milder conditions and that such fractions are very little contaminated with non-carbohydrate material. Structural

investigations on holocellulose polysaccharides must, however, be initiated with caution for quite extensive determinations are necessary to decide whether significant macromolecular rearrangements have taken place during the preparation of the holocellulose. That the polysaccharides associated with the cellulose are rendered more easily extractable in holocellulose is without question, for some are than capable of being extracted with hot water, but the reason for this change is not so obvious. There are at least three possible explanations: the first, which in view of modern theories of cell wall structure is most likely, is that the removal of the lignin and protein from the fibre simply allows greater penetration of solvents. Many of the hemicellulose fractions, however extracted, are eventually soluble in water, whether they are obtained from whole fibre or from holocellulose. The second is that in the preparation of the holocellulose the hypothetical linkage between the hemicelluloses and lignin has been broken by the removal of the lignin leaving part of the hemicelluloses more available. The third possibility is that removal of the lignin has somehow exposed the uronic acid groupings of the

hemicelluloses and that slight autohydrolysis follows, as in the gums. This last theory does not seem very likely as a large proportion of the arabinose units would be expected to be liberated at the same time for they are apparently present in the furanose form, as seen later by their ease of hydrolysis.

The use of chromatographic methods has made the estimation of hemicellulose fractions much more complete: instead of obtaining, as previously, one value for the total amount in any sample of grass, it is now convenient to calculate the amount of each of the individual constituent sugars which are released as a result of hydrolysis of the various hemicellulose fractions. The application of these techniques, in conjunction with holocellulose preparation does not seem to have been very frequent in grass studies, judging from published results. The analysis of one sample of cocksfoot grass was reported by Binger, Sullivan and Jensen (74) and an account of an investigation, which involved the preparation of holocellulose by four methods, (from straws and one grass sample), followed by analysis of the hemicelluloses was included in a thesis by Miss B.D.E. Gaillard (75). Much information

on the structural constituents of wheat and corn products has been published by workers in U.S.A. (76, 77, 78) and Canada (79, 80, 81, 82, 83, 84) but very little information of a quantitative nature is available.

The general result emerging from this work (74, 75) is that in the grass hemicelluloses, xylose is the predominant sugar with smaller approximately equal amounts of glucose, arabinose and uronic acids and very small amounts of galactose. The published results have been for mature grasses and in each of five species examined by Buston (28) the hemicelluloses amounted to about 17% of the grass dry matter and Binger et al (74) found about 12%. How seasonal variation and other factors affect either the quantity or nature of the hemicelluloses has not in the past been examined, but more recently Mackenzie and Wylam (85) published the results of an investigation of the changes in structural carbohydrates in one variety of rye-grass during the growing season.

Outline of biochemical relationships

Any attempt to follow the changes in composition of grasses must inevitably raise the question of how

Fig. 1 The outline of the photosynthetic cycle

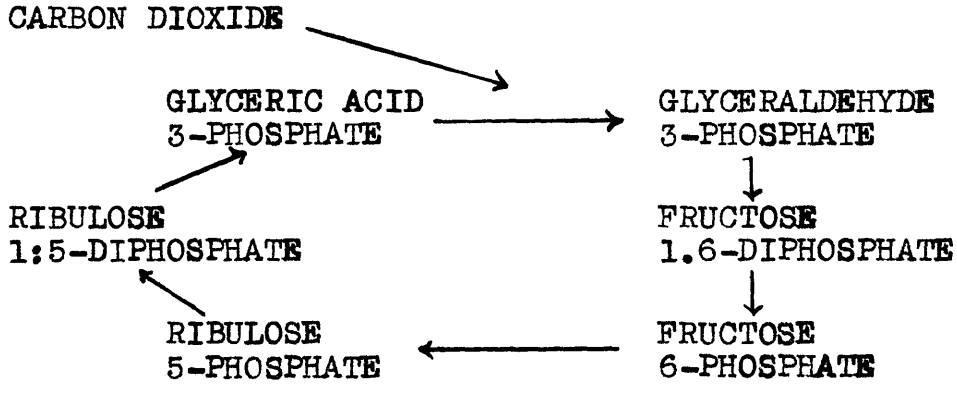
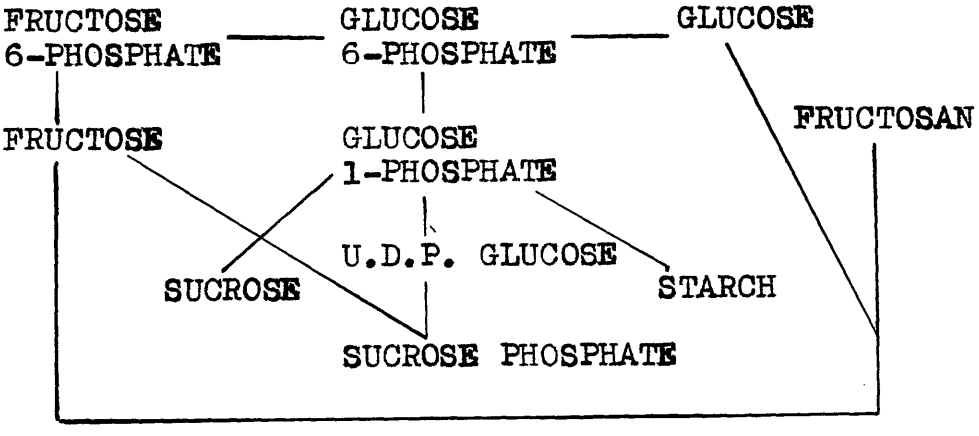


Fig. 2 The course of carbohydrate transformation



and why these changes occur. To try to answer these problems, even in a very general way, it becomes necessary to have some understanding of the normal course of plant metabolism. Very broadly, plant metabolism may be said to be the fixation of energy in photosynthesis in such a manner that it is then available either for the building up of reserve materials from carbohydrate or for the uptake of mineral matter. The carbohydrate may be further converted to fats, and nitrogen and sulphur are used to form part of the protein. Part of the stored energy is used in respiration and part is used for the formation of reserve and other materials. Only a very brief survey of the various chemical pathways that exist to produce the final metabolic pattern is desirable here, but sufficient will be presented to indicate how inter-related they all are.

Considering photosynthesis first (Fig. 1), only the carbon transfer will be dealt with, since this is all that is known with any certainty. It has been shown by Calvin and his colleagues (86) that the initial conversion of carbon dioxide from the atmosphere is by reaction with ribulose 1.6-diphosphate to form glyceric

acid 3-phosphate. This is then reduced to glyceraldehyde 3-phosphate which may be used for respiration or for conversion to glycerol for fats. If the glyceraldehyde 3-phosphate remains in the photosynthetic cycle it can be condensed to form fructose 1.6-diphosphate which is then converted to fructose 6-phosphate, and this is the start of carbohydrate transformation. The complete cycle from the hexose phosphates back to ribulose diphosphate has been traced and has been shown to allow the possible formation of ribose and sedoheptulose as intermediates. Each step in these and the following transformations is the result of an enzymatic process and the variations in distribution and activity of the various enzymes in different plant species causes differing effects on the final chemical composition of the plants. Thus sedoheptulose is found in Sedum species in amounts much greater than in most other plants (87).

Considering next the conversion of fructose 6-phosphate to the commonly identified free sugars and the various storage products (Fig. 2) there are two initial reactions which may be involved. One is isomerisation by the action of the enzyme phosphohexose isomerase to

form glucose 6-phosphate and the other is hydrolysis by a phosphatase to yield free fructose. The fructose can then react with glucose 1-phosphate to yield sucrose by the action of sucrose phosphorylase (88). Sucrose may also be formed, initially as the phosphate, by the action of fructose on uridine diphospho-glucose (89) which is produced from glucose 1-phosphate and uridine triphosphate. Glucose 1-phosphate is also the unit for the production of starch by the action of phosphorylases to form amylose (90) and by the combined action of phosphorylases and a branching enzyme (91) to form amylopectin. In grasses, however, these reactions seem to be restricted to the seeds and are replaced in the other parts of the plant by a system which produces fructosan. This involves the addition of fructose to sucrose in the presence of invertase or transfructosylases and can yield a series of straight chain polysaccharides by fructose addition to the sucrose moiety (92, 93). Glucose may be produced by the action of invertase on sucrose, when fructose is also liberated, or by dephosphorylation of the glucose phosphates.

It now appears fairly well established that respiration in plants involves the conversion of

Fig. 3A The course of respiration - Glycolytic system

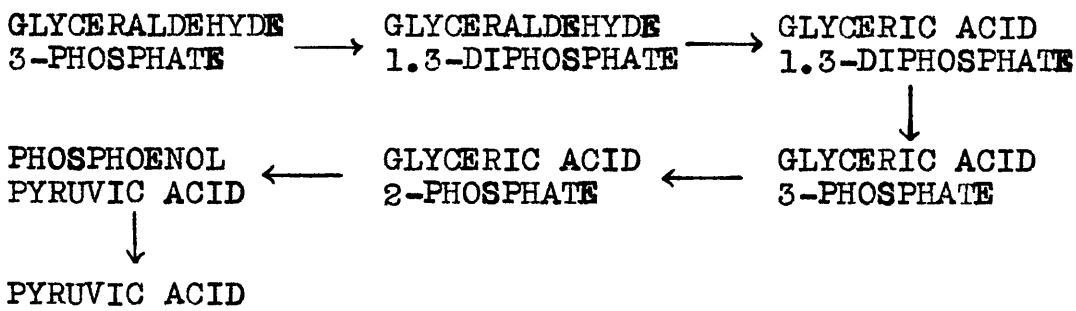
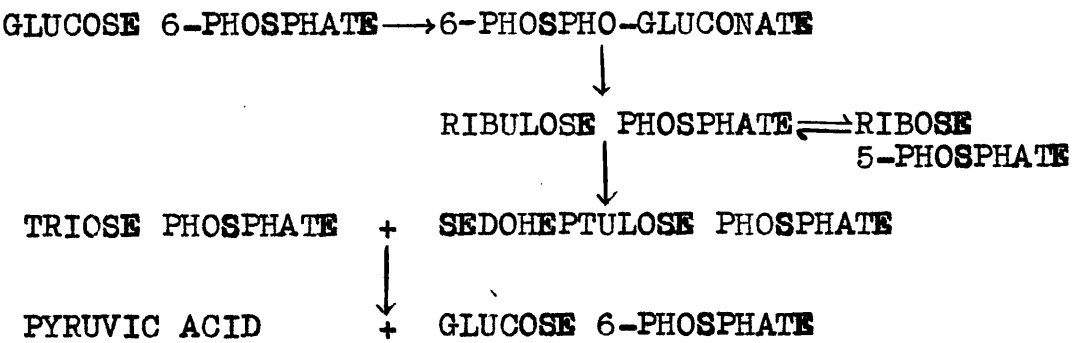


Fig. 3B The course of respiration - Pentose shunt



carbohydrates to pyruvic acid which may then enter the oxidative system of the Krebs carboxylic acid cycle (47) or some modification thereof (Fig. 4). The production of pyruvic acid may be achieved in two ways. The first (Fig. 3A), involving nucleotides of the adenosine series causes oxidation and phosphate transfer reactions to take place starting from glyceraldehyde 3-phosphate, which itself is formed, as necessary, by the reverse of the mechanisms outlined previously, from the hexose phosphates. This is the glycolytic system (94). The other series of reactions, known as the pentose shunt (Fig. 3B) is based on glucose 6-phosphate which is oxidised through gluconic acid 6-phosphate to pentose phosphate and pyruvic acid. Glyceraldehyde 3-phosphate is again an intermediate but the whole series is activated this time by pyridine nucleotides (95, 96). During the operation of the pentose shunt, sedoheptulose phosphate and half the original glucose 6-phosphate are reformed. Sedoheptulose is a precursor of shikimic acid which has been traced as a precursor of aromatic amino acids and lignin (97).

The pyruvic acid produced by degradation of the carbohydrate phosphates reacts with co-enzyme A yielding

Fig. 4 The organic acid cycle

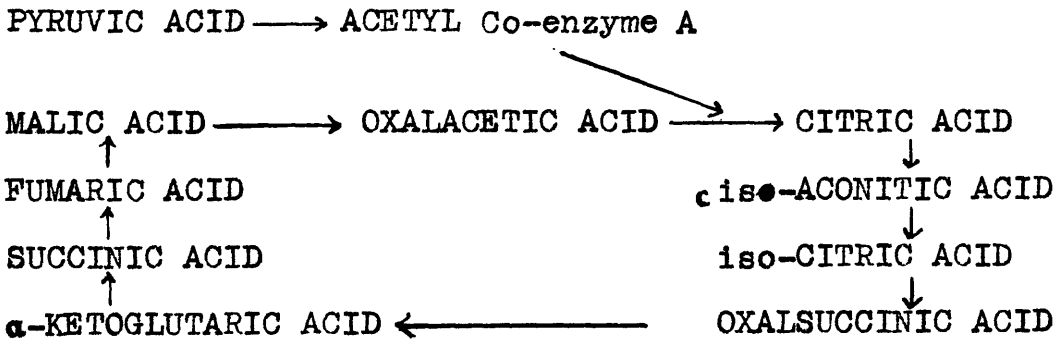
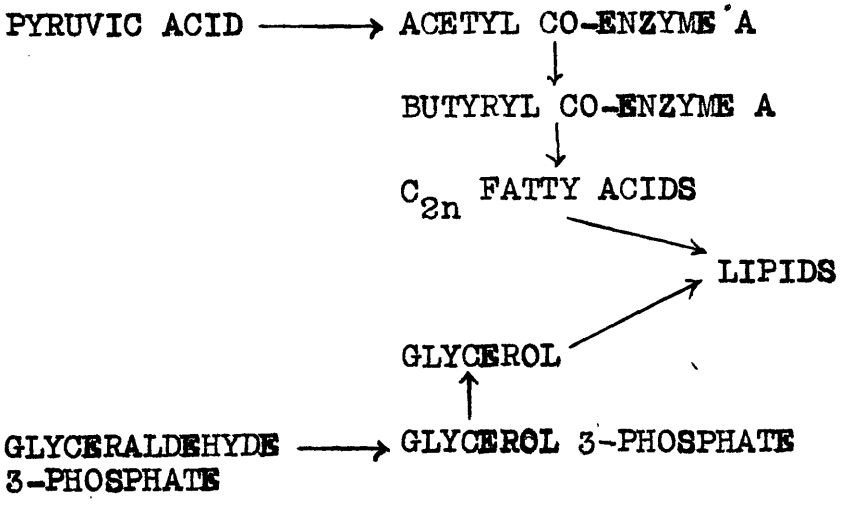


Fig. 5 The possible manner of production of lipids



acetyl co-enzyme A and liberating carbon dioxide (86). The acetyl compound then reacts with oxalacetic acid and enters the organic acid metabolic cycle (Fig. 4) as citric acid. It is not known exactly what reactions are involved in this cycle, since malonic acid, for example, which is an inhibitor of the normal Krebs cycle has been reported in a sample of grass (45) and the effects of other added inhibitors have not always been as predicted. However, during the course of this cycle, two carbon atoms, corresponding to the acetyl group, are liberated as carbon dioxide.

Acetyl co-enzyme A is also the major precursor of the long chain fatty acids (Fig. 5), condensing to form butyryl co-enzyme A and free co-enzyme A in the first stage and similarly to produce higher acids with an even number of carbon atoms by further condensations (98). The fats, as found in grasses, are synthesised by the action of the enzyme lipase on the fatty acids and glycerol, which appears to be formed by dephosphorylation of glycerol 3-phosphate, the product of the reduction of glyceraldehyde 3-phosphate.

The nature of protein synthesis is very uncertain but a number of the amino acids which are the probable

precursors of the polypeptides, may be formed by transamination of the various keto acids involved in the acid cycle: thus alanine, aspartic acid and glutamic acid can be produced from pyruvic, oxalacetic and α-ketoglutaric acid respectively. The aromatic amino acids are known to be formed from shikimic acid (99). The nitrogen required for such compounds originates in the nutrient solution that is absorbed through the plant roots in much the same way as the metal ions. The nitrogen can be taken up as ammonium ions, which are buffered by the organic acids or as nitrate ions which must then be reduced in the plant tissue.

It will be realised that the relationships that have just been described cover only a small part of the compounds found in grasses, the most obvious exceptions being the fibrous materials which constitute the cell walls. The reason for these omissions is that facts are much scarcer than hypotheses in regard to the mode of formation or subsequent changes of these materials. Some brief indications of what now seems probable will be given for some of these compounds.

Following the use of radioactive tracer techniques,

information is now accruing which shows that cellulose in wheat is largely synthesised from intact glucose molecules (100, 101, 102, 103) but the labelled atom distribution in the product consistently suggests that a significant part of the glucose is obtained by recombination of the triose fractions produced during glycolysis (Fig. 3A). Similar results were obtained from work with cotton cellulose (104) and by the examination of the production of bacterial cellulose (105). The studies of Neish (101) and Altermatt and Neish (102) included the investigations of xylan synthesis and it appeared that xylan was formed without degradation from glucose but not from xylose. It was considered likely that the C_6 of glucose was removed by oxidation and decarboxylation of the free sugar or a derivative thereof before condensation. Free xylose however was considered to be involved in the pentose shunt and the glucose structure was produced from it only after much rearrangement. These workers also showed that xylan was produced more rapidly than cellulose in old wheat plants although the rates were equal in young plants. Work (106) involving exposure of sugar beet leaves to $C^{14}O_2$ resulted in the cellulose

in the leaves taking up C^{14} before the free glucose and it was thought that a glucose phosphate was the active precursor of cellulose, but uridine diphospho-glucose appears to contain C^{14} after a very short time under these conditions. By analogy with sucrose synthesis, Altermatt and Neish (102) suggested a scheme for the formation of all the plant polysaccharides, based on uridine diphospho-glucose. The conversion of the glucose moiety to the required galactan, xylan and other polysaccharides was proposed to be accomplished through reactions of their uridine diphosphate (U.D.P.) derivatives. Some of the enzymes required for these changes have been shown to exist; and the reactions below are reasonably established:

UDP glucose $\rightleftharpoons$ UDP galactose (107)

UDP xylose $\rightleftharpoons$ UDP arabinose (108)

No evidence has been published yet as to the actual pathway by which the polysaccharides are formed.

Lignin biosynthesis is also being actively studied but the task is made especially difficult since the nature of lignin has not been established. Shikimic acid has, however, been shown to be a

precursor (97) and a connection is suggested here with the carbohydrates, for this acid is believed to be derived from sedoheptulose.

OUTLINE OF THE PRESENT WORK

The work described in this dissertation is the result of an examination of the chemical composition of some forage grasses with particular regard to two aspects of this very complex subject.

Part I describes the development of a detailed scheme of analysis undertaken to give more useful information than the Weende or other systems of analysis and to determine whether any materials, other than those normally expected, were present in the grasses in significant amounts. The first section of Part I is concerned with the analysis of various extracts of the grasses and was also directed towards obtaining a complete recovery of the grass dry matter in terms of known fractions. Most difficulty was encountered in the analysis of the water-soluble materials in which fractions were found which it is suggested were respectively some type of lignin compound and simpler phenolic material. A water-soluble polysaccharide other than fructosan was also isolated. Non-ionic substances which were not precipitated by ethyl alcohol were noticed in the extract from which it was intended to isolate the pectin complex.

The second section of Part I is an examination of the materials in the grass fibre, which is defined in terms of the applied treatments necessary for its isolation. The polysaccharides of the plant cell walls are the main constituents of the grass fibre and these have been examined in detail in holocellulose prepared from the fibre.

Part II describes the application of the analytical methods developed in Part I to a series of five grasses cut at four stages of growth. Particular attention was directed towards the composition of the structural polysaccharides. These were found to change in a regular manner, unlike the soluble carbohydrates, and a comparison between the different grasses showed only slight differences. The long recognised variations in such constituents as lignin and protein have been confirmed.

PART I

THE DEVELOPMENT OF A COMPREHENSIVE METHOD OF ANALYSIS OF DRIED GRASS

INTRODUCTION

As already explained the methods available for the analysis of dried grass samples have increased considerably since the Weende scheme of analysis was introduced (8). The faults of that analytical procedure have been discussed in the general introduction to this present work. So far as can be judged from published information the other methods that have been proposed are themselves not entirely satisfactory ways of determining the chemical composition of such materials. The relative advantages of the various analytical schemes in nutritional and digestibility studies are not necessarily dependent on the merits of the chemical analyses. An illustration of this distinction is afforded by strong arguments put forward by Nordfeldt and his co-workers (109) for the retention of the determination of crude fibre by the Weende method for

use in digestibility trials, even though they examined the fibre and found enormous variations in the chemical composition of that fraction, when it was prepared from different forages.

The most noticeable feature of all the analyses that have been introduced is that when these techniques have sought to measure all the components of the grasses or other forage crops in terms of chemical entities, a variable amount of the material is unaccounted for. One of the earliest reported analyses of this type was carried out by Headden (110) who examined clover samples by the determination of sugars, dextrins, starch, xylan, lignones, cellulose, ash, protein, ether extract and the sum of the ethyl alcohol and water extracts after deduction of the values for sugars and dextrins. By the knowledge gained later in this section it is considered that one of the more serious errors in that analysis was the partial duplication of the values for ash and protein, which were determined on the whole sample and part of which would also have been included in the alcohol and water extracts. Other errors could have arisen from the neglect of the organic acids as well as the

assumption that the material termed cellulose was pure cellulose. Even so the result of the summation obtained was that 4.8% of the sample was not estimated.

Considering some of the more recent work, the findings of three groups of investigators will be discussed. In 1942 Ferguson (111) analysed a sample of wheat straw and reported 13.7% of the dry matter to be missing after the following determinations had been made: ether extract, crude protein, ash, lignin, furfural yield and cellulose (60). A large part of the undetermined material would consist of polysaccharides which would be dissolved during the cellulose estimation, and also of water-soluble substances such as organic acids and soluble sugars. This work was followed (112) by the analysis of samples of a young and an old grass from which the recoveries were calculated to be respectively 95.5 and 84% of the dry matter, using the same methods.

This same variation in the amount of material which was undetermined was noticed by Reid (113) who found that the discrepancy was greatest with samples of mature grass for an analytical scheme which included the determination of lignin, cellulose,

hemicelluloses, pectin, starch, sugars, ash, crude protein and ether extract. Using the same technique the variation in the material not determined was from 2.5 to 4.5% of the dry matter for samples of young and old grasses (timothy). These analyses (114) were difficult to interpret, particularly with regard to the values for starch which were unusually large (8%) and those for soluble sugars which were somewhat small (5%).

Ely and his collaborators have also published results (115) which included very large values for the quantity of starch in cocksfoot samples; their method of analysis included the determination of crude protein, ash, ether extract, soluble sugars, starch, organic acids, pentosans, lignin and cellulose. Large discrepancies were reported, from 7.5 - 18.5% of the dry matter.

In none of the above results was there any recognition of the fact that the cellulose preparations might contain lignin or protein. Correction of the cellulose value for ash was sometimes made. The lignin values were frequently carefully corrected for ash and protein although the presence of a more soluble

form of lignin than that determined by the use of 72% sulphuric acid has frequently been postulated and never convincingly disproved (116, 117).

Scope of the work in this section

There was thus an obvious need for an investigation into the causes of the errors in the summations quoted even though the workers who published these results did not seem to express undue concern about their findings. The elimination of the causes of the errors of summation was the object of the first part of this thesis and the results were mainly satisfactory in that 95-99% of the dry matter of the grass samples were ultimately accounted for as determinable quantities. The materials studied were a sample of cocksfoot, which was used for most of the preliminary investigations, and samples of rye-grasses and timothy.

The general method of examination of the samples was the successive extraction of the grasses with neutral reagents including ether or ethyl alcohol and benzene, water and ammonium oxalate solution. The quantity and nature of the material extracted by these reagents was then determined as far as possible. The grass fibre residue which was then obtained was

examined for the structural components of the grasses and for the retained protein. The polysaccharides, in particular, were investigated in the holocellulose prepared from the fibre. The quantities of polysaccharides estimated in the samples by the methods developed in this work were compared with the values obtained by some of the more standard analytical techniques.

METHODS

Unless stated otherwise, in connexion with some specific purpose, the general methods of analysis used in the work were as follows.

Moisture determination of dried grass or grass residues

Quantities of 0.5 - 1.0 g. were heated at 100°C for 3 hr. in aluminium dishes.

The total solids contents of extracts

The extracts were evaporated in small, round bottom flasks, under reduced pressure to a small volume (2-5 ml.) and then on a sand bath at about 110°C. Ether solutions were evaporated on the steam bath. The flasks in both instances were then dried at 100°C for 3 hr.

Total nitrogen Macro-Kjeldahl procedure

Digestion was effected with 20 ml. sulphuric acid in the presence of copper sulphate-selenium catalyst mixture until the solution was clear and for 2 hr. after clearing. The ammonia formed was titrated in boric acid with N/10 hydrochloric acid.

Micro-Kjeldahl procedure

Digestion was effected with 2 ml. sulphuric acid in the presence of a mercury catalyst. Zinc dust was added to the digest before the addition of alkali. The ammonia produced was distilled and collected in boric acid for titration with N/100 hydrochloric acid.

Non-protein nitrogen (N.P.N.) in extracts

The extract was clarified with dibasic lead acetate and sodium phosphate and the nitrogen determined in the filtrate by the micro-Kjeldahl procedure.

It was decided in view of the results published by Synge (118) to assume that the N.P.N. values multiplied by 5.6 gave the amount of N.P.N. substances present.

Crude protein and true protein in extracts

These values were calculated as Total N x 6.25 and (Total N - N.P.N.) x 6.25 respectively.

Organic Acids

For the total organic acids in dried grass the method of Pucher et al. (42) was adopted with slight modifications. The acids were calculated as malic acid. This value was used for the organic acids in the combined water extracts in the analyses (p. 79).

The organic acids in the preliminary extractions with alcohol (p. 62) or water (p. 64) were determined as follows. The extract was passed down a column of

cation exchange resin (Amberlite IR 120) to remove amino acids and metallic cations. An aliquot of the eluate was made alkaline with sodium hydroxide solution and the solution titrated electrometrically between pH 7.9 and pH 2.5 using N/10 nitric acid with calomel and platinum/quinhydrone electrodes. Unknown errors were involved when the method was used on these solutions because of the presence of phosphates, but the sum of the organic acids obtained from the alcohol and water extracts was only about 0.4% greater than the value for total organic acids determined as described in the previous paragraph.

An attempt was made to identify some of the constituent acids by direct paper chromatography (119, 120). The mixture was too complex, without previous fractionation such as ion exchange chromatrography (45, 121) to yield useful information and a complete examination of the acids was considered to be outside the scope of this investigation.

Soluble sugars

The carbohydrates glucose, fructose, sucrose and fructosan were determined in whole grass or in the appropriate extracts of the grass by the methods used by Waite and Boyd (33, 34).

Ash

For dried grass samples the material was weighed directly into a silica basin and for solutions a portion was dried in the basin. After the residue had been charred over the luminous flame of a bunsen burner the contents were incinerated at 550°C for 2 hr. in a thermostatically controlled muffle furnace. The basins were then cooled, the contents moistened with dilute nitric acid and heated for a further hour at 550°C, cooled again and weighed.

THE GRASS SAMPLES EXAMINED

The dried grass samples used in the following

work have been referred to throughout the remaining pages by numbers to make cross reference easy. They were as follows:-

Grass No. 1. Cocksfoot (S 143), cut in August as a hay aftermath.

No. 2. Ryegrass cut in September after having been allowed free growth all summer.

No. 3. Ryegrass cut in May for the first time.

No. 4. Ryegrass cut in August but at a height of about 8". The plot from which this sample was taken had previously been cut three times at a similar height.

No. 5. Timothy cut in September after having been allowed free growth all summer.

No. 6. Ryegrass (S 24) cut in April for the first time.

No. 7. Ryegrass (S 23) cut in April for the first time.

SECTION A

THE ANALYSIS OF GRASS EXTRACTS

(i) PRELIMINARY EXTRACTIONS

The ether extract

In view of the results reported by Hellström (122) showing an apparent loss of dry matter when dried hay was extracted with benzene, the amount of material recovered after each extraction of dried grass was of particular interest. This operation was carried out in the normal manner by extracting 1.5 - 2 g. dried cocksfoot grass (Grass No. 1) in cellulose extraction thimbles with diethyl ether using an all glass Soxhlet apparatus in an electrically heated water-bath for 18 hr. The grass residue after ether extraction was very hygroscopic and to obtain the dry weight of the residue it was necessary to weigh the thimble and contents inside screw cap bottles.

2 g. dried grass No. 1 was added to each of five cellulose extraction thimbles (25 x 80 mm. Whatman single thickness) which had been extracted previously with ether and then cooled in a desiccator after drying for 3 hr. at 100°C. Each thimble was then extracted with refluxing diethyl ether (A.R.) for 16 hr. (until no more colour was visible in the extracting

solvent) using weighed flasks to receive the extract. After extraction the thimbles and grass residues were individually drained with suction and washed with more ether. The washings were added to the respective extracts and the solvent distilled from each flask. The flasks with their extracts were dried at 100°C for 3 hr. and then equilibrated to laboratory conditions for ½ hour before being weighed. The empty flasks were dried and weighed in a similar manner. The extraction thimbles and grass residues were dried for 3 hr. at 100°C., and after cooling in a desiccator they were placed in screw cap bottles (10 x 3 cm) before being weighed. When the grass residue was wanted for further examination, the ether was allowed to evaporate from the thimbles and their contents in air at room temperature and then in vacuo. The total residue was weighed and the true dry matter determined by oven drying a small portion of the material.

The results of these extractions are given in Table 2.

Table 2. The results of ether extraction - Dried Grass No. 1

Weight of sample (g. dry matter)	Weight of ether extract (g.)	Weight of residue (g. dry matter)	Recovery (% dry matter)
1.792	0.079	1.743	101.7
1.906	0.083	1.839	100.7
2.350	0.100	2.272	100.9
1.504	0.066	1.457	101.2
1.771	0.078	1.711	100.9

The average recovery was 101.1% of the dry matter sample, the material extracted by ether being 4.4% of the whole grass dry matter and the residue 96.7%. The apparent slight gain in material was presumably caused by failure to weigh the recovered fractions in a completely dry state.

It was later found that if the extraction period was extended to 30 hr. with this particular grass the quantity of the material extracted by ether was increased to 5.7% of the dry matter of the grass.

Unless otherwise stated all values obtained in this work and reported as percentages, refer to the dry matter of the original grass.

The nature of the ether extractives was investigated by the determination, in aliquots, of ash, nitrogen, carbohydrates, free acids and pigments.

For the determination of ash and nitrogen, suitable amounts of the ether solution were transferred to crucibles and micro-Kjeldahl digestion tubes respectively and the solvent then removed by heating on a water bath. The ash was determined by heating at 550°C in an electric furnace for 3 hr. and the N by the micro-Kjeldahl method.

The ash content of the ether extract was 0.07% of the original grass dry matter, and the nitrogen

0.025%, which if calculated as crude protein was 0.15% of the grass dry matter.

It was not expected that any significant amount of carbohydrates would be extracted from dried grass with ether. To confirm this a portion of the extract was washed with water and the aqueous solution deionised. The reaction of this solution after concentration was weakly positive to the Molisch test [(123) α-naphthol-H_2SO_4] but paper chromatographic examination revealed no carbohydrates.

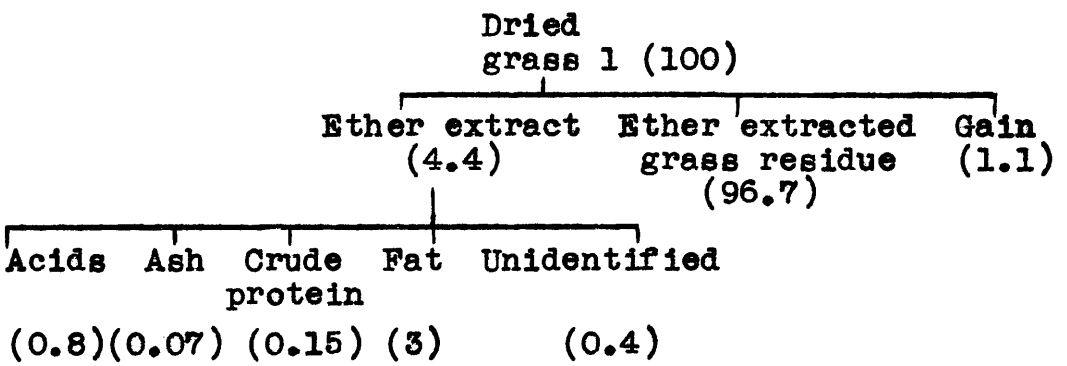
The free acids in the ether extracted material were determined by titration with acid after the addition of excess N/50 sodium hydroxide solution to the ether solution. The results for replicates varied widely for they were in the range 18-20 m-equiv. acid per 100 g. grass dry matter. The nature of the acids was not determined but when calculated as linoleic acid, the amount was 0.8% of the grass dry matter.

A general separation of the ether extracted material into fats and waxes, which by general considerations must represent the greater part of the extract, was carried out by the method previously used

by Smith and Chibnall (20). This was by precipitation of the fats from the ether solution by the addition of two volumes of acetone followed by cooling to 0°C. overnight. It was found that the fats represented about two thirds of the ether extract or about 3% of the grass dry matter.

The results of the ether extraction of Grass No. 1 were thus as follows:

Table 3. The ether extraction of dried grass No. 1 (% of the original grass dry matter)



The alcohol extract

Dried grass samples have previously (34) been extracted with 90% ethyl alcohol in this laboratory as the first step in the determination of the soluble sugars and in the present work this solvent was chosen as the next, more polar extractant to follow the ether extraction.

The method of extraction was gentle agitation of the ether extracted grass (No. 1) residue with 90% ethyl alcohol using 60 ml./g. grass residue for 18 hr. at 20°C. The mixtures were kept in stoppered tubes attached to the rim of a slowly rotating wheel.

The alcohol extracted grass from each tube was filtered off separately and well washed with further quantities of 90% ethyl alcohol. The alcoholic filtrate and washings from each grass aliquot were evaporated and weighed after drying *in vacuo*. The alcohol extracted grass residues were combined, dried *in vacuo* and weighed. The total weight of residue was corrected for residual moisture, which was determined on a small sample.

A considerable variation in the quantity of alcohol-extractable material was recorded but the average value was 17.7% of the original grass dry matter for Grass No. 1 (range 16.5 - 18.9%). The grass residue after ether and alcohol extractions was 77.9% of the original grass dry matter. Together with the alcohol extract this represented a recovery of 99.0% of the ether extracted grass.

The substances in the alcohol extract of other portions of Grass No. 1 were examined in a similar way to that described for the ether extract and it was found that the ash and crude protein contents were 4.0 and 1.4% respectively of the original grass. There was 4.6% soluble sugars, comprised of 1.4% free hexoses (glucose and fructose) and 3.2% sucrose. Paper

chromatograms prepared from the extract also revealed a spot suggestive of a very small amount of raffinose.

The most important observation at this stage was that after evaporation of the alcohol from the extract, a large part (4.4% of the original grass) of the alcohol-soluble material was soluble in ether. The nitrogen content (0.035% of the original grass) of this second ether-soluble fraction was of the same order as that of the original ether extract (0.025%) suggesting that both ether extracts had a common origin.

The ether soluble material obtained as a result of extraction of the grass with 90% ethyl alcohol presumably was an indication of the incomplete extraction achieved by the initial ether treatment. The efficiency of removal of ether-soluble material from dried grass was therefore compared (Table 4) for a number of solvent systems. After each extraction the organic solvents were removed and the residue partitioned between ether and water. It was found that the azeotropic mixture of ethanol-benzene was the most effective, both in respect of the time

required and the completeness of the extraction and the removal of green colour. The use of this solvent mixture was later adopted to obtain the total ether-soluble fraction from grasses and the water-extractable fraction combined with the main water extract.

Table 4. The extraction of ether soluble material from Grass No. 1 by various solvent treatments

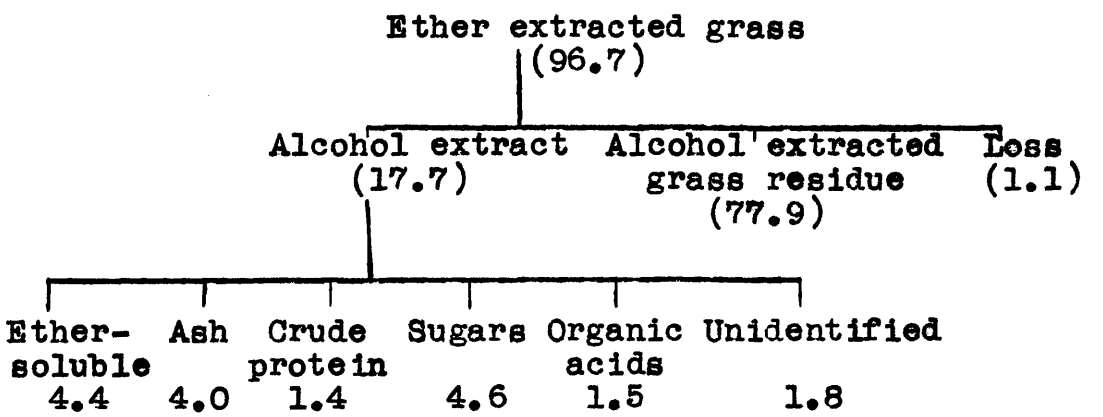
Solvent and conditions	Ether soluble material (% of the grass dry matter)
Ether Soxhlet 18 hr.	4.4
Ether Soxhlet 30 hr.	5.7
Ethanol-ether(1:1) reflux, 1 hr.	5.1
Ethanol-ether(1:1) reflux, 18 hr.	6.0
Shaking at 20°C. with 90% ethanol, 18 hr.	5.2
Acetone Soxhlet 18 hr.	6.0
Ethanol-benzene(1:2) Soxhlet 18 hr.	8.6
Ether followed by 90% ethanol 18 + 18 hr.	8.8

Organic acids were determined in the "true" alcoholic extract, by which is meant the material

that was extracted by 90% ethyl alcohol as described above and which was soluble in water when partitioned between ether and water. The quantity calculated as malic acid represented 1.5% of the original dry matter.

The results of the extraction of the ether extracted residue of grass No. 1 with 90% alcohol were thus as follows:

Table 5. The extraction of ether extracted dried grass No. 1 with 90% ethanol (% of the original grass dry matter)



Water extract

It has been found in this laboratory that for the determination of fructosan in dried grass it is sufficient to heat alcohol extracted grass with water at 60°C. for 30 min. to extract the fructosan

completely, and accordingly these conditions were a first choice in the present investigation.

1 g. portions of the residue of grass No. 1, after extraction with ether and 90% ethanol, were extracted with 75 ml. water in a water bath at 60-65°C for 30 min. The grass residue was obtained by filtration through weighed, previously dried filter papers (Whatman No. 1) and dried *in vacuo*. The yield of dry matter in the residues was calculated as before, after determination of the moisture content of a representative sample.

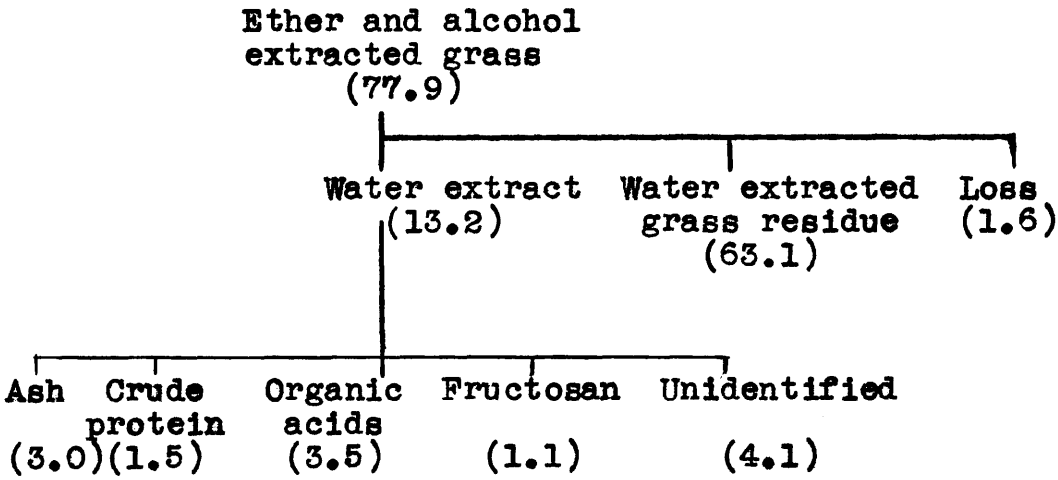
The total water extract from 3 g. of the ether and alcohol extracted grass residue was pooled and concentrated *in vacuo* in a final volume of 10 ml. To determine the total solids of the extract 1 ml. portions of this solution were evaporated on small weighed aluminium dishes, at first on a boiling water bath and then for 3 hr. in an oven at 100°C., and the total solids weighed.

The result of the water extraction of the ether and alcohol extracted residue of Grass No. 1 which was itself 77.9% of the original grass, was that the water extractable compounds amounted to 13.2% of the original grass and the water extracted residue was 63.1% of the grass. This was a recovery of 98.1% of the grass residue after treatment with ether and alcohol.

The water extracted materials were analysed in a manner similar to that for the alcohol extract and the results are presented in Table 6. It may be seen that quite a large part of the water-soluble fraction (4.1% of the original grass) was not identified: both this

and the material (1.8%) similarly unidentified in the previous alcohol extraction will be considered later.

Table 6 The water extraction of dried grass No. 1 which had been previously extracted with ether and alcohol (as % of the original grass dry matter)



It was stated (p.59) that the amount of material extracted with 90% alcohol from the ether extracted residue of Grass No. 1 varied considerably. However, the sum of the true alcohol-soluble extract (i.e. total alcohol extract minus ether-soluble material in the alcohol extract) and the subsequent water extract was reasonably constant (27-28% of the original grass dry matter). The total water-soluble extract obtained by extraction of Grass No. 1 with ethanol-benzene (the whole extract being then partitioned between ether and

water) and water at 60°C, was 26.5% of the original grass. This was considered to be fair agreement with the total water-soluble fraction obtained by extraction of the ether-extracted grass with alcohol and water. It was in accord also with general solubility considerations.

The amount of material extracted with water from a given alcohol-treated grass sample is dependent on temperature. Thus in a series of three extractions of samples of the same material at 4°, 60° and 100°C the quantity of water extract increased appreciably (Table 7). A broad separation of the substances present in each of these extracts into ionic and non-ionic fractions was made by using ion exchange resins. The resins Amberlite IR 120 (H form) and IR 4B (OH form) were used and after twice recycling the water extract solutions through each resin in turn, (until the pH of the eluate was unchanged), the resins were washed with water until the washings no longer contained carbohydrates as indicated by Molisch reaction (123). The quantity of material unabsorbed was then determined and presumed to be of a non-ionic nature. The absorbed substances, measured by difference, were

recorded as the ionic fraction (Table 7).

Table 7. Effect of temperature on the amount of material extracted by water from ether and alcohol extracted grass No. 1 (as % grass dry matter)

Temperature (°C)	Non-ionic	Ionic	Total
4	7.8	8.5	16.3
60	7.6	10.4	18.0
100	8.8	11.2	20.0

It will be seen that the principal changes in the amount of extract obtained at 4 or 60°C were the result of changes in the quantity of ionic compounds. Between 60° and 100°C the further increase was caused mainly by an increase in the quantity of non-ionic material. This suggested that some of the mineral matter in the grass was more difficult to extract at 4°C, possibly owing to the effect of temperature on the solubility of the salts and that at 100°C some degradation of normally unextractable materials was possibly taking place.

When the ether extracted residue (50 g.) of

Grass No. 1 was extracted with 90% alcohol (1 litre) at 20°C for 18 hr. with only occasional shaking, the extraction was not complete and there was subsequently a larger quantity of material extracted by water at 60°C than was found previously (p.62). This increase was mainly in the amount of the ionic fraction representing the material that was not extracted by alcohol (Table 8).

Table 8. The effect of type of alcohol extraction on the subsequent water extraction of Grass No. 1
(as % original grass dry matter)

	Ethanol extract	Water extract	Non-ionic material in water extract	Ionic material in water extract
Extraction with insufficient ethanol	10.2	18.0	7.6	10.4
Extraction with sufficient ethanol	13.3	13.5	7.8	5.7

The non-ionic material of the water extract could be accounted for as carbohydrates, protein, pigments and various other substances present in small amounts. These last substances have not been identified. The

ionic fraction included mineral salts, organic acids, non-protein nitrogen compounds and possibly protein: the protein appeared to have been absorbed on the resins. The water extract examined in detail below was obtained from the water-soluble fraction of the material removed by ethanol-benzene plus the water extract of the ethanol-benzene extracted grass residue. The water extract thus obtained will be referred to as the complete water extract.

Carbohydrates in the complete water extract

Fructosan, sucrose, glucose and fructose were the chief carbohydrates present in the complete water extract of the grass, but during earlier work concerned with the isolation and analysis of fructosan from New Zealand rye-grass it was discovered that a small quantity of a second polysaccharide was present in the material precipitated by alcohol from the water extract. Since this polysaccharide was not hydrolysed to alcohol-soluble units by N/100 acid at 100°C for ½ hr., conditions which cause complete hydrolysis of fructosan, the two substances were easily separated.

The second polysaccharide was hydrolysed to monosaccharides by heating with N sulphuric acid at 100°C for 2 hr. and the hydrolysate contained galactose, glucose, arabinose and xylose in the approximate ratios of 3:2:3:1. A similar galacto-araban was found in the complete water extract from Grass No. 1 and when determined by quantitative paper chromatography, it was found to represent 0.8% of the dry matter of the grass.

The ammonium oxalate extract

The residue of the grass after the successive extractions either with ether, alcohol and water or with ethanol-benzene and water was expected to represent the structural materials of the plant tissue, together with any other compounds that might have been rendered insoluble by the action of any of the previous treatments. Included in the residue is pectin which it was at first thought could be extracted without serious degradation of either itself or the remainder of the material. It was later learned (124) that some slight extraction of hemicelluloses might also be expected.

The laboratory reagent which has been used most often for the extraction of pectin (considering the whole pectic complex) from plant tissue is hot aqueous ammonium oxalate. There is said to be a disadvantage in the use of this substance in that the grass fibre retains some ammonia which cannot be removed easily (125). Other extractants were therefore considered, but dilute acids (126) are not suitable since they hydrolyse polysaccharides and alkaline solutions (127) usually extract polysaccharides. There are some reagents used in special circumstances for the extraction of pectin such as ethylene diamine tetra-acetic acid (128) but this requires alkaline conditions whereas the various polyphosphates (129) are most effective in acid solutions. None of these reagents was therefore thought to be satisfactory and the reported fault in the use of ammonium oxalate was then examined.

No evidence of the reported disadvantage of ammonium oxalate could be found provided reasonable washing of the residue with nearly boiling water was employed. This was shown experimentally as follows:-

Three samples (each being 4.00 g.) of water extracted grass residue (being 63.1% of the original dry matter of Grass No. 1) were heated under reflux in 200 ml. 0.5% aqueous ammonium oxalate for 24 hr. in a water bath at 80-85°C. The combined mixture was filtered and the residue washed with hot water (80°C) till the washings were colourless. The residue was then dried by washing with acetone and then ether and finally in the oven at 100°C for 1 hr.

The filtrate and washings were together made to 1 litre. The total solids in the extract were determined by the evaporation of 100 ml. portions under reduced pressure, the solids being finally dried for 3 hr. at 100°C. Most of this material was ammonium oxalate and by this treatment it was weighed as the anhydrous salt the nitrogen of which was estimated in solution by measurement of the ammonia distilled from the solution after making it alkaline.

The total nitrogen initially in the water extracted grass, and in the oxalate extracted residue was determined using the macro-Kjeldahl procedure and that in the extract by the micro-Kjeldahl procedure.

The results are shown in Table 9.

Table 9 The extraction of water-extracted Grass No. 1 with ammonium oxalate solution and the examination of the products for retention of ammonia

		Material expressed as % of original grass dry matter
Dry matter of water-extracted grass	10.63 g.	63.1
Total solids of extract solution	4.95 g.	
Ammonium oxalate in extract solution	4.21 g.	
Weight of material extracted by oxalate	0.74 g.	4.4
Dry matter of oxalate-extracted residue	9.78 g.	58.0
Total recovery	10.52 g.	98.9% of water extracted grass
Total nitrogen in extract solution	0.967 g.	
Ammonia nitrogen in extract solution	0.953 g.	
Protein nitrogen in extract solution (a)	0.014 g.	
Total nitrogen in oxalate-extracted residue (b)	0.306 g.	
Total of (a) and (b)	0.320 g.	
Total nitrogen in water-extracted grass	0.322 g.	

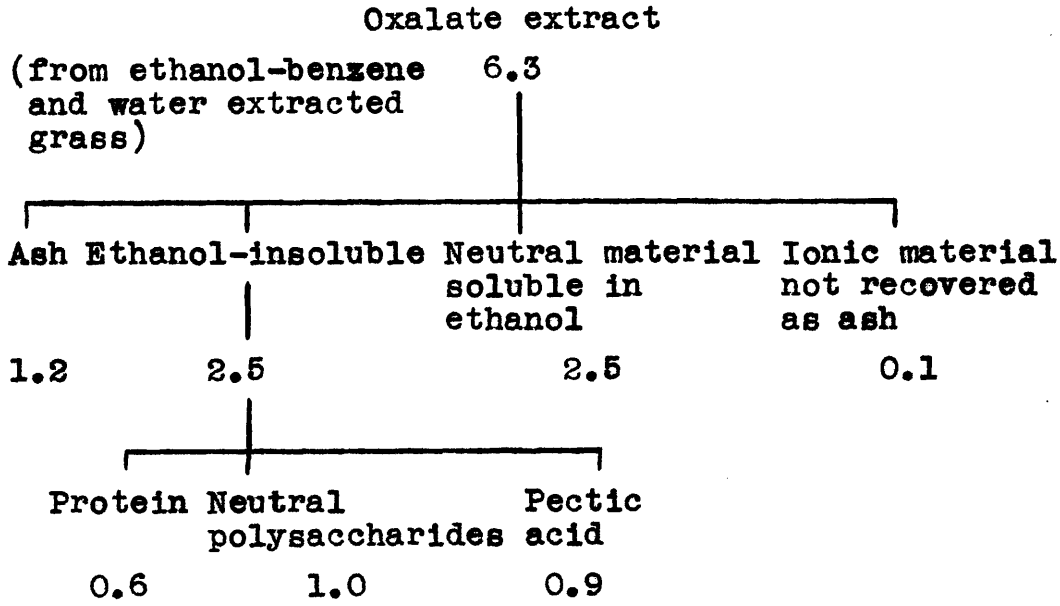
The quantity of crude pectin extracted by the ammonium oxalate solution was determined by precipitation from a concentrated solution with $3\frac{1}{2}$ volumes of 70% ethanol containing sufficient hydrochloric acid to make the final mixture decinormal. The precipitate was filtered through a Gooch crucible and washed first with ethanol containing hydrochloric acid and then with neutral ethanol. The weight of crude pectin was calculated by the loss in weight on heating the crucible and precipitate at 550°C for 3 hr. In this determination it was important that acid should be present in sufficient amount to prevent precipitation of the ammonium oxalate. The amounts of pectin recovered from grass extracts have been small and have not allowed its determination as calcium pectate.

The crude pectin thus isolated was expected to contain protein and this was determined by micro-Kjeldahl technique. The neutral polysaccharides were determined by paper chromatography on a sample hydrolysed for 4 hr. at 100°C with N sulphuric acid. The quantity of presumed pectic acid could then be calculated. The whole of the protein which was

calculated to be in the extract was recovered in the crude pectin. The acid alcoholic filtrate from the precipitation of crude pectin was deionised with ion exchange resins and the total solids of the deionised solution determined. A chromatographic examination of the deionised solution indicated the presence of at least four compounds which had mobilities similar to free hexoses and pentoses. There were however no free sugars present but each of the compounds detected reduced ammoniacal silver nitrate and contained groupings which reacted with sodium metaperiodate. None of the compounds was mannitol and by their ease of resolution from authentic mannitol it seemed unlikely that any of them were hexitols. The compounds were not further investigated.

The composition of this ammonium oxalate extract which had been examined in greater detail than before (p. 71) was as shown in Table 10.

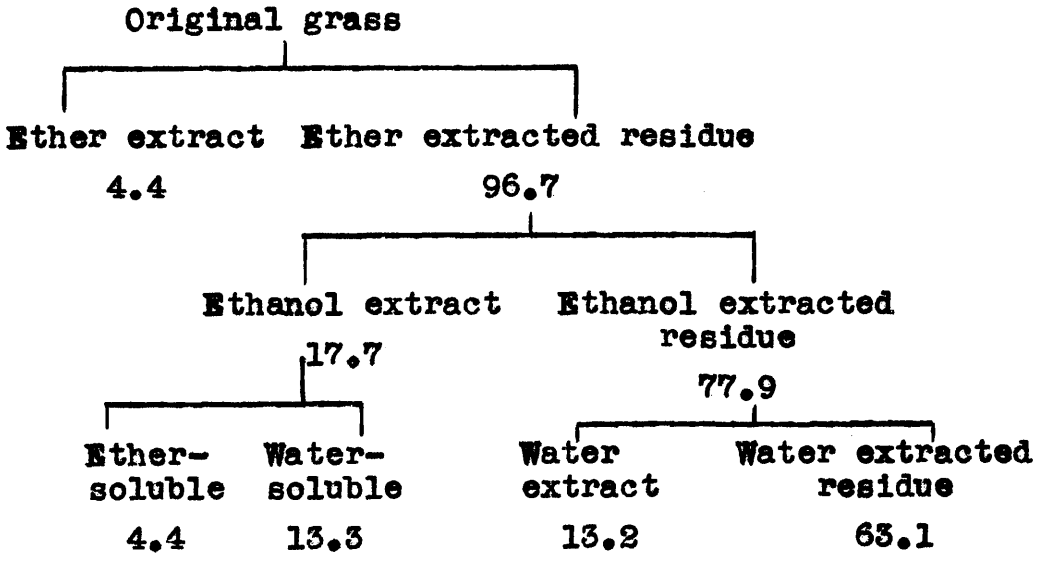
Table 10 The composition of the material extracted from Grass No. 1 by ammonium oxalate solution
(as % of the original grass dry matter)



Summary of the analyses of grass extraction

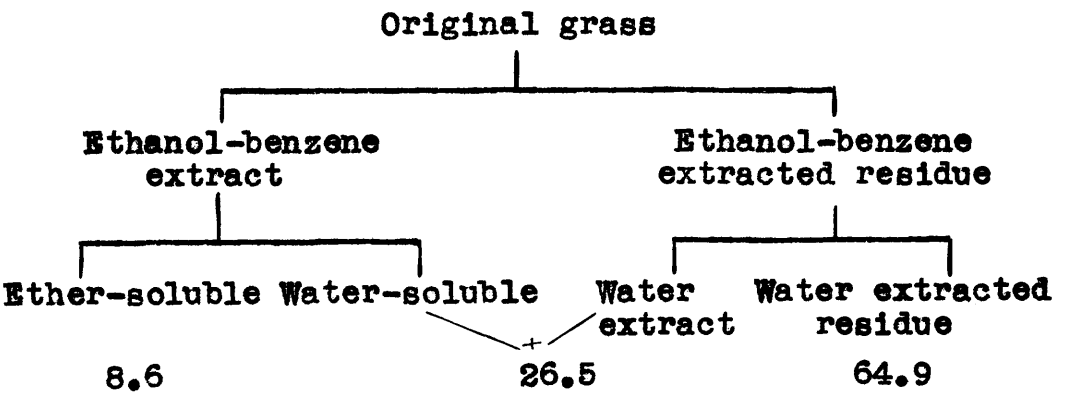
In the work so far reported and which has been concerned only with the results of various simple extractions applied to one sample of grass - a sample of cocksfoot - a fairly good recovery has been achieved at each stage. To start with the dried grass was extracted successively with ether, 90% ethanol and warm water. The amounts of the extracts and the grass residues were as shown in Table 11.

Table 11 The results of the successive extractions of Grass No. 1 with ether, alcohol and water
(as % original grass dry matter)



It was then shown that a very similar final result could be obtained by successive extractions with ethanol-benzene and water as in Table 12.

Table 12 The results of the successive extractions of Grass No. 1 with ethanol-benzene and water
(as % original grass dry matter)



Ammonium oxalate extraction of the water-extracted residue in Table 11, removed 4.4% of the original grass dry matter, leaving a residue which was 58.0% of the grass. Similar extraction of the residue in Table 12 yielded an extract which was 63% of the original grass. The difference between these values of the amount of extract may have been due principally to cummulative errors in recovering the various residues but may also have arisen by a slightly less efficient extraction having been achieved during the operations shown in Table 12. The final grass residue after ammonium oxalate extractions was 58.0% of the original grass for the sample referred to in

Table 11 and was probably slightly less than 58.6% for the sample in Table 12, so that by the end of the extractions there was very little difference between the two methods in percentage of residue obtained.

For the examination of other grass samples to be described in the next section the use of successive extractions with ethanol-benzene, water and ammonium oxalate was adopted and the residue from these extractions is termed grass fibre. This fibre contained most of the protein originally present and was therefore not the same as the crude fibre of the Weende system of analysis.

(ii) THE APPLICATION OF THE METHODS OF ANALYSIS OF THE EXTRACTS TO OTHER GRASS SAMPLES

The preceding results indicated a possible system of extractions of dried grass but left certain of the compounds undetermined and others not fully identified although determined. In an endeavour to clarify these points further experiments were made using the grass

used so far (No. 1 cocksfoot) and two ryegrasses (No. 2 and 3 p.53).

The grass samples were extracted successively with (a) the azeotropic mixture of ethanol and benzene in a Soxhlet apparatus for 24 hr., (b) water at 60°C for ½ hr., and (c) 0.5% ammonium oxalate solution in a water bath at 85°C for 24 hr.

(a) The total ethanol-benzene extract was evaporated and partitioned between ether and water. The water-soluble fraction was combined with the main water extract (b). A portion of the ether-soluble fraction was evaporated to give the amount of total solids.

(b) The combined water extracts were analysed for total dry matter content, ash, total nitrogen and non-protein nitrogen. A portion was deionised by treatment with ion-exchange resins (Amberlite IR 120 and Deacidite E), the passage of the solution over the cation exchanger being rapid to ensure that the fructose-containing carbohydrates were not hydrolysed. Dry matter, total nitrogen and non-protein nitrogen were re-determined on the deionised solution.

Two portions of the deionised solutions were treated with 10 volumes of ethanol to precipitate the alcohol-insoluble materials.

The alcohol filtrate was evaporated and the residue taken up in water. Total dry matter, total nitrogen and non-protein nitrogen were determined on portions of this solution. The material precipitated by monobasic lead acetate was also estimated - the total weight of precipitate corrected for its lead content was determined. The filtrate from this precipitate was deionised and evaporated to estimate its total solids content.

The total alcohol-insoluble product of one portion

was collected in a Gooch crucible and the amount determined by ashing. The other portion in which the alcohol-insoluble material had been precipitated was used for the determination of araban. The precipitate was filtered through a thin layer of celite filter aid on Whatman No. 52 paper and the mixture, which contained fructosan and araban as well as the celite, was heated in N/100 sulphuric acid for $\frac{1}{2}$ hr. at 100°C to hydrolyse the fructosan. The hydrolysate was treated with 10 volumes of alcohol and the precipitate filtered as before. The recovered material was well washed with alcohol, then heated with N sulphuric acid for $2\frac{1}{2}$ hr. at 100°C to hydrolyse the araban. The hydrolysate was filtered through a Gooch crucible to remove celite and any other material such as unhydrolysed protein. After washing with water the residual material was dried out and the amount determined by ashing. The quantity of the araban hydrolysis products was determined in the acid filtrate by paper chromatographic methods.

It was decided that it would be correct to include both the ash and organic acid values in the summation of the known ionic constituents of the complete water extract for their comparison with the total material retained on the ion exchange resins. The acids would presumably be present in the grass as a mixture of the free, partly neutralised and fully neutralised compounds. When ashed, the products would be metallic carbonates from neutralised acid - free acids having been completely removed. A calculation of the result of ashing an equimolecular mixture of the three forms of typical acids indicated only a slight excess recovery if the original mixture

was calculated as ash + organic acids. A slight decrease in weight occurs when phosphates are ashed (conversion to pyrophosphate) and this would tend to compensate for the small excess in recovery from organic acids.

(c) The ammonium oxalate extract was analysed for total solids content, ammonia nitrogen and ash. The crude pectin precipitated from the extract and the alcoholic filtrate from this precipitation were analysed as previously described (p. 73).

The grass fibre which was the residue of the extraction described above was dried with acetone and ether and then after 1 hr. at 100°C in an oven it was weighed. The dry matter was determined on a small sample of the fibre.

The results of these methods of analysis when applied to the three different grasses Nos. 1, 2 and 3 are shown in Tables 13-19. (below and pp. 84-89 ~~49-51~~).

Table 13. The amount of material extracted from three different grasses* by successive treatments (as % of the original grass)

	Grass 1	Grass 2	Grass 3
Ether-soluble	6.3	1.9	3.9
Water-soluble	26.3	15.1	47.3
Ammonium oxalate soluble	6.3	5.6	4.4
Residue - Grass fibre	60.7	76.2	45.1
Total recovered	99.6	98.8	100.7

* See p. 53 for a brief description of these grasses.

The recovery of the dry matter of the water and oxalate extracts of the three grasses varied considerably. With Grass No. 1, in the water extract only 0.6% of the original grass was not accounted for whilst a further 1.0% which was retained on celite after alcohol precipitation but not removed during acid hydrolysis of polysaccharides, was not identified. In the water extract of Grass No. 2 the actual amount of material retained on the celite after hydrolysis of the polysaccharides was not determined but the sum of the material which was either not accounted for or not identified was 5.4% of the original grass.

When analysing the water extract of Grass No. 3 there was 6.7% of the grass not accounted for and 1.0% of the original grass retained in the celite.

The large amount of unknown substances in the water extract of Grass No. 2 is particularly important since it represents over 30% of the extract. The fact that this was a very mature grass with a large lignin content leads to the suggestion that part at least of the unknown material might be a lignin derivative. This is referred to more fully in the discussion on

p. 151 .

The recovery of material in each of the oxalate extracts was complete within the experimental limitations but there was 0.9 - 2.5% of the original grass present as unknown neutral substances which were probably polyhydroxy compounds.

Table 14 The water-soluble extract of Grass No. 1
(as % of the original grass)

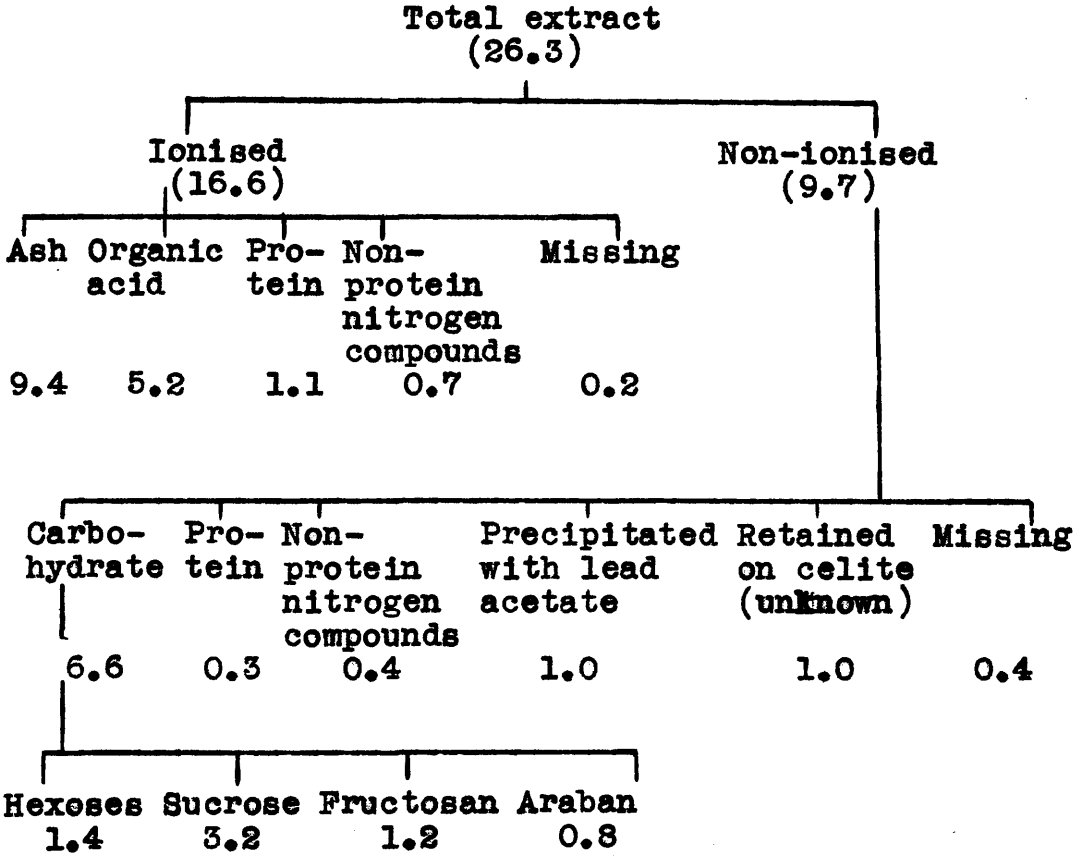


Table 15 The ammonium oxalate extract of Grass No. 1
(as % of the original grass)

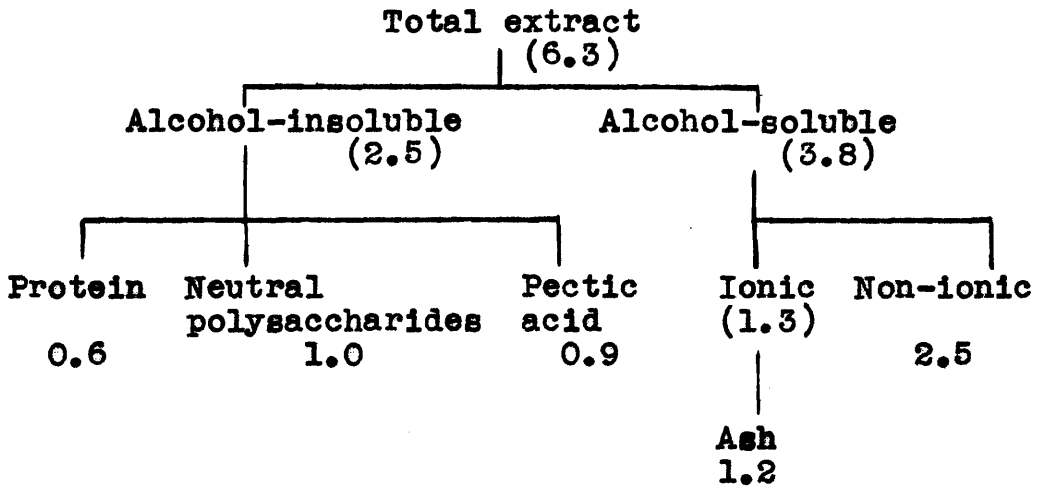


Table 16 The water-soluble extract of Grass No. 2
(as % of the original grass)

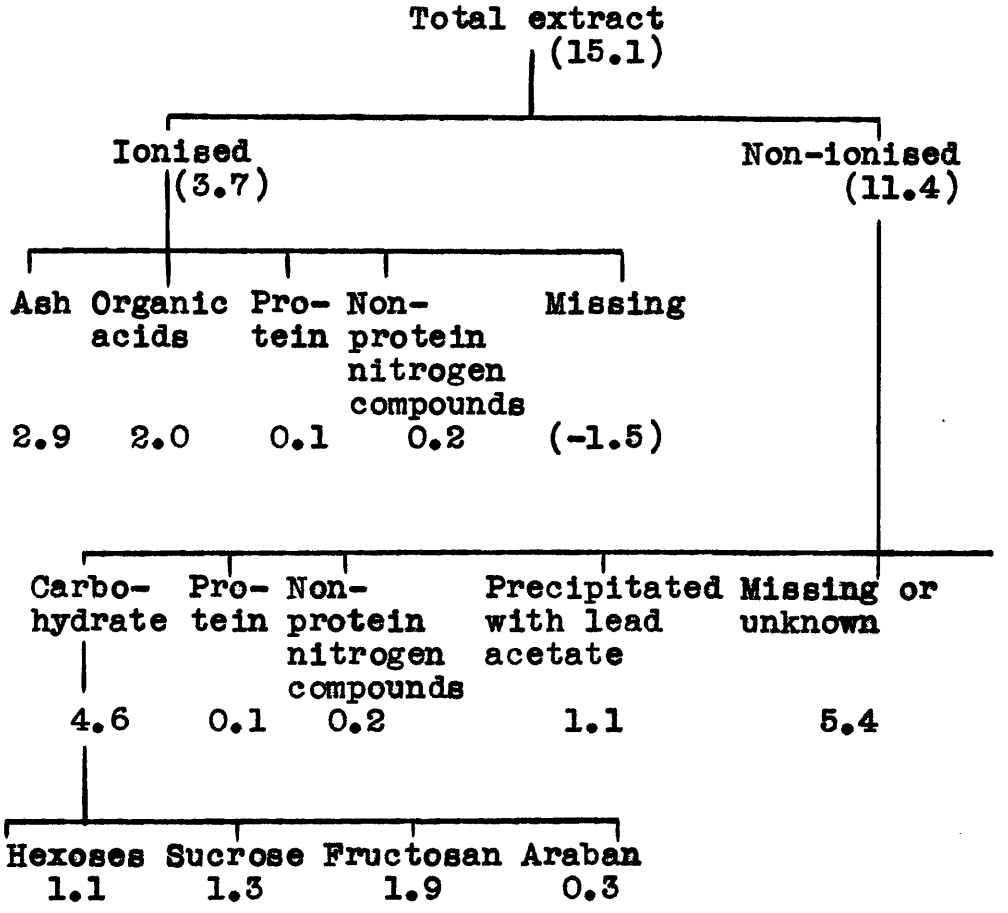


Table 17 The ammonium oxalate extract of Grass No. 2 (as % of the original grass)

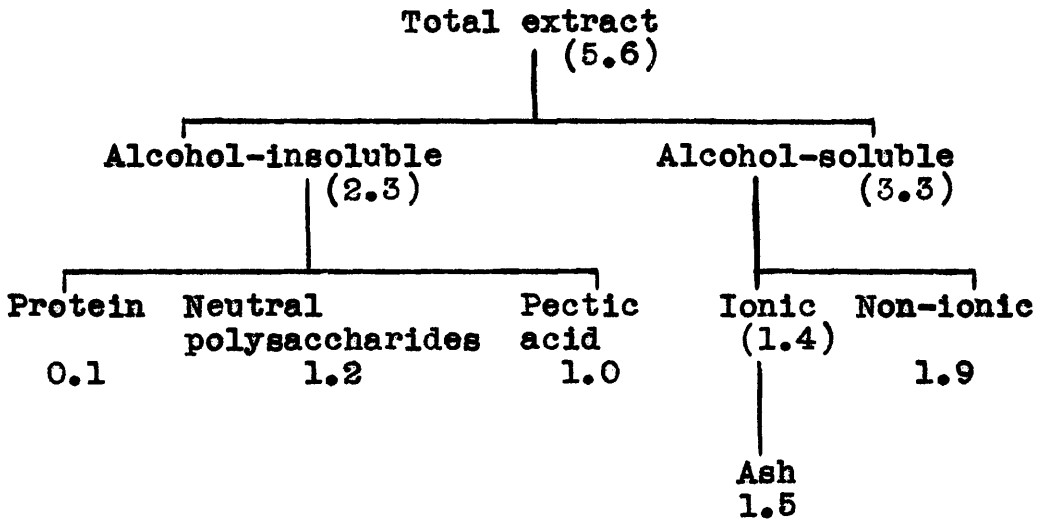


Table 18 The water soluble extract of Grass No. 3
(as % of the original grass)

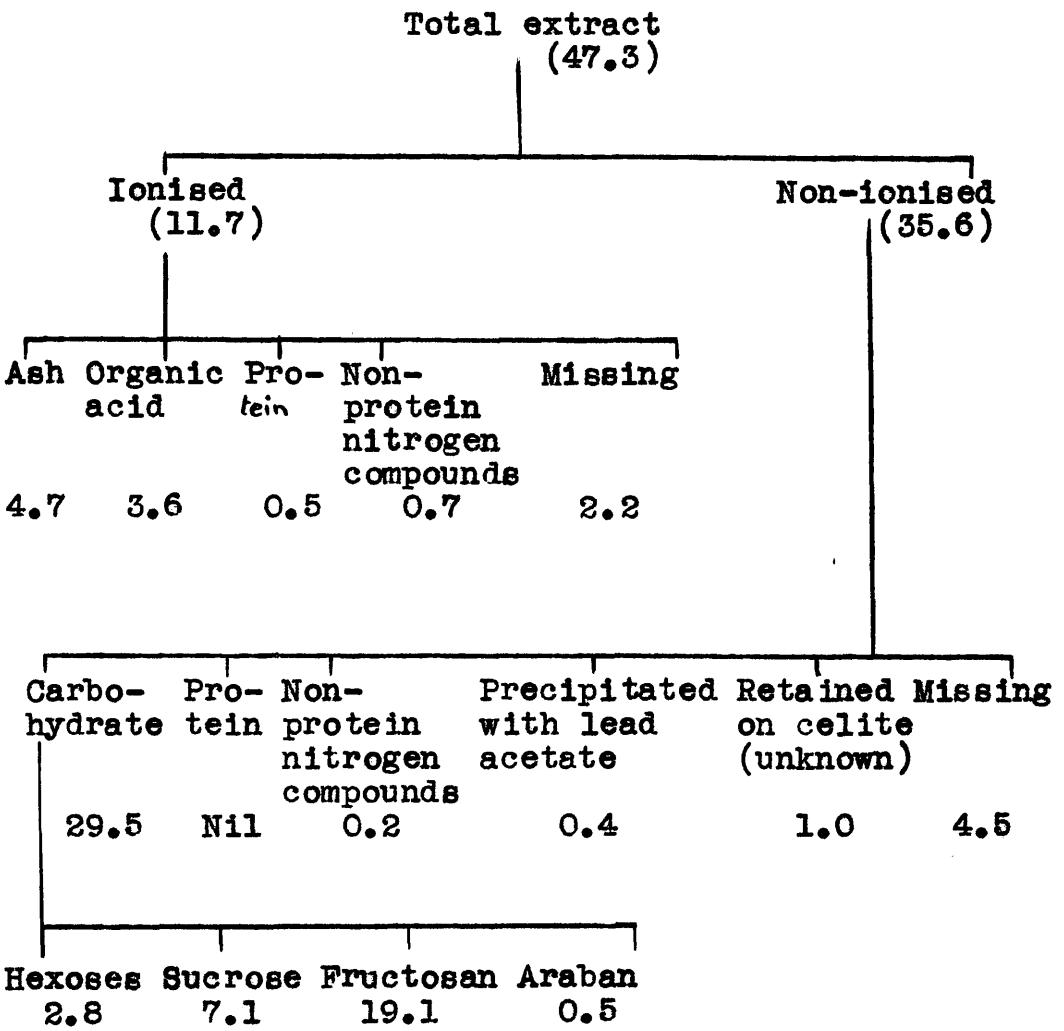
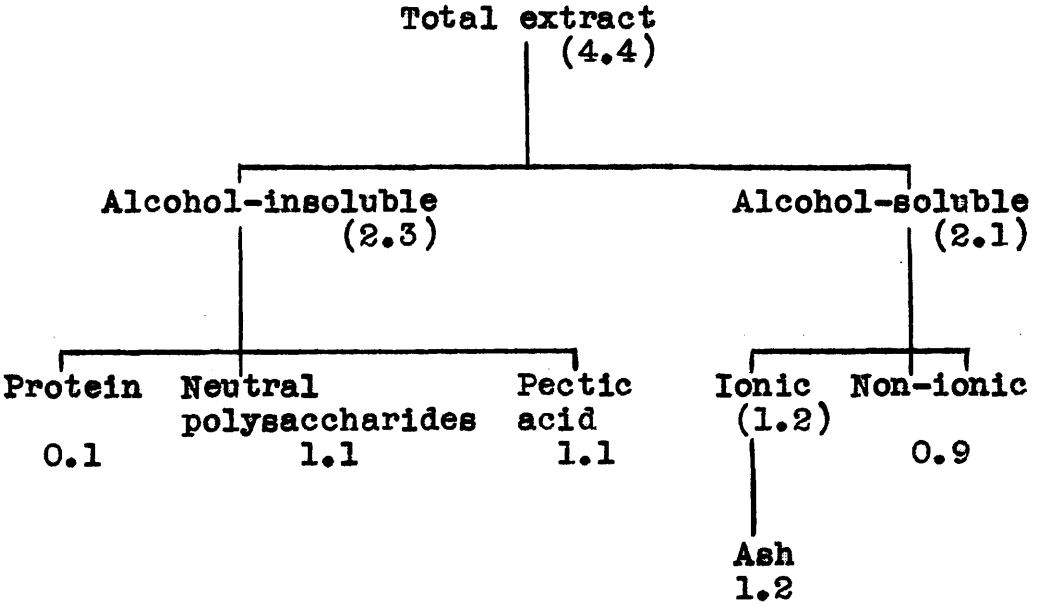


Table 19 The ammonium oxalate extract of Grass No. 3
(as % of the original grass)



SECTION B

THE ANALYSIS OF GRASS FIBRE

(1) The components of grass fibre

It must be emphasised that the term "grass fibre" used here and throughout the remainder of this thesis represents the grass residue produced by successive extraction of the original grass with ethanol-benzene, water and ammonium oxalate solution and that this grass fibre differs from the material usually called "crude fibre" chiefly by still possessing most of the protein of the original grass.

The botanical nature of grass fibre

So far as can be judged from general considerations of dried plant material the grass fibre isolated after treatment of the dried grass with only the neutral reagents referred to above consists principally of the original cell walls of the plant and in addition most of the protein of the cell protoplasm. Any materials unextracted by the various reagents, such as insoluble salts would also be present.

The development of the cell wall of land plants

begins during mitosis by the production of a very thin membrane. This membrane forms a middle lamella, on either side of which the primary cell wall is laid down. In mature tissue the middle lamella is mainly lignin (130) though in young tissue it contains a large proportion of pectin and protein. The primary cell wall has a plastic nature and is easily adaptable to the increasing size of the growing cell. It contains cellulose and pectic materials as well as other polysaccharides. The cell wall thickens during development until a secondary cell wall is formed. This is a much more rigid structure and its presence prevents any further change in the shape of the cell. The secondary cell wall is very strong and is laid down inside the primary cell wall in the form of a number of concentric layers. These layers contain cellulose, other polysaccharides and lignin.

The main content of the very young plant cells is protoplasm, which is, of course, rich in protein. As the cell enlarges the protoplasm is insufficient to fill the cell and splits into fibrils linking the cell nucleus with the cell walls. The spaces so left, the

vacuoles, contain water-soluble materials. In old cells much of the protoplasmic protein is deposited as a film on the secondary cell wall.

The grass fibre as isolated by successive extractions of the dried grass with ethanol and benzene, water and ammonium oxalate solution thus consisted of the primary and secondary cell walls, the adherant protein, the mineral constituents of the vacuole contents and part of the middle lamella. It was not expected that much of the pectin of the middle lamella would be present, since the pectin would be extracted by the ammonium oxalate.

The fibre has therefore been divided arbitrarily into the following constituents, ash (minerals), crude protein, lignin and polysaccharides and of these only the polysaccharides in the grass holocellulose will be considered in detail. Using a grass fibre preparation which represented 62.5% of the original dry matter of Grass No. 1 the following examinations were made.

The ash

The ash content of grass fibre No. 1 was determined at 550°C as previously described (p. 54) and

amounted to 1.4% of the original grass. It presumably contained any silica that contaminated the original grass or that was in the grass. Some calcium oxalate would be present, having been formed by the action of ammonium oxalate during the extraction of pectic acid from any calcium pectate in the grass. Other insoluble salts may have been in the grass originally and would not have been previously removed.

The crude protein

Most of the true protein of grass was not extracted by the various mild reagents used in the isolation of grass fibre but the ethanol-benzene and water extractions removed the non-protein nitrogenous substances. For the examination of the other substances in the fibre, especially the polysaccharides, it is desirable to remove the protein since it can cause artifacts on paper chromatograms used for determining sugars. The most suitable method for this seemed to be by enzymic digestion as developed for lignin estimations (61, 131, 132).

Two enzyme preparations are readily available which may be used for this purpose - trypsin and pepsin.

Trypsin however requires alkaline conditions (133) and these would almost certainly cause some extraction of the polysaccharides. Pepsin is normally used in dilute acid solution and even if used at its apparent optimum temperature of 40°C (117, 132) it would not be expected that the polysaccharides would be much affected provided the time of reaction was not unduly prolonged.

Accordingly the action of pepsin on cocksfoot grass fibre was examined at three different temperatures using a reaction time of 18 hr.

To each of six 1 g. samples of grass fibre No. 1 40 ml. pepsin solution [0.5% B.D.H. pepsin (1:2500) in 0.1N hydrochloric acid] were added and the mixtures, in lightly stoppered flasks, incubated in pairs at 20°, 37° and 46°C for 18 hr. A blank determination was made using the grass fibre in 0.1N hydrochloric acid only and incubating at 20°C for 18 hr.

After the digestion period the mixture was filtered and the grass residues washed with water until the washings were neutral. The nitrogen contents of the residues were then determined and expressed as crude protein in terms of the original grass. The results are shown in Table 20.

Fig. 6 The Action of "0·5 % Pepsin" in N/10 Hydrochloric Acid at 46°C on Cocksfoot Grass Fibre.

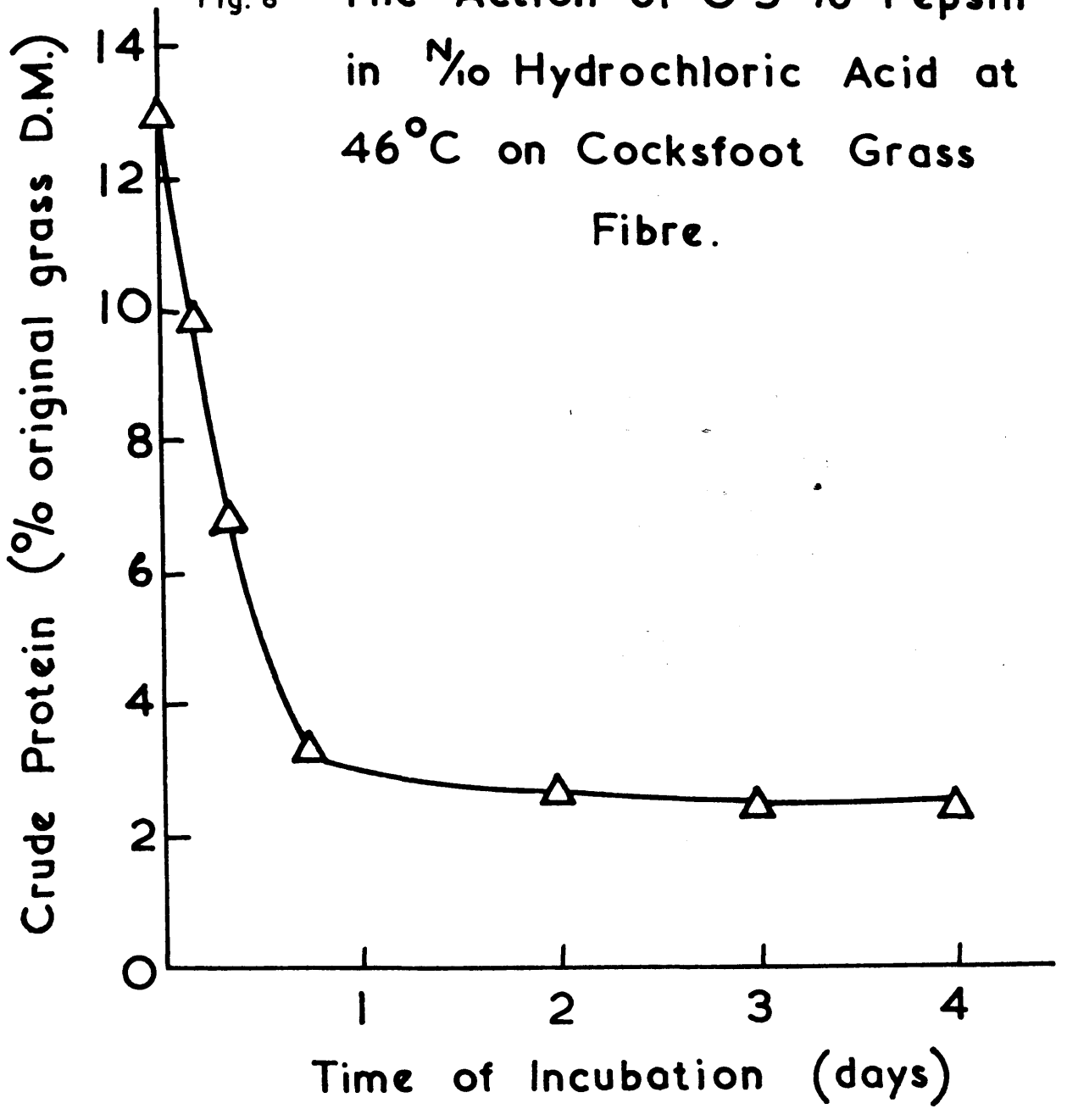


Table 20. The removal of crude protein from grass ~~protein~~ fibre by the action of pepsin

Treatment		Residual crude protein (as % grass)
Original grass fibre		13.0
N/10 hydrochloric acid at 20°C		13.0
Pepsin at 20°C	all in N/10 HCl	7.1
Pepsin at 37°C		5.0
Pepsin at 46°C		3.3

Although most crude protein was removed at 46°C it was expected that above this temperature the hydrolytic action of the acid on the polysaccharides would become significant and that rapid inactivation of the enzyme might take place.

The rate at which the crude protein was hydrolysed to peptides at 46°C by the pepsin solution was then investigated. The results are given in Fig. 6. They showed that a rapid dissolution of protein occurred during the first 18 hr. after which the reaction was much slower. This slower rate may have been caused in part by the exhaustion or inactivation of the enzyme.

For digestion periods of less than 24 hr. only traces of carbohydrates appeared in the extracts examined by paper chromatography. After 2 and 4 days the digests contained alcohol-insoluble polysaccharides, which when hydrolysed and examined (2N H_2SO_4 for 6 hr. at 100°) by paper chromatography appeared to contain galactose, glucose, arabinose, xylose and uronic acid. When more finely milled samples of grass were studied later, polysaccharide losses equal to 1% of the grass were common from grasses with a high protein content.

No carbohydrase activity was discovered in the pepsin used when tested against cellobiose, maltose, lactose, raffinose, starch, the water-soluble galacto-araban isolated in Part I A, a crude hemicellulose preparation obtained by extraction of grass fibre with dilute alkali, or the phenolic glycoside phloridzin.

The treatment therefore adopted was to incubate 1 g. grass fibre with 40 ml. pepsin solution (1% of 1:2500 in 0.1 N. hydrochloric acid) at 46°C for 18 hr.

Lignin and polysaccharides

Lignin apparently consists of polymeric aromatic substances, but despite the considerable amount of work that has been done on the subject it has never been

shown that any lignin preparation consists of a single compound. It is not therefore surprising that the structure of lignin is still unknown. Some of the structural units have, however, been determined, the main grouping for instance is the phenylpropane nucleus (134); lignin preparations obtained under mild chemical conditions are very labile, being readily changed to resinous materials, whereas stable lignin products are only obtained by methods that cause degradation before isolation (134)

For many years the methods available for the estimation of lignin have been the subject of arguments and recently a comprehensive review of the possible sources of error in the determination, using 72% sulphuric acid, was presented by Moon and Abou-Raya (117). Essentially, by the method used here, lignin is that part of the cell wall which is insoluble in 72% sulphuric acid. To isolate such a fraction satisfactorily it is necessary that no other substances are precipitated at the same time and that no lignin is dissolved by any of the treatments. It is on such aspects that most of the investigations of the lignin method have been done.

The method of determination of lignin in this work was that of Ellis, Matrone and Maynard (64), but the fibre used here as the starting material had received more extraction than was specified in their method. The quantity of lignin in the cocksfoot grass fibre was 6.2% grass.

The total polysaccharides of grass fibre can be isolated only after the substantial removal of lignin, and hence both these fractions are considered in this section. The removal of lignin from grass fibre will be discussed in the next section.

In the gross analysis of grass fibre in the present work the polysaccharides were not estimated directly but were calculated by difference, knowing the quantities of the ash, crude protein and lignin in the fibre as in the following example (Table 21) which shows the composition of the fibre of grass No. 1.

Table 21. The composition of the fibre of Grass No. 1 (as % original grass dry matter)

Ash	Crude protein	Lignin	Polysaccharides (by diff.)	Total grass fibre
1.4	13.0	6.2	41.9	62.5

(ii) Removal of lignin

For the removal of lignin from grass fibre numerous techniques have been developed ~~for the removal of lignin,~~ such as treatment with alcoholic potassium hydroxide (135) or with ethanolamine (136) but the methods that have proved most satisfactory involve treatment of the material with chlorine derivatives.

In many of these methods removal of protein is achieved at the same time, but it is often preferable to deproteinise the fibre first, as with pepsin, because of the better removal of lignin that is then possible. No account has been published of the complete removal of lignin and protein without loss of polysaccharides and it is now considered impossible to obtain a lignin-free product without some loss of polysaccharides. However quantitative and qualitative investigations of the crude polysaccharides have given valuable information.

The production of these partially delignigied materials, known as holocelluloses has allowed the recovery of practically the whole of the polysaccharides from certain materials. There was,

however, frequently contamination with ash, protein, lignin and other compounds. Sometimes these impurities have not been considered by the authors when the total recovery of the original polysaccharides has been claimed. The carbohydrate material in many of these products has commonly been referred to as "cellulose" although in fact it was a mixture of cellulose and other polysaccharides.

For a long time the classical method of preparing delignified fibre was that of Cross and Bevan (62) published in 1889 and used particularly in the paper industry. In this procedure, the fibre was boiled in alkali and then treated with free chlorine. The lignin degradation products were washed out with alkaline sodium sulphite and after other necessary washings the product was weighed.

A very much more convenient development of this method was introduced in 1933 by Norman and Jenkins (60) who used an acid solution of a hypochlorite as the source of chlorine. The amount of delignified product obtained by the Norman and Jenkins method agreed very closely with that resulting from the Cross and Bevan

procedure if the initial alkaline boiling was omitted from the latter. Both products were believed to represent all the "natural cellulose" except for the "encrusting polyuronides".

Starting in the normal way from whole grass the nature of the material isolated from a sample of young rye grass (No. 4) by the Norman and Jenkins technique, has been examined. Table 22 gives the composition of the cellulose isolated by this method and also that of the grass fibre prepared from the same grass.

Table 22. The composition of grass fibre and cellulose prepared by the Norman and Jenkins method from Grass No. 4
(as % original grass dry matter)

Material	Ash	Crude protein	Lignin	Polysaccharides	Total
Grass fibre	0.6	19.4	4.7	30.4	55.1
Norman-Jenkins cellulose	0.9	0.4	1.7	24.3	27.3

These results show that there was effective removal of protein and considerable displacement of lignin but at the expense of a significant loss of polysaccharides. The Norman and Jenkins method is therefore too severe

to be used for the preparation of delignified grass fibre if all the polysaccharides are required.

The preparation of holocellulose

The development of methods for the production of holocellulose was a great advance in polysaccharide chemistry, for ideally holocellulose is the entire group of cell wall polysaccharides without any contaminating substances. In practice, the products prepared contain small amounts of non-carbohydrate material, a compromise which has so far been found essential if a complete recovery of polysaccharides is desired.

The first satisfactory isolation of holocellulose was reported in 1931 when Schmidt and his co-workers published a method (137) based on a technique previously used (71) for the isolation of cellulose. The preparation of the holocellulose took 3-4 weeks and entailed treatment of the fibre with chlorine dioxide in pyridine followed by treatment with water. The original name applied to the product was "Skelettsubstanzen" which aptly described a carbohydrate structure of such fundamental importance.

A later method for the preparation of holocellulose

was devised by Kurth and Ritter (138) and as finally modified (139) consisted of successive treatments of the fibre with water, chlorine, ethanol and alcoholic ethanolamine, the cycle of operations being repeated till no colour was produced by the ethanolamine reagents, indicating complete removal of lignin. This procedure was applied to wood products, which had a very low nitrogen content and it was found to cause an increase in the nitrogen content of the product (140, 141). In other respects it was an improvement over Schmidt's method, for the reagents were less hazardous and the time required was a matter of hours rather than days.

The most convenient process now available for the production of holocellulose had its inception in a technique by Jayme (72) for the delignification of microscopic sections of plant tissues. The method was adapted by Wise and co-workers (142, 143) who used it to make bulk preparations from extractive-free wood using sodium chlorite in dilute acetic acid at 75°C as an <u>in situ</u> source of chlorine dioxide. The treatment was continued for 1 hr. and repeated three times, using, in all, a weight of sodium chlorite equal to the weight

of wood. For more porous fibres such as those from cereals and grasses, the total time of reaction may be reduced to 1 hr. adding one quarter of the reagents every 15 min. (73). It has been shown (144, 145) however that this chlorite technique allows large amounts of protein to be retained when applied to materials like grass.

The value of the chlorite method for the preparation of holocellulose lies not only in the convenience of the procedure but also in the very slight degree of structural degradation suffered by the polysaccharides (143).

The recovery of polysaccharides after treatment with sodium chlorite has rarely been perfect but the losses are usually very small. Holmberg and Jahn (146, 147) showed that a progressive loss of pentosan occurred during the reaction especially when the lignin content was reduced too far. Even with mild reaction conditions Bublitz (148) found polysaccharides in the reaction liquor, however Harwood (149) stated that the loss of carbohydrates from the holocellulose was less if the 1 hr. method (73) was used instead of the method of Wise (141, 142) which required a longer reaction

time.

It had been claimed by Jahn (72) that complete recovery of polysaccharides was achieved during the chloriting prodedure if the residue was allowed to contain a small amount of lignin but later Jayme, Eser and Hanke (150, 151) showed the presence of polysaccharides in the reaction liquor. This led to their postulation of the "excess polysaccharides" hypothesis since it was thought that all the original polysaccharides were in the holocellulose product. The apparent excess of polysaccharides was found to pass through a maximum value when the ratio of chlorite to wood was changed (152, 153). The excess material was later shown to be not polysaccharide but lignin products (153). These results were corroborated by Wise and his colleagues (143) who achieved a complete recovery of polysaccharides in the preparation of holocellulose from softwoods, but when working with hardwoods they noticed a slight loss of carbohydrates. They demonstrated in the chlorite holocellulose the presence of alcohol-soluble material which gave rise to errors in the lignin estimation on the product if the estimation was applied directly. If the lignin analysis

was done after treatment of the holocellulose with alcohol, satisfactory summations were obtained, although these were usually slightly high, (101 - 102%). It is interesting to note that the alcohol-soluble material in the holocellulose gave the same colour reactions for lignin as did similar material from grass which had not been delignified (117). The substances extracted from chlorite holocellulose with alcohol were considered to be lignin degradation products which could not be recovered as insoluble products during the normal lignin estimation. Herbst (154) found neither normal lignin nor any alcohol-soluble lignous material in holocellulose prepared from wood by a prolonged chlorite method.

Soluble lignin

It was found, particularly in examining the holocellulose samples reported in Part II of this thesis, that satisfactory agreement was possible between the amount of polysaccharides in the holocellulose calculated by difference (and hence including all analytical errors) and the quantity of polysaccharides calculated from the chromatographic analyses (to be discussed later) when samples originating from fibres

with a low lignin content were examined. In samples of older grasses having a high lignin content there was an appreciable difference which was thought to be caused by errors in the lignin estimation. This seemed especially probable in view of the reports mentioned above of "soluble lignin" formation during the treatment of lignified tissues with sodium chlorite. Accordingly the lignin determination employed (64) was examined, using three grasses in which the polysaccharides found were less than, similar to and more than those calculated by difference. The results for these samples are given in Table 23.

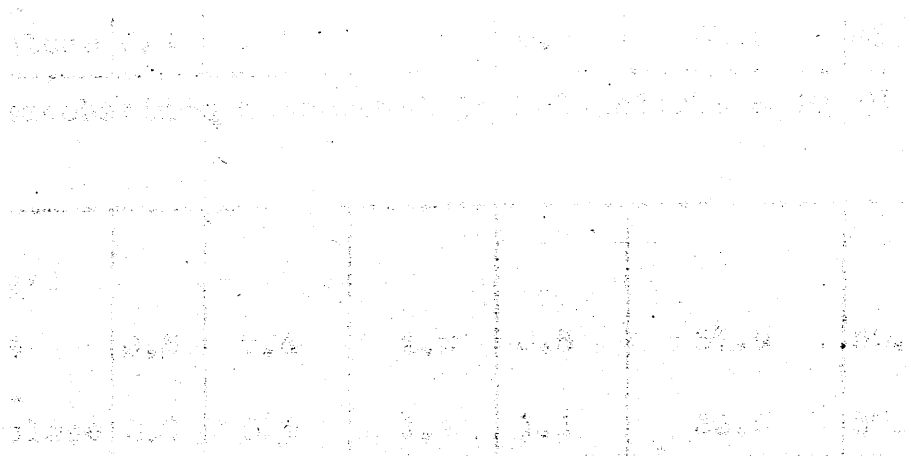


Table 23. The analysis of the fibre and holocellulose of three grasses to be examined for soluble lignin products
(as % original grass dry matter)

Grass	Ash	Crude protein	Lignin	Acetyl groups	Poly-saccharide (by difference)	Total
A (Rye-grass)						
Fibre	0.8	1.5	11.5	2.5	66.9	83.2
Holo-cellulose	2.1	0.5	2.5	3.2	66.7	75.0
Polysaccharides determined in holocellulose 61.6%						
B (Cocksfoot) (Grass No. 1)						
Fibre	1.4	13.0	6.2	0.8	41.6	63.0
Holo-cellulose	0.9	1.7	2.4	0.4	40.6	46.0
Polysaccharides determined in holocellulose 40.5%						
C (Timothy)						
Fibre	0.6	7.4	4.2	0.8	37.9	50.9
Holo-cellulose	0.6	0.6	1.4	1.1	33.5	37.2
Polysaccharides determined in holocellulose 34.0%						

The fibre and the holocellulose samples of grass were both examined by the same method which is described below, except that the grass fibre was first treated with pepsin. Only small amounts of material that might possibly have been lignin products, in addition to the lignin normally isolated, were obtained from the fibre and their inclusion did not increase the lignin value significantly. The holocellulose samples however yielded results which were pertinent to the problem.

The method of examination was to extract the holocellulose samples thoroughly first with cold and then with boiling ethyl alcohol [as suggested by Wise and his coworkers (148)], and then to carry out the treatments of the lignin determination method of Ellis, Matrone and Maynard (64) washing the solid residue after each step with alcohol. The acid extracts and alcoholic washings were allowed to stand at room temperature for 3 days, when small amounts of material were deposited. The first alcohol extract was evaporated under reduced pressure to a small volume, then diluted with ten volumes of water. A white flocculent precipitate was obtained, which was initially soluble again in absolute alcohol or water but which on standing became granular and insoluble in alcohol, water, dilute acid or dilute alkali. Neither the precipitate nor the concentrated solution before precipitation gave a positive reaction for carbohydrates (Molisch test) or lignin (Maule test), and after heating the precipitate with 6N hydrochloric acid for 48 hr. no amino acids were detected on paper chromatograms sprayed with ninhydrin.

The recovery of ash-free material from each of

the extracts of the three grasses is shown in Table 24.

Table 24. The recovery of possible lignin derivatives from grass holocelluloses (as % original grass dry matter)

Extract	Grass		
	A	B	C
Preliminary ethanol from holocellulose	2.4	0.5	Trace
5% sulphuric acid from residue	0.1	0.1	
Ethanol wash of residue from 5% H_2SO_4	1.1	0.1	
72% sulphuric acid solution	0.3	Trace	
3% sulphuric acid extract of lignin residue	0.3	0.1	
Lignin recovered in normal manner	2.8	2.4	(1.4)
Total	7.0	3.2	

In view of the fact that the bulk of the extra material obtained from grasses A and B was isolated from the preliminary ethyl alcohol extract, only this treatment was performed on the holocellulose of grass C of which only a small quantity was readily available. The amount of precipitate obtained was less than 0.5% of the grass dry matter.

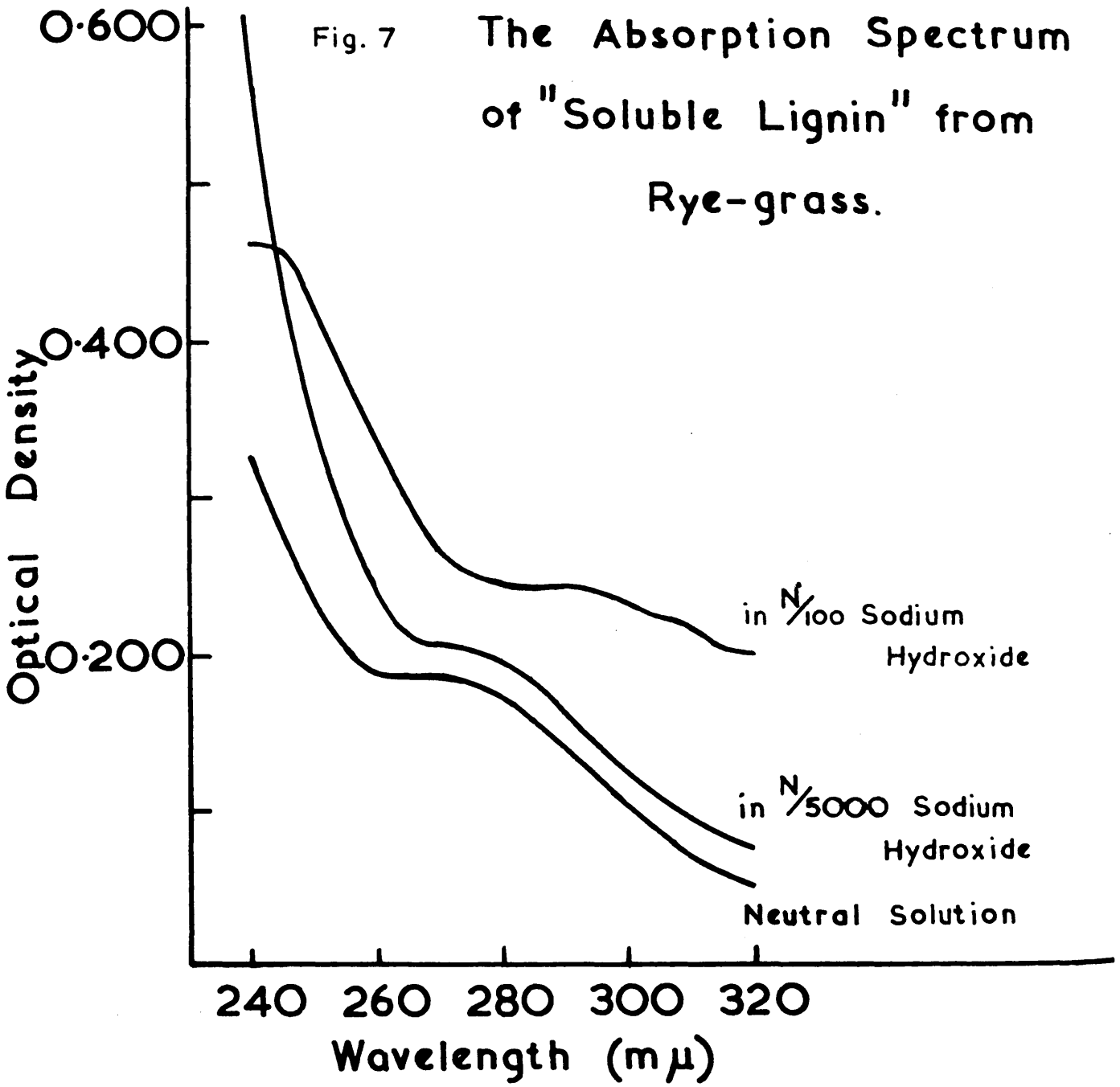


Fig. 7
The Absorption Spectrum of "Soluble Lignin" from Rye-grass.
0·600
0·400
0·200
Optical Density
in N/100 Sodium Hydroxide
in N/5000 Sodium Hydroxide
Neutral Solution
240 260 280 300 320
Wavelength (mμ)

The composition of the material isolated from any of the extracts described above is not known. The precipitate obtained from the first ethyl alcohol extraction was almost certainly material that is not normally estimated in the holocellulose as ash, crude protein, lignin or polysaccharides and for the want of a better name it will be referred to as "soluble lignin" since it has at least slightly resinous properties and the absorption spectrum (Fig. 7) of the solution has characteristics similar to those described by Herbst (154) and Ritter et al (155) for lignin derivatives.

The variation in the amount of the soluble lignin is just what is required to correct the value of the polysaccharides (calculated by difference) in the three holocellulose samples and produce agreement between the determined polysaccharides and the polysaccharides estimated by difference.

The analyses of grass holocellulose

Having considered the development of the sodium chlorite procedure and its possible faults, an account can now be given of the examination of holocellulose preparations from grass in the present work.

The first trial was to determine what effect an excess of reagents would have on the holocellulose product when prepared by the shorter method of Whistler et al (73), in case the ratio of the quantity of reagents to the amount of lignin was critical.

6 g. cocksfoot fibre (obtained as previously described) was suspended in 100 ml. water in an open flask and maintained in a water-bath at 70-75°C. The mixture was stirred mechanically and a stream of CO_2 passed over the surface continuously. The whole of the reaction was conducted in an efficient fume cupboard because of the toxic nature of the chlorine dioxide evolved. For the normal reaction 7.5 g. sodium chlorite (BDH 80% pure) was added in four equal portions at 15 min. intervals, each portion being preceded by the addition of 0.5 ml. glacial acetic acid. After 1 hr. total reaction time the mixture was filtered through filter cloth, washed rapidly with ice-water and finally dried with acetone and ether. The product was weighed and the dry matter content determined in the usual way.

Similarly 6 g. fibre were treated with double the previous quantity of reagents, i.e. 15 g. sodium chlorite and 4 ml. acetic acid.

In each holocellulose preparation the ash, crude protein and lignin contents were determined and the polysaccharide content calculated by difference. The results are shown in Table 25.

Table 25. The composition of grass holocellulose prepared with different quantities of reagents
(as % original grass dry matter)

	Ash	Crude protein	Lignin	Poly-saccharide	Total
Fibre of Grass No. 1	1.4	13.0	6.2	41.9	62.5
Holocellulose (Normal treatment)	1.7	8.5	3.0	42.5	55.7
Holocellulose (Double reagents)	1.7	8.4	3.2	42.3	55.6

It appeared that an excess of reagents had no apparent effect on the composition of the holocellulose produced. As discussed previously, the quantity of residual crude protein by this method was still very large and the lignin removal not entirely satisfactory. The slight increase in the amount of polysaccharides was probably due partly to experimental errors and possibly also to "soluble lignin".

It was known that the crude protein content of the fibre could be reduced to a much lower value than that obtained in this holocellulose simply by treatment with pepsin. Accordingly two samples of holocellulose were

prepared from a batch of rye-grass fibre (No. 4) of high protein content. One was prepared in the normal way whilst the other was treated with pepsin before sodium chlorite treatment. The normal quantities of reagents were used for each holocellulose preparation (7.5 g. sodium chlorite and 2 ml. acetic acid per 6 g. fibre) and the products analysed as before (Table 26).

Table 26. The effect of pepsin pretreatment on holocellulose composition
(as % original grass dry matter)

	Ash	Crude protein	Lignin	Poly-saccharides	Total
Fibre of Grass No. 4	0.6	19.4	4.7	30.4	55.1
Holocellulose - pepsin treatment	0.6	1.9	2.1	29.7	34.3
Holocellulose - normal treatment	1.4	14.5	3.2	29.8	48.9

Using the pepsin pre-treatment, not only was the removal of protein very satisfactory but the reagents seemed to cause a much greater reduction in the lignin content, and the amount of the polysaccharides was unaffected. If the composition of the pepsin pre-treated product is compared with the Norman and Jenkins

cellulose (Table 22 p.101), the far better recovery of polysaccharides in the holocellulose is evident.

The method of preparation of holocellulose chosen for the following work was a pre-treatment of the grass fibre with pepsin in hydrochloric acid followed by delignification with sodium chlorite and acetic acid as described on page 114. The product was recovered by centrifuging the mixture after it had been cooled in ice and the solid residue was then washed on Whatman No. 31 filter paper with ice-water, followed by washing with acetone and ether to dry the holocellulose.

Acetyl groups associated with polysaccharides

Many natural non-nitrogenous polysaccharides contain a small proportion of acetic acid, presumably combined to hydroxyl groups by ester linkages. Hemicelluloses in particular have amounts greater than average, maple wood hemicellulose (156) having been reported to possess 9%. The acetyl radicles are very labile and are lost in most methods of extraction of the polysaccharides, especially when alkaline reagents are employed.

The technique of holocellulose preparation using sodium chlorite and acetic acid, however, allows the

complete recovery of the acetyl groups (142). It is recognised that acetylation is possible in the reaction mixture but this is not considered likely. A more probable source of error lies in the possible estimation of acetic acid not washed out of the holocellulose. For this reason an alkali saponification method was employed in the present work using an oven dried sample from which any free acetic acid would have been expelled. Lignin, protein and true cellulose have not been reported to contain any acetyl groups so it was considered that the presence of these substances would cause no trouble in the calculations.

The method of acetyl estimation used was developed from the publication by Scott and Golberg (157) in which the acetyl content of acetylated filter paper was estimated by reaction with 0.2 N potassium hydroxide in 80% ethanol. Various conditions including temperature, time of reaction and method of determination of uptake of the alkali were investigated using a sample of acetyl cellulose prepared in the laboratory. It was concluded that the following method was satisfactory for the present work.

The sample (50 mg. acetyl cellulose or 500 mg. grass fibre) was stirred with 25 ml. 0.2N potassium hydroxide in 80% ethanol. The flask was then stoppered and left at room temperature for 4 hr. with occasional swirling. After this time 10 ml. 0.5N hydrochloric acid were added and the mixture filtered through Whatman No. 31 filter paper. The residue was well washed with water until the washings were neutral and the combined filtrate and washings then titrated with 0.1N sodium hydroxide using phenolphthalein as the indicator. A blank determination was made under exactly the same conditions but without any sample and the uptake of alkali, by the hydrolysis of the acetyl groups, was measured by the difference between the titres.

The acetyl content of the fibre was then calculated (as a percentage of the grass) as:

$$\frac{\text{(Uptake of 0.1N NaOH)} \times 4.20 \times \text{(fibre as \% of the grass)}}{\text{Dry weight of fibre}}$$

The value of 4.20 is used for the conversion factor for the acetyl group (M.W. 43) since on hydrolysis such as:

$$R\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3 + H\text{-}OH \longrightarrow R\text{-}O\text{-}H + CH_3COOH$$

the liberation of the CH_3CO-group is accompanied by formation of the alcohol (sugar residue) R-OH. The sugars produced on hydrolysis (see later) are estimated without any allowance for this reaction so the factor for the acetyl group is reduced to prevent it being measured twice in any summation.

The acetyl content of the fibre of Grass No. 1 was found by this method to be 0.8% of the original grass

but larger values are common in other grass samples (See Part II of this thesis).

(iii) The carbohydrates in grass holocelluloses

In the following work the holocellulose was extracted with a number of reagents and the composition and amount of polysaccharides in each extract estimated from the sugars liberated after hydrolysis of the extract. The cellulose residue was weighed directly and corrected for ash and lignin contents. The ratio of the constituent sugars in the cellulose residue (glucose and xylose) were then estimated in the hydrolysate of a small portion.

Before reporting the composition of the holocellulose, the method of estimating the polysaccharides in the extracts is given below.

Characterisation of the neutral sugars

The neutral sugars obtained by hydrolysis of the grass cell wall polysaccharides were characterised by direct isolation in only one instance.

A sample of holocellulose (6g.) of old autumn rye-

grass (variety S 24) was hydrolytically extracted for 3 hr. with N sulphuric acid at 100°C and the extract further hydrolysed for 3 hr. The solution was neutralised with barium carbonate and the filtrate deionised. After concentration the sugar solution was treated with activated charcoal and finally evaporated to a syrup (1.5 g.)

The sugar syrup was put on a cellulose column (158) (300 x 30 mm) and developed with n-butanol, half saturated with water ~~as the developing agent~~. The column was in a room maintained at 35°C.

Good resolution of the following four sugars was obtained and each was obtained crystalline.

Sugar	M.P. (°C)	Mixed M.P. (°C)	$[\alpha]_D^{21}$ (°) (equilibrium value in water)
D - xylose	144	143	+23
L - arabinose	154	152	+95.5
D - glucose	146	143	+52
D - galactose	164	164	+80

No other sugars were detected in the eluate.

Quantitative estimation of sugars in hydrolysates by paper chromatography

The polysaccharide extract was hydrolysed with N sulphuric acid, cooled and a known weight of L-rhamnose added to serve as a standard sugar in the following procedure. This was justified only if no rhamnose was present in the hydrolysates under examination and this sugar has not been detected in any of the polysaccharides

from the grass samples used in this work. The method assumed that during the preparation of the hydrolysates for chromatography there was no preferential absorption of any sugar on the various solid reagents or precipitates.

The acid hydrolysate was neutralised with a slurry of barium carbonate and after filtering through a thin pad of celite, the filtrate was treated immediately with cation-exchange resin (IR 120) to remove barium ions. The solution, which was then slightly acid (by reason of uronic acids), was evaporated *in vacuo* to a syrup which was taken up in methyl alcohol and filtered. The alcoholic solution was again evaporated and the thin syrup applied to a sheet of Whatman No. 1 paper as a narrow band. After air-drying the sheet was placed in a chromatographic tank and irrigated with the basic mixture *n*-butanol-pyridine-water (10:3:3 v/v) for 3 days at room temperature.

After 3 days the paper sheet was removed and dried in a forced draught cabinet at 80°C until only a slight smell of pyridine was noticeable. Strips 1" in width, were cut from the paper in the direction of the solvent flow, two being at least 2" from the edge and the third approximately at the centre of the sheet. These guide strips were sprayed with a solution of 2% p-anisidine hydrochloride dissolved in *n*-butanol containing some water and a trace of stannous chloride. From the positions of the coloured zones thus produced the areas of the remaining sections of the paper sheets corresponding to the separated sugars could be determined and cut out. The use of a solvent mixture containing pyridine resulted in the retention of uronic acids near the line of application. A clear separation of glucose and galactose was obtained under these conditions provided the weight of syrup applied was not too great; 10 mg. of each sugar could be separated easily on a single sheet of paper.

The paper strips were hung from wires fitted inside water condensers and the sugars were extracted from the paper section for ¾ hr. by the condensate from 10 ml. water refluxing in Pyrex tubes (260 x 40 mm). A

corresponding section of the chromatogram selected to contain no sugar was similarly extracted, to act as a blank. The sugars from the paper areas were estimated by oxidation with sodium meta-periodate (159), titrating the formic acid produced with N/100 sodium hydroxide. Quantities as small as 0.2 mg. monosaccharide could be estimated with good duplication but with amounts greater than about 10 mg. liberation of iodide occurred during the oxidation. It was found that residual pyridine on the chromatogram caused no appreciable effect provided the paper was dry to the touch.

From the ratio of the weights of rhamnose to those of the other sugars estimated in the paper sections, the amounts of each sugar originally in the hydrolysate could be calculated using the relationship:

Original weight of sugar =

$$\frac{\text{wt. of sugar estimated} \times \text{wt. of rhamnose added}}{\text{wt. of rhamnose estimated}}$$

The extraction of polysaccharides from holocellulose

Various methods are available for the extraction of polysaccharides from natural material, the choice depending upon the polysaccharide under examination and the purpose for which the extraction is being performed. Only very rarely can a homogeneous polysaccharide be obtained as a result of a simple extraction technique. Even the nature of what constitutes a homogeneous polysaccharide preparation is a matter of discussion and the increased use of physical methods of examination

in recent years has led to more exacting working standards. Any degradation caused by the methods of isolation of the polysaccharides will lead to heterogenity of the preparation and some attempts at purification may actually defeat their intended purpose.

The polysaccharides of plant cell walls were originally extracted from grass fibre by dilute solutions of alkali but by such methods the polysaccharide products were seriously contaminated with lignin which was extracted at the same time. If polysaccharides are extracted from holocellulose instead of from fibre, much milder conditions may be used. It is often found that a considerable proportion of the polysaccharides are then soluble in hot water and that more rigorous treatment, as with acid or alkali, achieves greater extraction when applied to holocellulose than when similar reagents are used with grass fibre.

From previous work (85, 160, 161) it was known that grass fibre contained polysaccharides which on hydrolysis yielded glucose and xylose with lesser amounts of arabinose, galactose and uronic acids. In the present

investigation the first extractions were designed to attempt to remove the galactose and arabinose units and leave a residue of cellulose and xylan. It was considered likely that the arabinose and galactose residues were associated in some highly branched structure with part of the xylose residues and that as a result these sugar residues might be more readily extracted. Arabinose units in polysaccharides are frequently present in the furanose form and hence are readily hydrolysed by dilute mineral acids, so this property too, was expected to make feasible the ready isolation of an arabinose-free product.

It was decided to examine the effect of acid and alkaline extractions on grass holocellulose to determine the manner in which the polysaccharides could be extracted. Extraction of the holocellulose of Grass No. 4 with sulphuric acid was investigated first, the preliminary examination being qualitative and subsequently quantitative. Two series of alkaline extractions were then performed on samples of the same batch of holocellulose. The first alkaline extractions were done in a manner similar to the quantitative acid

extractions except that the alkaline extractions included an initial treatment with hot water. The second alkaline treatment was for the preparation of α-cellulose in view of the interest shown in the fraction when prepared from other materials (84, 162, 163).

Acid extraction - Qualitative

Holocellulose (1 g.) prepared from Grass No. 4 was heated under reflux in a boiling water bath with 200 ml. N/20 sulphuric acid for 2 hr. The mixture was filtered and the solution neutralised with barium carbonate. After re-filtering the barium was removed from the solution using a cation exchange resin. The final extract and other portions of the acid solution removed during the hydrolysis were examined on paper chromatograms using a solvent mixture of ethyl acetate, acetic acid, formic acid and water (18:3:1:4 v/v).

The holocellulose residue was similarly extracted in succession with N/10 and N/2 sulphuric acid and each of the extracts examined as above on paper chromatograms.

Part of the final holocellulose residue was dissolved in 72% sulphuric acid and hydrolysed for 2 hr. at 20°C before the solution was diluted with 15 volumes of water and heated for 2 hr. at 100°C. This final hydrolysate was also examined chromatographically.

The results of these acid extractions are shown in Table 27.

Table 27. The examination of the acid extracts of holocellulose (Grass no.4)

Acid	Extraction time (hr.)	Arabinose	Xylose	Uronic acid	Glucose	Galactose	Pentose oligosaccharides
N/20	½	+	-	-	-	-	-
	1	++	+	-	-	-	±
	1½ & 2	++	+	-	-	-	±
N/10	½	±	+	±	-	-	-
	1	±	+	±	-	-	-
	1½ & 2	±	+	±	-	-	-
N/2	½ & 1	-	+	-	±	-	±
	2	-	++	-	+	-	±
	3	-	++	-	+	-	-
72%		-	±	-	++	-	-

- Not detected on chromatogram. ±, +, ++ Detected, in increasing amounts

It appeared that all the arabinose units had been hydrolysed after the treatment with N/10 sulphuric acid. The failure to detect galactose, a sugar repeatedly found later, in any of the solutions was probably because of its slow rate of hydrolysis and because the small amount present may have been distributed over many of the fractions. Uronic acids were detected only in the N/10 acid extracts, suggesting that these too are fairly readily extracted, though likely to be present only as aldobiuronic acids or even larger acid oligosaccharides.

The presence of glucose in the N/2 sulphuric acid extracts was rather surprising since it was thought that this sugar would be associated only with the true cellulose or some similarly constructed polymer, which would be hydrolysed with difficulty. Treatment of cotton wool with N/2 sulphuric acid for $1\frac{1}{2}$ hr. at 100°C produced a solution in which no sugars could be detected on paper chromatograms and which gave only a very weak reaction with the Molisch test (α-naphthol and H_2SO_4). The glucose noticed in the

holocellulose extracts was therefore not considered to arise from a cellulose degradation product, unless this had been formed as a result of the chlorite treatment.

Acid extraction - quantitative

To determine the amounts of the various neutral sugars present in the acid extracts of grass holocellulose a sample of holocellulose (No. 4) was extracted successively with N/2 sulphuric acid (twice) and N sulphuric acid for 3 hr. at 100°C. The extracts were filtered from the residue and adjusted to normal acid concentration. They were then hydrolysed for 2 hr. at 100°C. The final residue was hydrolysed with 72% sulphuric acid as above.

The composition of each hydrolysate was determined by paper chromatography as previously described (p. 119). The results are shown in Table 28.

Table 28. The fractionation of grass holocellulose by extraction with acid (as % original grass dry matter)

Extract	Galactan	Glucosan	Araban	Xylan	Total
N/2 H_2SO_4 1st	0.5	1.2	1.8	4.8	8.3
" " 2nd	-	-	-	-	trace
N H_2SO_4	-	0.1	-	0.4	0.5
Residue	-	15.2	-	2.0	17.2
Sum	0.5	16.5	1.8	7.2	26.0

Alkaline extraction

A sample of holocellulose (No. 4) (1 g.) was extracted first with 100 ml. water at 85°C for 20 hr.

The residue was then extracted successively with 1% and 10% potassium hydroxide solutions under nitrogen at 20°C for 24 hr. The final residue was dissolved in 72% sulphuric acid and hydrolysed as above.

The aqueous extract was hydrolysed directly, after the solution had been acidified to make it normal with respect to sulphuric acid. The alkaline extracts were neutralised by the addition of cation exchange resin and then hydrolysed at the same acid concentration in the presence of the resin. Each extract was hydrolysed for 4 hr. at 100°C and then the composition of the hydrolysate was determined.

The results of the extractions are given in Table 29.

Table 29. The fractionation of grass holocellulose by extraction with alkali (as % original grass dry matter)

Extract	Galactan	Glucosan	Araban	Xylan	Total
Water	0.2	0.8	1.2	1.6	3.8
1% KOH	0.2	0.7	0.1	1.3	2.3
10% KOH	0.1	0.5	0.1	2.1	2.8
Residue	-	15.8	-	0.4	16.2
Sum	0.5	17.8	1.4	5.4	25.1

The first result of interest from these extractions was that approximately a third of the extractable polysaccharides (i.e. 3.8% from a total of 8.9%) was obtained in the water extract, a solution which also contained most of the araban.

Over 90% of the extractable polysaccharides (i.e. 8.3% from a total of 8.8%) were liberated during the first extraction with N/2 sulphuric acid (Table 28) whereas the total amount (6.1%) obtained by extraction with water and 1% potassium hydroxide solution (Table 29) did not rise to this quantity. The composition of the residue after only acid treatment was interesting since it contained much more xylan than the residue after the alkaline extractions. This suggested that solutions of potassium hydroxide were better able to penetrate the cellulose structure than the sulphuric acid, a suggestion that seems reasonable in view of the known swelling effect caused by alkalies on cellulose, as in the process of mercerisation (164, 165).

It should be noted that the amount of lignin in the holocellulose samples was small and when any alkaline extractions were made it was assumed that the lignin then remaining in the cellulose residue was negligible. However, if only acid was employed in the extractions, the residue was corrected for lignin (the amount determined in the complete holocellulose)

as it was not expected to have been removed by the acid.

The isolation of α-cellulose

0.9 g. holocellulose was stirred with 10 ml. 17.5% sodium hydroxide and after 25 min. the mixture was diluted with 30 ml. water. After a further 5 min. it was filtered through fine cloth and the filtrate received in acetic acid. The residue, α-cellulose, was washed with acetic acid, ethanol and ether and dried in an oven at 100°C and weighed.

A portion of the α-cellulose was dissolved in 72% sulphuric acid and hydrolysed as previously. The ratio of the amounts of the constituent sugars in the hydrolysate was found to be

Glucose: xylose: arabinose = 95: 2.5: 2.5

The remainder of the α-cellulose, was ashed at 550° and the total weight of ash-free cellulose calculated, 0.474 g. representing 16.7% of the original grass dry matter.

The acetic acid solution of the alkaline filtrate was made to 20 ml. and a 10 ml. portion treated with cation exchange resin before being hydrolysed with 2N sulphuric acid. The composition of the neutral sugars is given in Table 30.

Table 30. The fractionation of grass holocellulose by the preparation of α-cellulose (as % original grass dry matter)

	Galactan	Glucosan	Araban	Xylan	Total
α-cellulose	-	15.9	0.4	0.4	16.7
Alkali extract	0.4	1.1	1.2	5.4	8.1
Sum	0.4	17.0	1.6	5.8	24.8

It may be seen in Table 30 that the α-cellulose obtained from this grass was mainly composed of glucosan (cellulose) but that some araban as well as xylan was also present, in contrast to the cellulosic product obtained previously with the dilute acids or alkali.

Comparison of acid and alkaline extractions

The acid and alkaline systems of extraction of holocellulose were applied to three other grasses using the same conditions as previously described. The results are presented in Table 31 where any slight variations in the methods are noted in the first column.

Table 31. The extraction of polysaccharides from grass holocelluloses using either alkali or acid
(as % original grass dry matter)

Reagent	Galactan	Glucosan	Araban	Xylan	Total
Grass No. 1					
Water	0.4	0.9	1.9	3.4	6.6
1% KOH	0.2	0.8	0.4	2.2	3.6
10% KOH	-	-	-	1.8	1.8
Residue	-	22.9	-	1.2	24.1
Sum	0.6	24.6	2.3	8.6	36.1
Water(4hr.)	0.2	0.4	0.6	0.9	2.1
N H_2SO_4	0.5	1.7	1.7	5.6	9.5
Residue	-	22.8	-	2.1	24.9
Sum	0.7	24.9	2.3	8.6	36.5
Grass No. 5					
Water	0.4	0.5	1.5	3.6	6.0
1% KOH	0.3	0.3	0.8	4.8	6.2
10% KOH	-	0.7	-	2.1	2.8
Residue	-	23.4	-	1.2	24.6
Sum	0.7	24.9	2.3	11.7	39.6
N/2 H_2SO_4	0.5	0.7	1.9	9.2	12.3
N H_2SO_4	-	0.1	-	0.9	1.0
Residue	-	23.5	-	1.6	25.1
Sum	0.5	24.3	1.9	11.7	38.4
Grass No. 2					
Water	0.3	0.4	1.5	5.6	7.8
1% KOH	0.2	0.3	0.5	5.0	6.0
10% KOH	-	0.2	0.3	5.9	6.4
Residue	-	29.4	-	0.8	30.2
Sum	0.5	30.3	2.3	17.3	50.4
Water(4hr.)	0.2	0.3	0.9	2.0	3.4
N/2 H_2SO_4	0.5	0.8	1.4	10.0	12.7
N H_2SO_4	±	0.2	0.1	1.3	1.6
Residue	-	27.9	-	3.5	31.4
Sum	0.7	29.2	2.4	16.8	49.1

The general pattern of these results agreed with that found for holocellulose (No. 4) namely that approximately half of the extractable polysaccharides were extracted by water, that treatment with N/2 sulphuric acid was equivalent to extraction with water and 1% potassium hydroxide as judged by analysis of the hydrolysates, and that the cellulose residues after treatment with alkali contained least xylan.

In view of the results in Table 31 it seemed that it might be useful to examine the effect of extraction with N sulphuric acid following extraction of the holocellulose with 1% potassium hydroxide solution. This was done using holocellulose of Grass No. 2, applying the extractants as previously except that the sulphuric acid extraction was for 2 hr. The results of these extractions are shown in Table 32.

Table 32. The extraction of grass holocellulose with water, alkali and acid (as % original grass dry matter)

Reagent	Galactan	Glucosan	Araban	Xylan	Total
Water	0.2	0.7	0.9	2.2	4.0
1% potassium hydroxide	0.3	1.1	1.1	8.4	10.9
N sulphuric acid	0.1	0.7	0.4	6.2	7.4
Residue	-	25.9	-	1.8	27.7
Sum	0.6	28.4	2.4	18.6	50.0

It will be seen from these results (Table 32) that the effect of the alkali was as desired in that the proportion of xylan in the cellulosic residue was less than that recorded for the residue from the acid treatment in Table 31. The proportion of xylan was, however, greater than when the holocellulose was treated only with alkaline reagents (Table 31).

This compromise in the extraction system thus achieved seemed very satisfactory for more general use, since the extraction with acid required less time and the need for only one set of operations in an

atmosphere of nitrogen instead of two was a considerable practical advantage.

The series of operations carried out to yield the results in Table 32 were therefore adopted as the standard procedure for the analyses of the samples to be reported in Part II of this thesis.

The method of preparation of the hydrolysates of the extracts and the cellulosic residue as finally decided was as follows:

Approximately 0.9 g. grass holocellulose was stirred with 50 ml. water in a conical flask until it was thoroughly wetted but without leaving any material on the walls above the liquid. The flask was then heated under reflux in a water bath at 85°C ± 2° for 20 hr. The residue was filtered through a Whatman No. 52, 7 cm. paper in a Buchner funnel and washed thoroughly with water. The combined extract and washings were cooled and diluted to 200 ml. Part of the water extract (100 ml.) was hydrolysed with 3 ml. concentrated sulphuric acid for 4 hr. (i.e. in approximately N sulphuric acid) and the hydrolysate analysed by the standard chromatographic method (p. 119).

The holocellulose residue was dried on the filter by washing with acetone and ether and then brushed into a 100 ml. flask. This flask was fitted with a dropping funnel and a lead for a stream of nitrogen which was used to flush out the flask, taking care not to blow out the sample. With the nitrogen still flowing 30 ml. 1% potassium hydroxide were run in from the funnel and the holocellulose well mixed with the liquid. The delivery heads were then removed and the flask quickly stoppered. The flask was swirled occasionally during 24 hr. and the contents then filtered as before. The whole of the

filtrate and washings (100 ml.) were neutralised with Amberlite IR 120 resin and hydrolysed in the presence of the resin with 3 ml. concentrated sulphuric acid.

The residue of the holocellulose after the alkali extraction was again dried with acetone and ether and transferred to another flask, where it was extracted for 2 hr. with about 50 ml. N sulphuric acid at 100°C. The acid solution was filtered, the residue washed with water and the combined solutions adjusted to normality again and heated for a further 4 hr. at 100°C.

The final cellulose residue was dried with acetone and ether, transferred to a weighed moisture dish and finally dried for 3 hr. at 100°C before weighing. The ash content was determined as previously described. A small portion (30 mg.) of the dried material was hydrolysed in 1 ml. 72% sulphuric acid at 20°C for 2 hr. with occasional stirring. The solution was diluted with 12 ml. water (to approx. 2N) and heated at 100° for 4 hr. The sugar ratio was determined in the hydrolysate and thence in the organic matter of the "cellulose". Only a trace (less than 1 mg.) of undissolved material, presumably lignin, was found in the hydrolysates making a correction unnecessary.

The water extractable polysaccharides of grass holocellulose

It seemed quite certain that the grass fibre isolated in the work reported here and from which holocellulose samples were prepared, did not contain any water-soluble materials, since the preparation of the fibre included extraction with warm water and a hot solution of ammonium oxalate. It has been shown above that the holocellulose samples contained a

large proportion of water-extractable polysaccharides. The problem of how this fraction became soluble was a matter of great interest since it was felt that its elucidation might well indicate something of the complex nature of the grass cell walls. The fraction did not contain simple sugars for only traces of free arabinose were detected when the extract was analysed on paper chromatograms.

Two possible causes of the greater availability of this fraction were that a protective covering layer had been removed from the surface of the polysaccharides or that some chemical bonding had been cleaved during the production of the holocellulose, or perhaps both. The second possibility could be determined only with difficulty but the first was examined further and the results obtained agreed with the theory that there were protective covering layers. If it is assumed that the polysaccharides as originally present in the dried cell wass are covered by or intermingled with other compounds the most likely substances for this purpose are lignin and protein. Most of the protein is removed by pepsin digestion and much of the lignin during the treatment with

sodium chlorite. With young grass, the amount of protein in the fibre is much greater than the amount of lignin. Removal of only the protein from such a sample should therefore have approximately the same effect on the availability of the polysaccharides as the application of the complete treatment for the preparation of holocellulose to a more mature grass fibre, in which the amount of lignin far exceeds the amount of protein.

To test this hypothesis a sample of rye-grass fibre (No. 6) having a high crude protein content was treated with pepsin in hydrochloric acid in the normal way and the residue extracted with water at 85°C for 20 hr. The water extract was hydrolysed and analysed by the standard procedure to give the results in Table 33.

Table 33. The extraction of high protein grass fibre with hot water after pepsin digestion (as % original grass dry matter)

Composition Rye-grass (No. 6) fibre

Ash	Crude protein	Lignin	Acetyl groups	Polysaccharides	Total
2.2	14.1	3.6	0.3	32.2	52.4

Analysis of water extracted polysaccharides

Galactan	Glucosan	Araban	Xylan	Total
0.28	0.75	0.84	0.93	2.8

Treatment of the fibre with both pepsin and chlorite produced a holocellulose which yielded only 1.9% of water-soluble polysaccharides. The removal of protein alone had therefore opened the grass structure sufficiently to make available more water-soluble polysaccharides than were available after chloriting. The difference of 0.9% polysaccharides between the two extracts was found to be a normal value for the polysaccharides appearing in the chlorite liquor.

Irrespective of the discussion of the possible

losses of polysaccharides in this sample of rye-grass (which had a high protein content), the result of removal of most of the protein agreed with the suggestion that their apparent insolubility when in the fibre was because the water could not remove them through the protein covering.

Uronic acids

The analysis of the polysaccharides from the holocellulose extracts discussed so far, have included only the neutral sugars liberated after 4 hr. hydrolysis with N sulphuric acid at 100°C. It is known however that hemicelluloses contain uronic acids and they were, in fact, indicated in the hydrolysates in this work by the acidity of the solutions after removal of the sulphuric acid and by their non-migration in the basic solvent mixture on paper chromatograms.

In view of the great resistance to hydrolysis of the uronosidic linkage, the relatively mild conditions used for the hydrolysis of the extracts would not be expected to produce free uronic acids, but rather aldobiuronic acids. When chromatograms of the

hydrolysates were prepared using an acidic solvent mixture, in which uronic acids were well separated, two major sugar acids were usually detected corresponding in rates of movement to aldobiuronic acids. No recognisable free uronic acids were detected and the biuronic acids were not identified.

Methoxyl groups are often associated with the uronic acids in plant material (166) but they were not determined in this work and this must necessarily make a small discrepancy in the summations (approx. 0.2 - 0.5% of the grass if all the uronic acids were monomethylated).

The estimation of uronic acids is not possible with the same accuracy as that of the neutral sugars, since chromatographic methods are more or less unsuitable because of the degradation that accompanies any attempt at complete hydrolysis and because other methods are not completely specific. Most methods for the estimation of uronic acids are based on the decarboxylation of the acids by hot mineral acids and are modifications of the original method by Lefèvre and Tollens (167). The basis of this method is that the sample is refluxed with 12% hydrochloric acid and

the carbon dioxide evolved is measured by any suitable method. Furfural is also produced in the reaction but unlike the production of carbon dioxide the yield of it is not quantitative. The main criticism of the method is that nearly all carbohydrates as well as many other compounds are slowly degraded to carbon dioxide under these digestion conditions, but the principal sources of interference are the pentoses. The results however are not seriously affected but corrections can be applied if the course of evolution of the carbon dioxide is determined since the production of carbon dioxide from the two sources (168) can be identified. Calorimetric analyses are also available, such as reaction with carbazole (169, 170) or naphthoresorcinol (171) but these are even more influenced by interfering substances and are only of use in limited circumstances, such as pectin assay (172), in which the uronic acids constitute the greater part of the sample.

The modification of the acid digestion process used in this work to estimate the carbon dioxide evolved was a semi-micro method described by Johansson, Lindberg and Theander (173) in which the carbon

dioxide was recovered as barium iodate. It seemed justifiable to calculate the uronic acids so estimated in terms of aldobiuronic acids because of the moderate nature of the hydrolysis conditions for the determination of the neutral sugars. The neutral sugar combined in the biuronic acids was assumed to be xylose, and would not be estimated with the other neutral sugars.

The distribution of the uronic acids among the various extracts of the holocellulose samples was believed from visual examination of the paper chromatograms of the hydrolysates to be such that the water extract had the greatest amount. This was confirmed by estimating the uronic acids in the holocellulose residue after each of the extractions. These results are shown in Table 34.

Table 34. The extraction of uronic anhydride from holocellulose
(as % original grass dry matter)

	Rye grass S 23
Uronic anhydride in holocellulose	3.5
" " " water extracted residue	0.7
" " " potassium hydroxide extracted residue	Trace
" " " N sulphuric acid extracted residue	Nil

In the results presented in Part II of this thesis it will be shown that the range in uronic anhydride content of the grass samples examined was 1.4 - 4.3% of the grass dry matter.

Estimation of pentoses as furfural

Until the introduction of chromatographic methods, the pentose constituents of hemicelluloses were estimated together in terms of the furfural produced by digestion of the material with 12% hydrochloric acid in a manner similar to the uronic acid determination already described. It was considered of interest briefly to compare a value arrived at by

the furfural method with one obtained by the present extraction technique. The bromate oxidation method for the estimation of furfural of Kullgren & Tyden (174) was first used but was found to be inaccurate in the presence of relatively large amounts of hexose material. Typical values are shown in Table 35.

Table 35. Pentose estimation as furfural using bromate oxidation

Xylose present (mg.)	Additions (mg.)	Xylose estimated (mg.)
161	Nil	168
190	Nil	189
198	200 cellulose	197
133	207 cellulose	133
198	143 starch	199
169	65 fructose	168
189	242 glucose	187
36	1095 glucose	81
45	1680 cellulose	67
53	1060 fructose	67

A calorimetric method introduced by Stillings and Browning (175) and modified by Adams and Castagne (176) did not suffer from this defect; results by this and the extraction methods are given in Table 36.

Table 36. The analysis of pentosans in a sample of dried rye-grass
(as % of the original grass)

By furfural production (colorimetric method of Adams and Castagne) :	
Pentosan in whole grass = 11.3%	
By chromatographic analysis of holocellulose extracts :	
Araban	= 2.1%
Xylan	= 10.1%
Uronic anhydride	= 2.0%

The value for pentosans by the furfural estimation was too high (by approximately 1%) in comparison with the analysis of the holocellulose by the chromatographic method because furfural produced from water-soluble araban and from pectin was included. Moreover, for grasses, the factors used to calculate the amount of pentosans from the yield of furfural appears open to question. When the factors were used to calculate the expected furfural yield from the individual values for araban and xylan (obtained chromatographically) and this furfural yield then converted to a value for total pentosans, the result was 13.3% (cf. 11.3% by

the colorimetric furfural method). Even if the uronic anhydride, from which the furfural yield is uncertain, was neglected, it is apparent from the above results that the method of estimating pentoses in grass by the formation of furfural, yielded low values.

Composition of some cellulose preparations

The composition of a sample of cellulose prepared by the method of Norman and Jenkins (60) from a sample of rye-grass has already been mentioned (p. 101). It was seen that a loss of polysaccharides occurred during the preparation of the cellulose but the nature of the polysaccharides which had been extracted was not investigated. This question was examined for two other samples of grass, the polysaccharide compositions of which were already known.

Cellulose preparations were also isolated from these two grasses by the method of Crampton and Maynard (15).

These two methods of cellulose preparation are commonly employed in forage analysis, a result obtained by Norman-Jenkins' method often being taken as a measure of practically all the structural carbohydrates and a result by Crampton-Maynard's

method being considered a reasonable measure of true cellulose.

The results of the analyses of the cellulose preparations, as well as of the corresponding analyses of the fibre and holocellulose prepared from the grasses are shown in Table 37. It will be seen that neither method of cellulose preparation really approached the standards expected since with Norman and Jenkins' method a large part of the polysaccharides are removed and with Crampton and Maynard's method there was still contamination of the true cellulose with other polysaccharides.

Table 37. Composition of some cellulose preparations
(as % of original grass dry matter)

Grass No. 4

	Ash	Crude protein	Lignin	Polysaccharides	Total
Grass fibre	0.6	19.4	4.7	30.4	55.1
Holocellulose	0.6	1.9	2.1	29.7	34.3
Norman-Jenkins cellulose	0.9	0.4	1.7	24.3	27.3

Grass No. 7

	Ash	Crude protein	Lignin	Acetyl groups	Polysaccharides	Total
Grass fibre	2.3	15.8	3.0	0.6	31.6	53.3
Holocellulose	1.4	1.0	1.8	0.8	29.3	34.3
Norman-Jenkins cellulose	1.1	0.1	1.3	0.1	18.5	21.1
Crampton-Maynard cellulose	2.7	0.4	1.1	1.2	16.8	22.2

Polysaccharides

	Hemicellulose					Cellulose		
	Galactan	Glucosan	Araban	Xylan	Uronic Anhyd.	Glucosan	Xylan	Total
Holocellulose	0.5	1.6	1.8	5.4	2.0	15.5	1.5	28.3
Norman-Jenkins cellulose	0.1	0.3	0.4	0.9	-	15.3	1.7	18.7
Crampton-Maynard cellulose	-	0.1	-	0.8	0.3	14.6	0.8	16.6

Grass No. 5

	Ash	Crude protein	Lignin	Acetyl groups	Polysaccharides	Total
Grass fibre	0.5	1.8	11.9	1.8	55.6	71.6
Holocellulose	1.0	0.5	2.8	2.4	56.3	63.0
Norman-Jenkins cellulose	0.2	0.1	1.3	-	38.9	40.5
Crampton-Maynard cellulose	1.0	0.3	1.0	1.4	32.8	36.5

Polysaccharides

	Hemicellulose					Cellulose		
	Galactan	Glucosan	Araban	Xylan	Uronic Anhyd.	Glucosan	Xylan	Total
Holocellulose	1.2	1.7	2.1	10.7	3.5	25.7	5.6	50.5
Norman-Jenkins cellulose	0.2	0.6	1.0	4.3	0.7	26.7	5.9	39.4
Crampton-Maynard cellulose	-	0.2	0.1	2.1	0.8	27.2	2.5	32.9

DISCUSSION OF THE COMPREHENSIVE ANALYSIS OF GRASSES

It is not claimed that the detailed analysis of dried grass samples as developed in this work is directly applicable to the routine examination of such materials. The purpose of the analysis was to try to determine the whole of the constituents of the grass samples in terms of groups of substances which if necessary could be specifically characterised. The choice of reagents and the procedure adopted has, however, afforded a satisfactory distribution of the components of the samples between the various fractions. Apart from the ash and nitrogen containing compounds no substance appeared in significant amount in more than one fraction.

The recovery of the original dry matter of the grass samples was reasonably satisfactory. If the summation was calculated in terms of the major fractions i.e. ether-soluble, water-soluble, ammonium oxalate soluble and grass fibre (as in Part I A of this thesis) the recovery was 99-101% of the original grass but in the analysis of the water-soluble fraction a further 0.6 - 6.7% of the grass was not recovered in terms of known groups of substances. Of

the material fractionated in the water and ammonium oxalate extracts there was a total of 3-5% of the grass which could not be characterised except in so far as it was apparently non-ionic. The principal component of these unknown materials was precipitated from the water-soluble fraction along with the fructosan. This precipitation seemed to change its solubility unless the material had a very strong absorptive affinity for the celite filter-aid employed, for it was not subsequently removed by dilute acid. Simply by reason of this peculiar change in property it is suggested that the substance might be some form of lignin. A converse change in solubility was recorded in Part I B of this thesis, for material which might have been a modified form of lignin extracted from holocellulose. Campbell and McDonald (177) were able to absorb an acid soluble lignin fraction on a cation exchange resin, a reaction which seems to have been physical rather than chemical, so a similar absorption on celite is not impossible. Small amounts of "native" lignin have been isolated from various woods by extraction with a large number of hydroxyl-containing solvents and it is stated that

the extracted products are insoluble in dilute acids (134).

The use of ammonium oxalate solution for the extraction of pectin had two possible disadvantages. The first, the retention of ammonia in the residual grass fibre was shown not to be operative in this work. The other consideration, which was only realised at a late stage (124), was that the neutral sugars obtained after acid hydrolysis of the crude pectin might have originated from certain of the other structural polysaccharides that might have been extracted from the grass fibre. Only the pectic acid component of the pectin might have been recovered in the ammonium oxalate solution. The analyses of the hydrolysates of the polysaccharides from the ammonium oxalate extracts obtained in this section and from various samples of the grass heads in the latter part of this thesis indicated the presence of sugars which are not usually part of the pectin complex. Galactose, glucose, arabinose and xylose were detected in all these fractions.

In the analysis of Grass No. 1 (cocksfoot) it was found, as expected, that much of the ash (80%) appeared

in the water fraction; much smaller amounts (10%) were in the oxalate extract and in the fibre. In the latter the ash was probably present mainly as insoluble silica.

Most of the nitrogen compounds (expressed as crude protein)(80%) were present in the fibre. There was much less (16%) in the water extract and only a small amount (4%) in the oxalate solution. Of the crude protein in the water extract of the grass approximately half was in the form of non-protein nitrogen compounds. A similar distribution of the ash and crude protein was found in the extracts of the other grasses studied.

Regarding the analysis of the grass fibre samples, the method proposed (Part I B), involving the preparation of chlorite holocellulose after a digestion with pepsin, seemed very satisfactory. The amount of the polysaccharides found by analysis in the holocellulose showed a good recovery of the amount calculated to be present in the original fibre. It was only later in this work when more finely milled samples were employed, to pass a sieve with holes 0.5 mm. instead of 1.0 mm. diameter that

significant losses were apparent. Similar losses have been reported for pepsin pretreated holocellulose prepared from forages and faeces by Ely and his associates (178).

The nature of the polysaccharide separation achieved by the three extractions of the holocellulose samples can best be understood if the arrangement and structure of the cell wall polysaccharides are first considered. The basic structure of plant cell walls is pure cellulose, in the form of a long chain glucosan with 1:4 β linkages. Part of the cellulose is arranged in molecular bundles or fibrils which have a regular spatial orientation (179). Very closely associated with the cellulose are shorter, straight chain xylan molecules of a similar configuration to that of cellulose, except that the C_6 groupings of the glucose residues are present in the latter (180). Such a structure would allow very tight packing of adjacent molecules of the two series, perhaps as mixed crystals (181) and except in a few special preparations (182), "cellulose" isolated from plant materials has always contained xylan as did the cellulosic residues in the present work. The

suggestion has also been made that "mixed" molecules may be present (183, 184), containing both glucose and xylose units, as the steric distortion produced by such a combination would be negligible.

Cellulose is usually considered to be obtainable in a pure state only from cotton but Hirst, Isherwood, Jermyn and Jones (182) prepared cellulose from pear cell walls which, when hydrolysed contained no sugars other than glucose. The method of isolation of this pear cellulose involved only mild conditions (by chemical considerations) and the product was not appreciably degraded. Hydrolytic treatments have not proved capable of yielding pure cellulose, as seen in the present work. The first stage (e.g. N sulphuric acid) by such methods yields a resistant structure containing both glucose and xylose residues, while further, more drastic treatment (e.g. 72% sulphuric acid) hydrolyses both units simultaneously.

Surrounding the cellulose fibrils is a group of polysaccharides of varying properties together with the polymeric material lignin. It is these substances which introduce complexities into the structure of the cell walls and as Jermyn observed (185) to call them

encrusting substances conceals our present ignorance of their chemical structure and botanical function.

It seems to be fairly generally assumed that the encrusting polysaccharides are constructed from xylose and other sugar residues and that they are highly branched molecules. A branched structure could result in a greater solubility, although the same effect would be observed with small linear polysaccharides. The encrusting polysaccharides and hemicelluloses have been estimated in the present work by hydrolysis of extracts of the holocellulose and by the determination of the uronic acids in the complete holocellulose.

No attempt was made in this work to determine the nature of the individual polysaccharides and it is possible that part of the araban and galactan found in the hemicellulose fractions may have been derived from part of the pectin complex which was resistant to extraction by ammonium oxalate solution.

There was in the hydrolysates of the water extracts of the holocelluloses a smaller proportion of xylose and a greater proportion of uronic acid than in the acid or alkaline extracts. This suggests that the more

readily extracted polysaccharides are more highly branched than those materials which need more vigorous extractants. A polysaccharide preparation from the water extract of wheat straw holocellulose has previously been shown to be highly branched (79, 80, 186) whereas a fraction isolated from wheat straw fibre by alkaline extraction was much less so (187).

The encrusting role of lignin in plant material is generally accepted as rendering some constituents unavailable to the action of both solvents and digestive juices. This effect is well seem from the amount of water-soluble polysaccharides in the holocellulose after lignin removal. It is not perhaps so obvious that protein may also act similarly, particularly in an aqueous medium.

After the newer methods of analysis of the grass samples had been considered an examination was made of the results obtained by older methods. In particular the composition of the residues isolated as "cellulose" by the methods of Norman and Jenkins (60) and of Crampton and Maynard (15) was of interest. It was found that by either of these methods very little protein (0.1-0.6%) was left in the products and the

lignin content of both young and old grasses was reduced to about 1.2% of the original grass. The hemicelluloses, as compared with those determined in holocellulose preparations, were largely removed by these cellulose methods, moreso from the Crampton & Maynard cellulose than from the Norman & Jenkins a cellulose, although the uronic acids were absent from the latter product. It was interesting to find that the cellulose residue obtained from the Crampton & Maynard cellulose, when extracted by the methods developed (p. 135) contained less xylan than corresponding residues from holocellulose and Norman & Jenkins cellulose.

The comparison of the determination of pentosans in grasses by the method of furfural formation and by chromatographic methods showed that the result by the furfural method was lower than the more accurate value obtained by chromatography. This despite the fact that the value by the furfural method included furfural also arising from water-soluble araban, pectin and the uronic acids in the hemicelluloses and the values by the chromatographic method included only the araban and xylan in the holocellulose.

As a result of the work reported here it is considered that methods are now available by which a detailed chemical composition of dried grass may be determined with reasonable accuracy.

SUMMARY OF PART I

Part I of this thesis consists of two sections. In Section A the analysis of grass extracts has been considered and in Section B the analysis of the grass residue from these extractions has been dealt with.

Section A

1. The extractions of dried grass examined were those obtained by the use of ether, 90% alcohol, a mixture of ethanol and benzene (1:2), water and 0.5% ammonium oxalate solution.
2. It was found that direct extraction with ether did not remove all the ether-soluble material, whereas extraction with ethanol and benzene did.
3. Extraction of the ether-extracted grass with alcohol followed by water or of the original grass

with ethanol-benzene followed by water, removed practically the same amount of water-soluble material.

4. The water-soluble material included all the soluble sugars (hexoses, sucrose and fructosan), all the organic acids, a large part of the ash and some of the crude protein.

5. The water-soluble fraction obtained as in (3) above also contained material which was not identified, but of which some was phenolic and some may have been lignin compounds.

6. When the residue obtained by either of the extraction systems in (3) above was extracted with ammonium oxalate solution a mixture containing pectic acid and neutral polysaccharides was recovered. Small amounts of ash, crude protein and unidentified polyhydroxy compounds were also present in the extract.

7. The total amount of material recovered from the ethanol-benzene, water and ammonium oxalate extracts plus the residual grass fibre accounted for 95-99% of the dry matter of the grasses but the amount of material identified was only 92-96%.

Section B

1. The grass fibre examined was the residue of the grass after the extractions in Section A and contained mainly polysaccharides together with some crude protein, lignin and ash.

2. Holocellulose was prepared from the grass fibre by digestion with pepsin in hydrochloric acid followed by treatment with sodium chlorite in acetic acid.

3. It was found that digestion of the fibre with pepsin was necessary if the protein content was to be suitably reduced. Only when finely milled samples were studied was it found that some of the polysaccharides were dissolved during this treatment under the conditions chosen.

4. It was found that in holocellulose prepared from grass fibre which originally had a high lignin content there remained lignin compounds which were soluble in hot ethanol.

5. Methods of extraction of the polysaccharides from holocellulose were studied and it was found that cellulosic residues with a small xylan content could be obtained by extraction with water, alkali and acid.

6. A substantial proportion of the extractable polysaccharide (hemicelluloses) was soluble in hot water. These hemicelluloses were made accessible by the removal of protein from a high-protein young grass or by the removal of protein and lignin from older grass.

7. The composition of the polysaccharides from samples of grass were studied and in all of them xylose was the major sugar in hydrolysates of the extracts. Smaller, approximately equal amounts of glucose and arabinose, were also found together with very small quantities of galactose. The sugars were separated by paper chromatography and estimated by periodate oxidation.

8. Each of the sugars from the hemicelluloses of one grass sample were obtained crystalline for characterisation.

9. Uronic acids were detected in all the holocellulose extracts but were estimated, in terms of biuronic acids, in the unextracted holocellulose.

10. The cellulosic residues of the holocelluloses contained mainly glucosan (true cellulose) and a small amount of xylan.

11. A comparison was made of the estimation of pentosans by measurement of the furfural produced by distillation with acid and by chromatographic methods. It was found that the furfural method yielded low values.

12. An examination was made of the composition of two cellulose products prepared by methods frequently used in agricultural chemistry. Both methods were found to remove a large part of the hemicelluloses but the recovery of the true cellulose was good. There was, however, more lignin in the products than had been expected.

PART II

THE APPLICATION OF A METHOD OF ANALYSIS DERIVED FROM THE WORK DESCRIBED IN PART I TO FIVE GRASSES CUT AT FOUR STAGES OF GROWTH

INTRODUCTION

The chemical composition of grass varies greatly according to its stage of growth. The stage of growth of a grass may be influenced by several factors including the nature of the soil, climatic conditions, time of year, application of fertilisers, age of the plants and the nature of any previous cutting or grazing. All of these conditions may be controlled or selected with the exception of climate.

Before the effect of different methods of management of grasses can be assessed, it is important to know the variations which normally occur as a result of the natural uninterrupted growth under average conditions. The gross changes in composition of grasses associated with the age of the plant have

been detailed by many investigators, with reference to such constituents as crude protein, lignin, ether extract, fibre and ash and the changes were found to be regular and progressive. The changes in the soluble sugars have been found to be much more complex (31, 33, 34).

There remained, amongst other variations yet to be examined, the nature of the changes undergone by the grass fibre and the structural polysaccharides and these have been investigated in the present work. The large and rapid changes in the quantity of the soluble sugars must be reflected by complementary changes in the other grass constituents, especially when these are expressed on a percentage basis. For this reason the grass samples were collected at growth stages which from previous work were known to be associated with distinct phases in the variations experienced by the soluble sugars.

The analysis of the samples was made in two stages, the first being the use of neutral extractants to isolate the grass fibre and the second the analysis of the polysaccharides in holocellulose prepared from the grass fibre. The changes in the amounts of

hemicelluloses and cellulosic polysaccharides were determined as well as their compositional variation. The analyses have been made on the whole serial parts of five common agricultural grasses and on the leaf, stem and head samples where these fractions could be separated.

EXPERIMENTAL

Soil

The grasses were grown in small plots in an overall area of 90ft. x 80ft., the geological nature of which was quite uniform. The land was in open country adjoining the Hannah Dairy Research Institute in Ayrshire. There was no shelter from sun or rain and only a slight shelter from north-south winds. The soil was a sandy loam without mineral deficiencies.

Grasses used

The grass samples were collected during the summer of 1955 from four plots of perennial grasses sown in 1953 and one plot of an annual grass sown in the spring of 1955.

Perennial grasses	Annual grass
Cocksfoot S143	Rye-grass, Western Wolths
Timothy S48	
Rye-grass S23	
Rye-grass S24	

The perennial grasses had been sown in April 1953 and cut and fertilised regularly in the succeeding years.

Management of the grasses

In March 1955 the perennial grasses were cut back to ½" and rolled; they were then treated with a mixed fertiliser that had a nitrogen:phosphorus: potassium (N:P:K) ratio of 12:4:12. The fertiliser was applied at the rate of 4 cwt./ac. The plot for the Western Wolths rye-grass was treated with 6 cwt./ac. mixed fertiliser and sown on April 30th. The weather was wet for the first week after sowing and generally suitable for germination. The summer was warm and dry but the annual grass was well established before any shortages of rainfall occurred.

Sample collection

The samples were cut at four stages of growth:

1. as soon as the grass could reasonably be handled,

i.e. about 10 cm. high.

2. just before the rapid growth phase began - this was determined by the examination of the growing points of a number of tillers and corresponded to an average height of 25 cm.

3. at the end of the rapid growth phase when an average height of 50 - 80 cm. had been reached.

4. after seed-shedding and approaching senescence, average height 60-90 cm.

All the samples were cut with hand shears close to the ground. They were always cut between 9 and 10 a.m. to minimise diurnal variations which are known to occur in soluble carbohydrate composition and may occur in the other constituents. The bulbous portion at the base of the stem of the timothy was not included in the samples.

The fresh grass sample was weighed, and, knowing the area of plot that had been cut, the approximate yield was calculated. The grass was then dried rapidly in a forced draught oven maintained at 100-110°C. This usually took about 40 minutes. From the loss in weight on drying the yield of dry matter was calculated.

The length of the samples was recorded as the average of 100 tillers of the fresh, cut material.

Some of the dried grass was separated into leaf, stem and head fractions and the ratio of the three fractions determined by weight. In the first two cuts from each grass the "stem" was in fact the tightly rolled leaf sheaths. The dried materials were ground in a hammer mill, to pass a sieve with holes 0.5 mm. in diameter and the powder then stored in brown bottles closed with wax corks.

The essential feature of the scheme for collecting and preserving the samples was that not more than 1 hr. should elapse between cutting the grass and reducing its moisture content to about 1-2%. It was considered (33) that this method would prevent enzymic changes from occurring in the samples but would not be drastic enough to cause chemical changes of any significance. A comparison of the results of the analysis of the soluble sugars in grass that had been treated with boiling ethyl alcohol with those for grass that had been oven dried by this equipment was made by Waite & Boyd (33). Typical results obtained by them are given in Table 38. They show that there

was no appreciable difference in the results by the two different methods.

Table 38. The analysis of soluble sugars in grass preserved by two methods (as % grass dry matter)

Treatment	Hexoses	Sucrose	Fructosan
Boiling alcohol	2.3	4.7	7.7
Oven dried	2.0	4.8	7.7

Analytical methods

Moisture, soluble sugars, crude protein and ash were determined on the grass samples as previously described (pp. 50-52).

Similarly the quantities of the ether-soluble, water-soluble and ammonium oxalate soluble fractions, as well as the weight of residual grass fibre were determined as previously (pp. 79- 81). There was one important modification in that only a portion of the ethanol-benzene extract was evaporated and partitioned for the determination of the ether-soluble fraction. The quantity of the water-soluble fraction was calculated as the sum of the water-soluble material from ethanol-benzene and the quantity determined

in the actual water extract.

For the determination of the water-soluble araban, the polysaccharides in the water extract were precipitated directly from a portion which had been concentrated and hydrolysed for ½ hr. at 100°C with N/100 sulphuric acid. The extract was not deionised before precipitation.

The crude pectin content of the ammonium oxalate extract was determined as previously (p. 73). It was found that when analysing samples of the heads of the grasses starch, if present, appeared to be almost completely extracted by the ammonium oxalate solution, and precipitated at the same time as the pectin. The starch was estimated simply by hydrolysing it in a 200 ml. portion of the ammonium oxalate extract. A period of 4 hr. at 100°C with the solution made normal with respect to sulphuric acid was satisfactory. The glucose in the solution was then estimated after paper chromatographic separation. The neutral polysaccharides associated with the pectin were also hydrolysed under these conditions but they do not normally contain any appreciable amount of glucose.

A comparison was made of the results obtained by the direct hydrolysis of the extract and by hydrolysis of a separated starch-iodine precipitate prepared by the method of Pucher et al. (188) from a sample of heads of Western Wolths. Determined by direct hydrolysis 1.8% of starch was found in the dried grass compared with 1.6% by hydrolysis of the isolated starch precipitate.

The sum of the ether-soluble, water-soluble and ammonium oxalate soluble materials plus the grass fibre (as a percentage of the original dried grass) was usually slightly less than 100 but serious discrepancies were rare.

The ash, crude protein, lignin and acetyl contents of the grass fibre were determined as described previously (pp. 98, 115), and the remaining material presumed to be polysaccharides.

After pepsin treatment the chlorite holocellulose was prepared as already described (p. 115) and the ash, crude protein, lignin and acetyl contents re-determined. The polysaccharides were again calculated by difference.

The holocellulose was extracted successively with water 1% potassium hydroxide and N sulphuric acid as

Table 39. The stage of growth and production of grass at each cut

Cut no.	Date	Height (cm.) (Average of 100 tillers)	Stage of growth	Ratio (dry wt.) Leaf:stem:head	Moisture (% of fresh wt.)	Yield of dry matter (g./sq. yd.)
Rye-grass S24						
1	22.4.55	11.1	Growing point just floral	5 : 1 : 0	74.6	100
2	19.5.55	17.7	Growing point floral	1.2 : 1 : 0	74.8	300
3	4.7.55	52.2	Anthesis commenced	1 : 5.6 : 1.9	67.8	550
4	12.9.55	54.0	Seed set	1 : 10.4 : 1.8	57.3	200*
Rye-grass S23						
1	22.4.55	10.7	Growing point vegetative	10 : 1 : 0	72.7	100
2	14.6.55	23.5	10% of tillers headed	1.1 : 1 : 0	74.4	300
3	19.7.55	62.8	Anthesis	1 : 10.9 : 3.6	61.6	325*
4	13.9.55	57.0	Seed set	1 : 4 : 1.1	65.1	230*
Timothy						
1	25.4.55	11.9	Growing point vegetative	4 : 1 : 0	73.3	100
2	30.5.55	27.3	Growing point floral	2 : 1 : 0	73.4	350
3	12.7.55	76.5	Anthesis	1 : 3.5 : 0.7	62.7	410
4	14.9.55	88.0	Much of the head material blown away.	1 : 4.9 : 0.1	45.5	680*
Cocksfoot						
1	25.4.55	11.7	Growing point vegetative	5 : 1 : 0	78.0	100
2	26.5.55	26.4	50% of tillers headed	1.6 : 1 : 0	78.4	300
3	6.7.55	81.5	Anthesis	1 : 8.2 : 1.7	65.8	200*
4	16.9.55	86.5	Seed set	1 : 6.3 : 0.9	39.8	210*
Western Wolths						
1	21.6.55	18.4	15% of tillers headed	0.5 : 1 : 0	83.1	65
2	1.7.55	35.5	90% of tillers headed	1 : 4.8 : 1.2	82.6	170
3	18.7.55	61.0	Anthesis	1 : 8.2 : 3.1	68.0	450
4	12.8.55	60.5	Seed shedding	1 : 16.7 : 7.3	56.8	270

* These samples also included new growth which was not included in the yield.

already described (p. 135) and the amounts of the sugars in the hydrolysed extracts determined by paper chromatography. The extracted residue was dissolved in 72% sulphuric acid and the amount of glucose and xylose determined. The unextracted holocellulose was also analysed for uronic acid.

RESULTS

The stage of growth characteristics of the grasses at each cutting are given in Table 39. It will be seen that the date of collection of the 2nd and 3rd samples of each grass had been judged fairly accurately to correspond with the beginning and end of the period of rapid growth, i.e. the change of the growing point from a vegetative to a floral state and the production of anthers by the flowering head.

The yield of dry matter per sq.yd. from each of the perennial grasses shows the presence of what was recorded as "new growth". This description may have been essentially correct in that the material, which was shorter and at an earlier stage of growth than the main sample, might have been the growth of secondary

tillers. In view of the decrease in measured yield at the later cuts, it seems possible also that the new growth may have been part of the main production which had been retarded by competion by the grass that had developed faster. For the purpose of this work, only the principle growth from each plot was used.

Another apparent anomaly was in the values of the leaf-stem ratio. In general the proportion of stem increased with the age of the tillers but with the last cut from the S23 rye-grass and cocksfoot the amount of stem had decreased. This was most likely caused by marked withering of the leaves, which brought about the utilisation of the available nutrients from the active stem but without any replacement being supplied by the dying leaves.

The results of the proximate analyses by the present system are given in Table 40. (Tables 40, 41 and 42 are on pp. 202 - 204). As expected, the amounts of ether-soluble material and crude protein in the leaf samples were greater than in the stem samples and the amount of both in all portions of the grasses generally decreased with age.

A particularly interesting observation during

the analysis of the water-soluble araban was the detection and estimation of small quantities of ribose in the hydrolysates of the soluble polysaccharide preparations from the cocksfoot samples. The quantities of the araban are not shown as they were all small (0 - 1.0% of the grass).

The ammonium oxalate extract had only slight irregular variations with stage of growth though the values for the leaf samples were consistently higher than those for the stem. Almost every grass yielded at least one sample which appeared to have an unusually large oxalate extract. This might have been caused by an error in the previous water extraction, but in each of these instances the value for crude pectin had risen correspondingly. The amount of starch determined in the oxalate extract from the head samples was never very great, about 1% of grass dry matter, since such samples included much structural material other than the actual seeds.

The differences in the quantities of the extracts of the different grasses examined at corresponding stages of growth were very slight, the only significant variations being between the water extracts

which were caused by the expected variations in the soluble sugars.

The total amount of the grass fibre did not vary markedly between the different grasses, but in the whole samples the cocksfoot had generally the most whilst the annual rye-grass had the least. In the latter grass the increase in fibre during the growing period was much less than in the other grasses. The variation in the values of the amount of the grass fibre was made up mainly of the simultaneous variations of its protein, lignin and polysaccharide constituents as shown in Table 41. The changes in the ash and acetyl groupings were comparatively small. In the grass fibre the quantity of crude protein decreased rapidly with the age of the grass but was always directly related to the total crude protein in the grass. The lignin and the polysaccharides increased in a regular manner in most samples and both had their largest values in the cocksfoot samples which resulted in the amount of the fibre being greatest in this grass. In all the grasses the fibre from the leaf fractions had less lignin and more crude protein than the stem fibre.

The distribution of the polysaccharides was much more complex. For example the fibre of the leaf and stem of the S23 rye-grass had very similar amounts of polysaccharides but in the S24 rye-grass the leaf fibre had slightly more. The analyses of the cocksfoot samples, and by calculation those of the Western Wolths, indicated that the stem contained slightly more polysaccharides than the leaves and that this tendency increased with the age of the grass. The timothy samples were again different, for in the second cut the amount of polysaccharides in the leaf fibre was less than in the stem, at the third cut the amounts were very similar and at the fourth cut the stem had the greater amount.

The analyses of the holocellulose preparations obtained from each of the grass fibres are also shown in Table 41 where it may be seen that the lignin content had generally been reduced to 1.5 - 3.0%, with the larger values remaining from grass fibre samples having most lignin. The presence of material which was possibly a soluble lignin derivative has already been reported (p.109). With very few exceptions the crude protein values were

Fig.8 POLYSACCHARIDES IN GRASS HOLOCELLULOSE

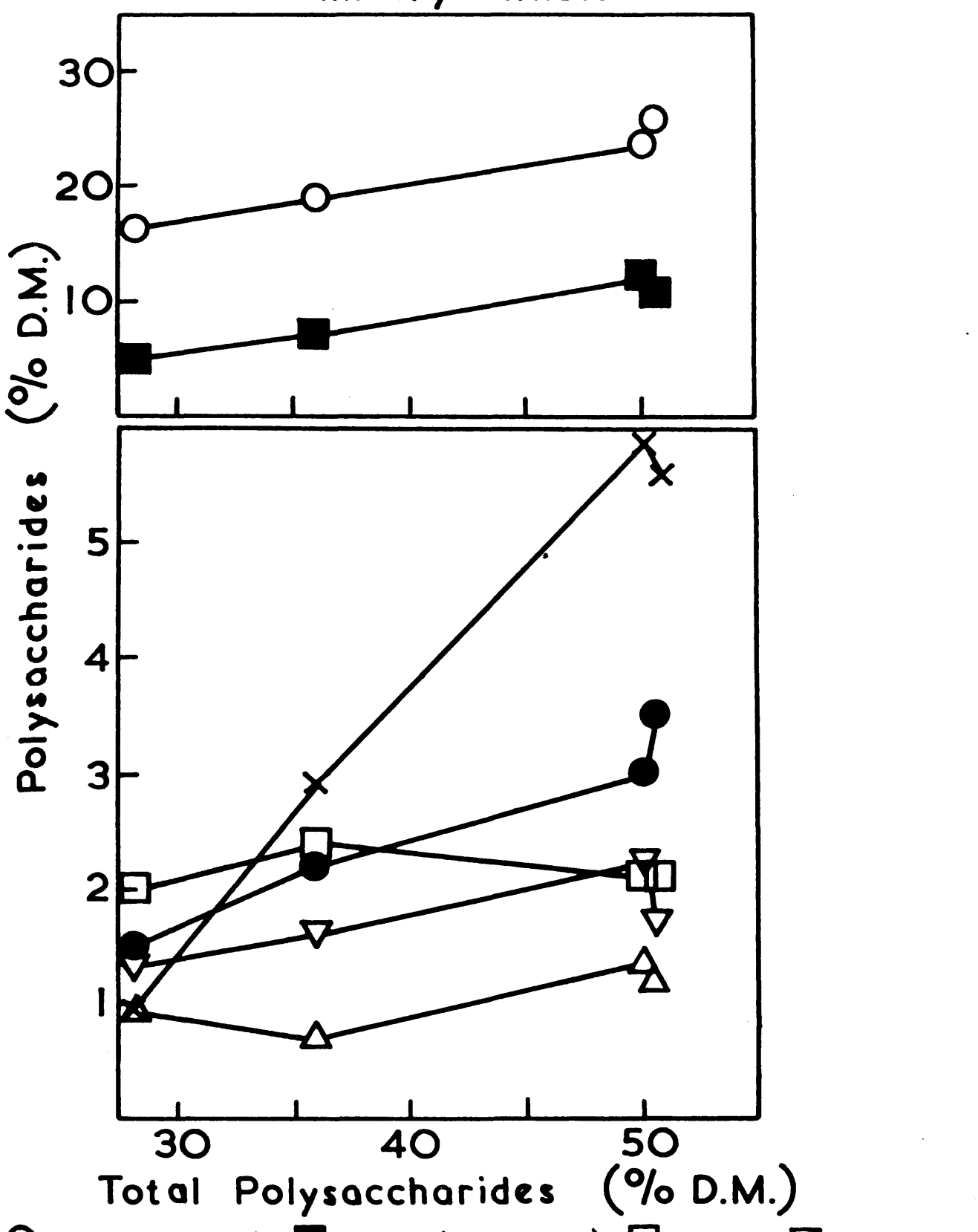
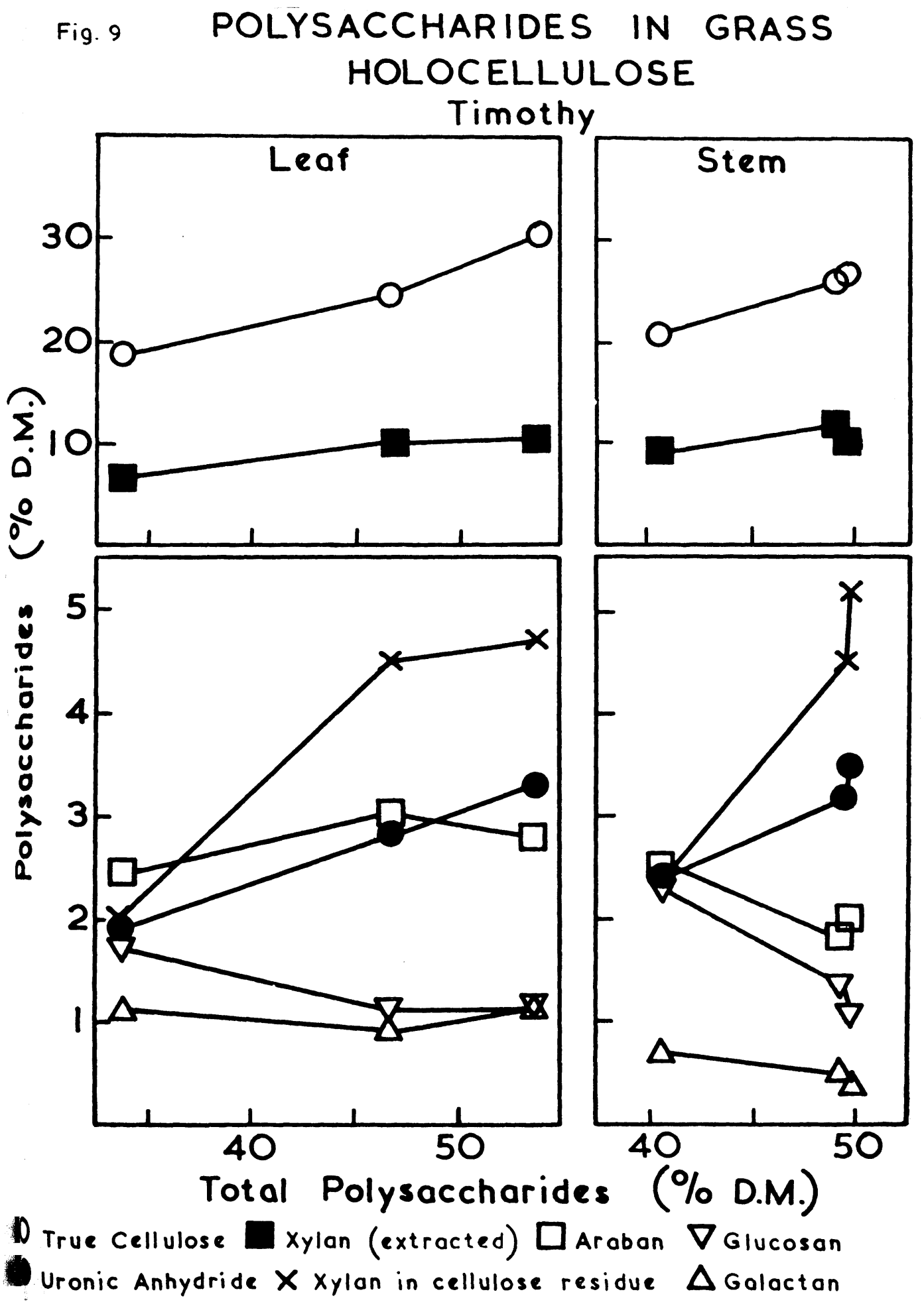


Fig. 9


POLYSACCHARIDES IN GRASS HOLOCELLULOSE
Timothy
Leaf
Stem
30
20
10
5
4
3
2
1
Polysaccharides (% D.M.)
40
50
40
50
Total Polysaccharides (% D.M.)
True Cellulose
Xylan (extracted)
Araban
Glucosan
Uronic Anhydride
Xylan in cellulose residue
Galactan

reduced to less than 1.0% whilst the amount of ash and acetyl groupings were largely unchanged. The losses of polysaccharides appear to be quite small except in the holocelluloses prepared from some high protein, low lignin fibres. These recoveries, however, were very dependent upon the accuracy of the lignin estimation as has already been shown (pp. 106 - 111).

The polysaccharides in each of the holocellulose fractions were analysed by the progressive extraction methods previously described (p. 135). The detailed results obtained from the individual extractions are given in the Appendix but the total carbohydrates so determined are shown in Table 42 and Figs. 8 - 13. The values for total polysaccharides in these Figures are those actually determined in the holocelluloses. The polysaccharides have been reported as the quantities of the anhydro sugar residues in the arbitrarily separated hemicelluloses and cellulose residue.

The total polysaccharides in the holocellulose determined analytically show good agreement with the amounts calculated by difference except for the last cuts of each grass and in particular for the stem

Fig.10 POLYSACCHARIDES IN GRASS HOLOCELLULOSE

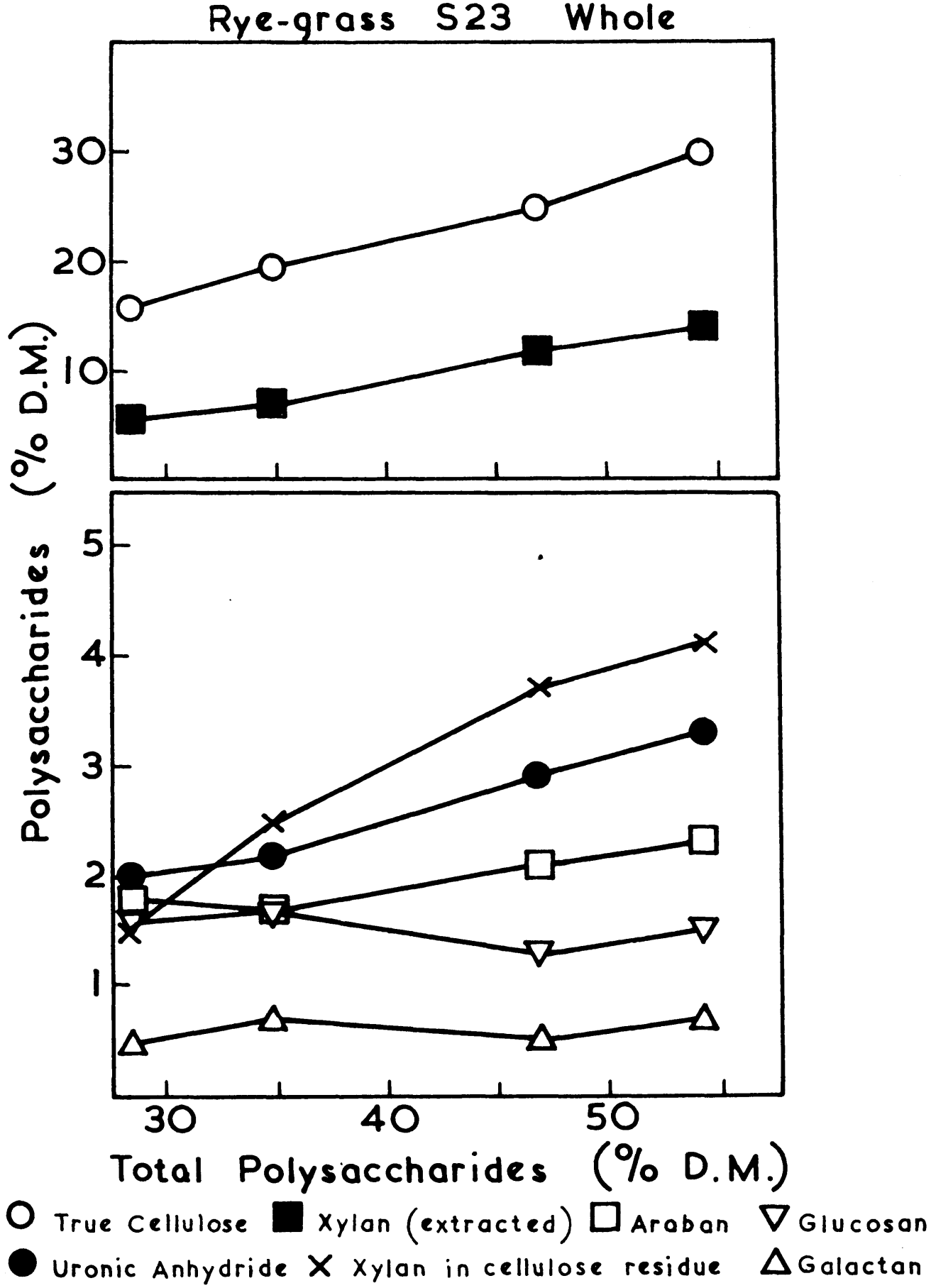
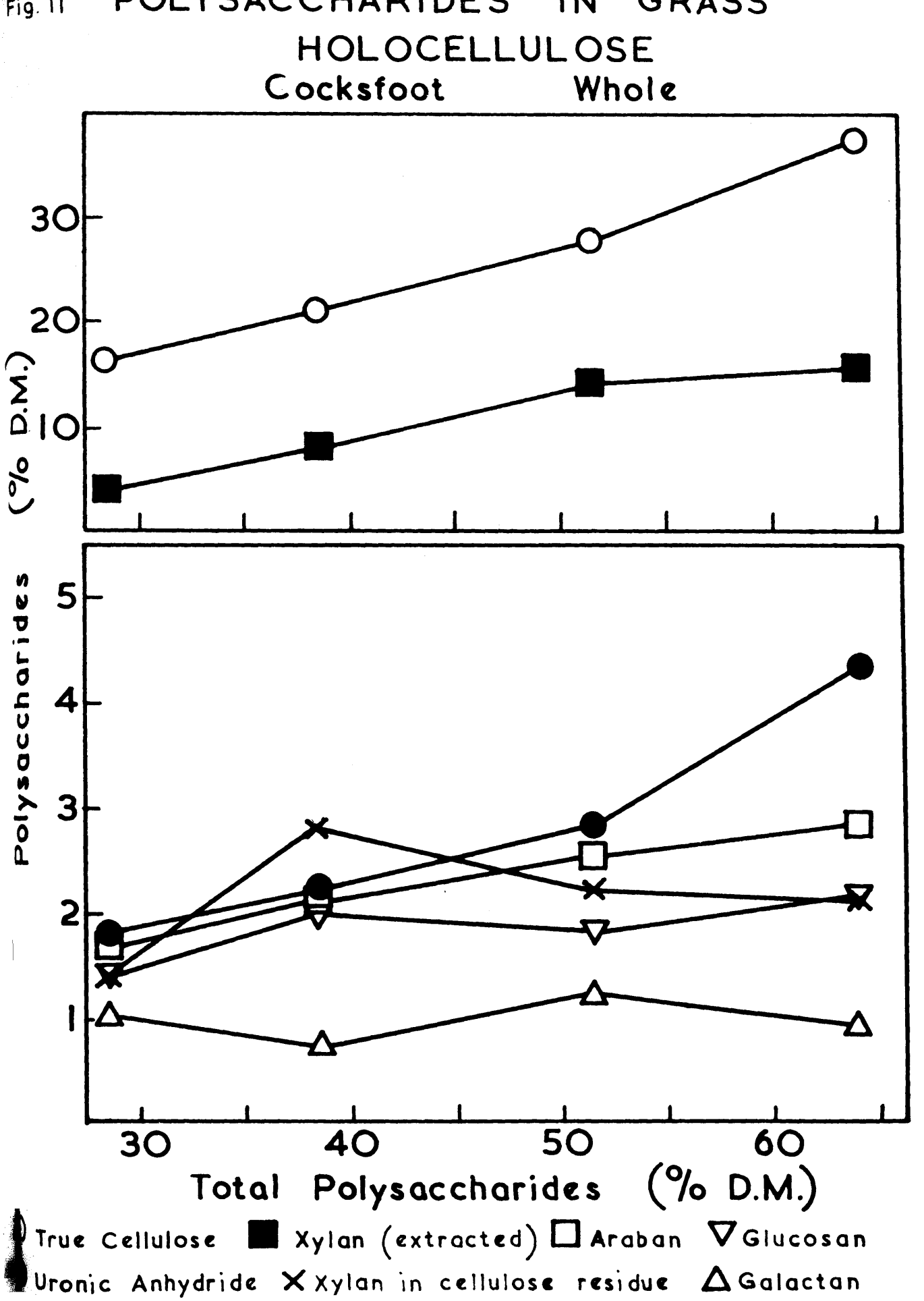


Fig. 11


POLYSACCHARIDES IN GRASS
HOLOCELLULOSE
Cocksfoot Whole
30
20
10
(% D.M.)
Polysaccharides
5
4
3
2
1
30
40
50
60
Total Polysaccharides (% D.M.)
True Cellulose
Xylan (extracted)
Araban
Glucosan
Uronic Anhydride
Xylan in cellulose residue
Galactan

samples. These discrepancies probably arose from the losses of alcohol-soluble lignin (cf. pp. 106-111) formed during the preparation of the holocellulose and hence they occurred particularly in the results for the most lignified materials. The amount of cellulose residue from each holocellulose after the three extractions was found to be directly related to the total polysaccharide of each grass, the highest proportion being found in the annual rye-grass. Only glucose and xylose were found in the cellulose residue in determinable amounts and the proportion of xylose to glucose increased in successive cuts. The xylose-glucose ratio was usually greater in the stem than the leaf and appeared to show very little difference between the different species. Thus the results for rye-grass (S23) samples were as shown in Table 43.

Fig. 12

POLYSACCHARIDES IN GRASS HOLOCELLULOSE

Rye-grass S24 Whole

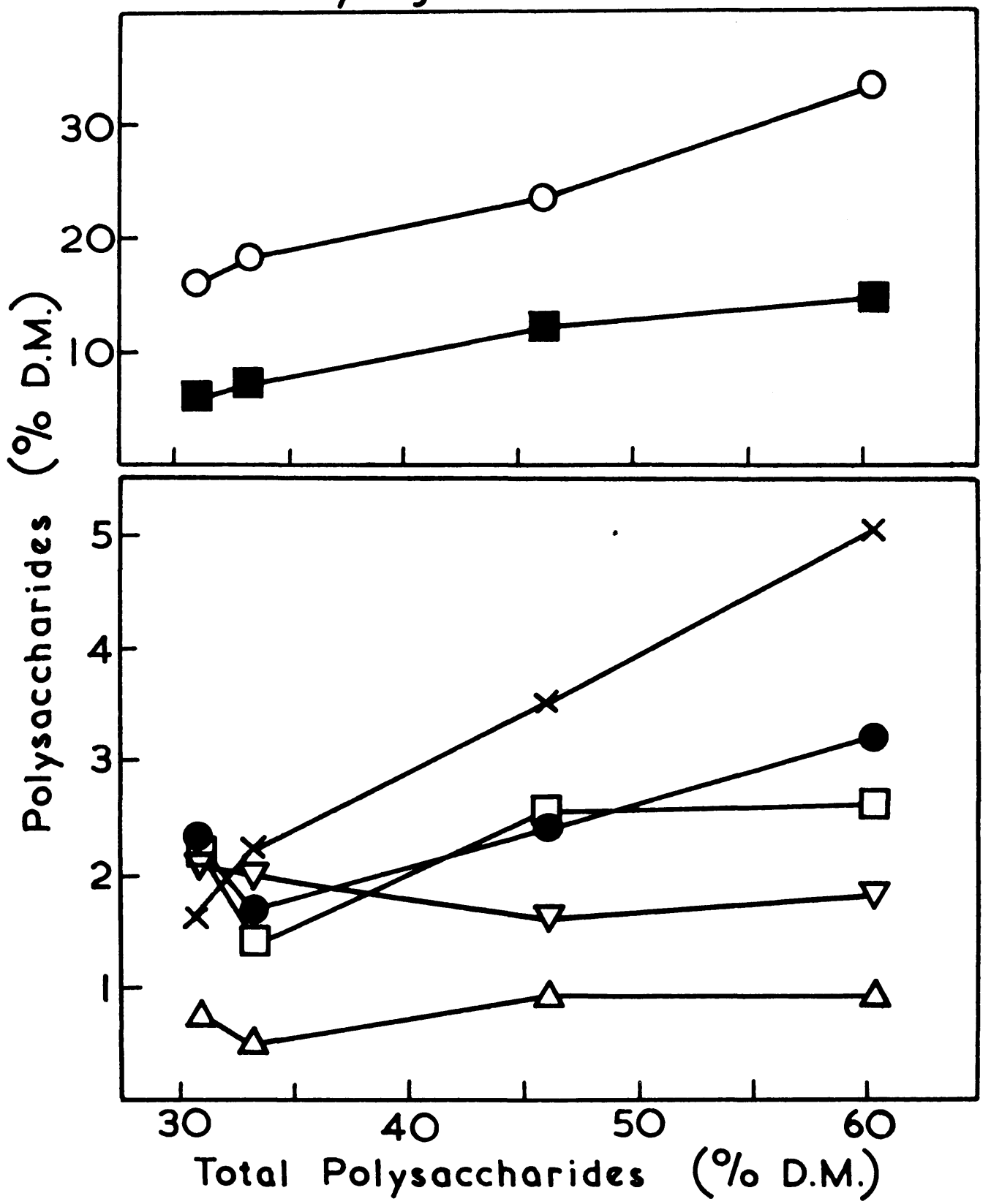


Fig. 13 POLYSACCHARIDES IN GRASS HOLOCELLULOSE

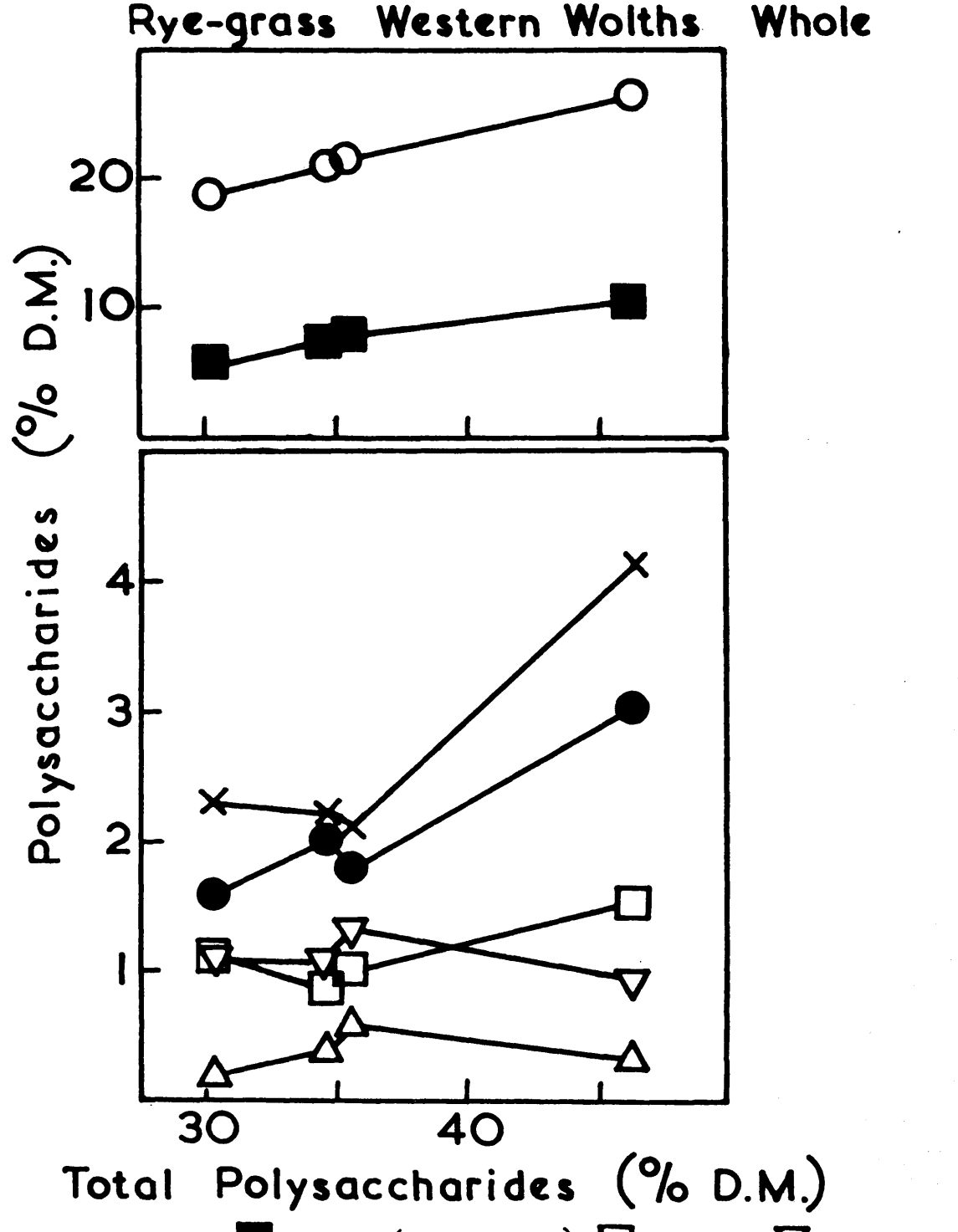


Table 43. The ratio of xylan to glucosan in the cellulose residue of rye-grass S23 holocelluloses

Cut no.	Whole samples	Leaf samples	Stem samples
1	1:10.0	-	-
2	1: 7.7	1:11.4	1: 7.7
3	1: 6.7	-	1: 8.9
4	1: 7.1	1:10.8	1: 5.8

The hemicellulose hydrolysates of the holocelluloses all contained galactose, glucose, arabinose, uronic acids and xylose with the last sugar being the major component. The principal change as the age of the plant increased was the rise in the xylan content. The quantities of galactan, glucosan and araban on the other hand, remained almost constant throughout but the uronic anhydride exhibited a slight though definite increase with increasing age of the grass.

DISCUSSION

The present view of the metabolism of plants during their normal life cycle is that all the

soluble constituents are in a dynamic state. The intermediates in the photosynthetic cycle are, for example, continuously being made and degraded. The position with regard to the insoluble materials is not so apparent. These compounds are essentially of a structural nature and whilst it has been comparatively easy to demonstrate that cellulose and lignin are formed readily from soluble precursors, it has as yet been impossible to show directly whether these materials, once formed, can or do take any further part in the metabolism of the plant while the plant tissues are still active.

The amount of structural carbohydrates in pears has been shown to decrease after picking (189) and it seems quite reasonable to assume that the liberated material is used for respiration.

Other fruits have been examined and similar findings have been reported by other workers and discussed by Jermyn and Isherwood (189) and it has been known for many years that germinating seeds utilise their cell wall polysaccharides as though they were a food reserve comparable to starch (190). Jermyn and Isherwood's (189) work strikingly

illustrated the active relationship between the cell wall polysaccharides and the soluble constituents of pears stored after picking. After a period during which the quantity of polysaccharides decreased, a stage of over-ripeness was reached at which there appeared to be re-synthesis of polysaccharides. The cell wall polysaccharides of beans have also been shown to be used during starvation of the plant (191). No such results, however, have been recorded for intact grass plants although fermentative changes after cutting, such as those which occur during ensilage, are known to affect the cell wall polysaccharides (192).

Soluble constituents

The grass constituents which are easily extracted (e.g. with water) are associated with the cell juices and it appears that their quantity in a grass is influenced by the ratio of the number of active growing cells to the number of mature cells, for as the grass ages the percentage of readily soluble materials in the dried sample decreases. In this connection it is important to remember that although only a small part of the protein content of a grass sample may be extracted after drying (as in the methods used in the

present work), if the fresh tissue is given plasmolytic treatment (193) a larger recovery of protein is possible. It therefore seems advisable to consider the protein constituents of grasses as being part of the soluble components, despite the fact that when the dried samples are analysed as in the present work, the majority of such material is retained in the grass fibre.

The ether-soluble material, organic acids, ash and protein are thus all part of the soluble fraction of grasses and the concentration of each of them in the dried samples has been found to decrease with increasing age of the grass, i.e. as the relative proportion of mature or dying cells has increased. The function of each of these groups of compounds is closely connected with active metabolism of the plant. One apparent purpose of the ether-soluble materials is to render the outer cells of the tissue water-proof and prevent leaching of their contents by rain, but the fraction also includes pigments such as chlorophyll which is essential for photosynthesis. It has been suggested also that other pigments may be protective agents, absorbing harmful radiation from

the solar spectrum (25).

The variations in the values for the water extract with age of the grass are mainly caused by the fluctuations in the amount of the total soluble sugars. The water extract has been shown (Part I A) to be almost entirely composed of soluble sugars and ionic material, the latter including part of the normal ash and the organic acids. Both the ash and the organic acid contents vary with age in a manner analagous to that of the ether extract, decreasing steadily with development of the grass. This decrease corresponded to the difference between the quantity of water extract and the soluble sugars in the present results. The amounts of ash and of organic acids were greater in the leaf samples than in the stem samples possibly because of the more structural nature of the stem.

The organic acids and the ash are not entirely unrelated. There are two reasons for this. Firstly, the total ash of plant material includes a certain amount arising from the conversion of the organic acids to carbonates on incineration. Secondly, the presence

of organic acids requires the presence of an equivalent amount of cations (194), which although they may be composed in part of basic nitrogenous compounds are also very largely metal ions.

The acids are presumably required by the plants for the later stages of the processes of respiration (some form of the Krebs cycle) though Brummond and Burris (195) have suggested that this mechanism may not be of great importance in all plants, and the isolation of malonic acid in grasses, an inhibitor of such systems, by Hulme and Richardson (121) might suggest that grasses are a particular instance of this lesser dependence.

The quantity of soluble polysaccharide other than fructosan was determined in all the water extracts but the values have in general been insignificant, less than 1% of the dry matter of the grass. The hydrolysates of this polysaccharide from the cocksfoot samples all contained a very small amount of ribose, which was absent from all the other grasses studied.

This sugar is not thought to be formed by epimerisation of the arabinose in the solution, since

by trial this reaction required much more rigorous treatment than was encountered during the neutralisation of the hydrolysates, and the epimer would have been expected to have been produced in many more instances.

Measurement of the absorption spectrum of the solution of the polysaccharide hydrolysate (N sulphuric acid at 100°C, 2 hr.) was consistent with the presence of compounds of the ribonucleotide group and the ribose may have been combined before hydrolysis, by some labile linkage. This hypothetical bonding would need to allow hydrolysis of the attached ribose far more easily than the hydrolysis of the constituent ribose of the nucleotide.

Ribose was detected in the samples of cocksfoot at all stages of growth and hence does not appear to be associated only with young high protein material.

The total crude protein in the grasses showed the usual changes, in that the leaf had more than the stem fractions and that the amount in all parts of the plant decreased with age. Most of the crude protein (70%) was determined in the grass fibre and slightly greater amounts were found in the fibre from leaves than in the fibre from stems.

Pectin is often associated with actively growing tissue, although it has been suggested (59) that pectin may act as a precursor of both hemicelluloses and lignin. In the present work the percentage of pectin remained substantially constant at the four stages of growth. This suggests either that the pectin associated with each cell was not utilised for other compounds or that there was a constant usage and replacement.

The soluble sugars, which so far have been omitted from this discussion are both intermediary metabolites and temporary storage products. The marked variations that occur in the concentration of the soluble sugars in grasses have been examined in particular by Waite and Boyd (33, 34) and Wylam (31) and have been found to be dependent upon the relative synthetic power and energy requirements of the plants at any particular time. Thus periods of reduced sunlight cause a decline in synthetic activity and hence in fructosan content, whilst in the period prior to the rapid elongation of the stem of established perennial grasses the energy requirements are low and an accumulation of fructosan occurs which

disappears as the rate of growth increases.

Insoluble constituents

It has already been mentioned that most of the protein found in the isolated grass fibre is present because the previous conditions of extraction were unsuitable for extraction of protein. For that reason its presence will not be considered in the following attempt to elucidate the nature of the cell wall components, in the light of the results obtained.

The function of the cell wall changes with increasing age. In young cells the wall probably acts as a semi-permeable membrane but later the wall thickens as more polysaccharides and lignin are produced. The cell wall is then able to confer rigidity to the plant. The total amount of grass fibre as a percentage of the original dry matter changed with advancing age in a very similar way in each of the grasses examined and in the leaf and stem fractions of each grass. The variation was made up in general of a regular increase in the polysaccharides and the lignin as the tissues aged, with a corresponding decrease in the protein content. In the samples from the rye-grasses S23 and S24 the main change in

composition from the first to the second cuts was the large increase in the soluble sugars. This was reflected in the grass fibre by the low percentage of polysaccharides, which did not increase as much with age as in the samples of timothy and cocksfoot. The amount of grass fibre in the rye-grasses was in fact less in the second cut than in the first. The amount of fibre from later cuts of all the perennial grasses increased in a regular manner. The greater proportion of soluble sugars in the rye-grasses resulted also in a greater fall in protein percentage in the fibre between the first and the second cuts but the yield of protein was very similar for these cuts.

It might have been thought that the stem fraction would contain more fibre than the leaf but in fact equal percentages were found. This is understandable if it is accepted that structural strength is conferred more by lignified polysaccharides than by polysaccharides *per se* and more lignin and less protein were associated with stem fibre than with leaf. Using the yield data there was an increase in the weight of stem fibre per unit area as the grass

aged but a decrease in the amount of leaf fibre. For example, in the leaf of timothy there was a decrease in the weights of fibre between the second and third samples from approximately 100 g./sq.yd. to 50 g./sq.yd., whereas in the stem samples there was an increase from 60 to 200 g./sq.yd.

The proportion of cellulose to the other polysaccharides remained substantially constant at each of the four cuts. This constant ratio was achieved by a slightly greater increase in the amount of xylan than in the amount of cellulose, to compensate for the only slight changes in the values of the extracted galactan, glucosan and araban.

In the perennial grasses the amount of extracted xylan (as a percentage of the original grass) was always large in proportion to the total hemicelluloses and in many samples it was more than doubled during the growing period. In all the whole grasses, for example, the ratio of xylan to all other hemicelluloses was about 2:1 in the first cuts and in the fourth cut the ratio had risen to 3:1. The xylan was present in the leaf and stem holocelluloses in about equal amounts when compared at the same total polysaccharide content (Fig. 8 and Table 42). At any particular cut,

however, the stem had a slightly greater proportion of xylan than the leaf, even although the amount of total polysaccharides was occasionally greater in the leaf fraction, as, for instance in the S24 ryegrass. The amount of xylan in the holocelluloses of each grass and grass fraction at corresponding cuts was very similar.

It had been hoped that the use of three different extraction reagents might have resulted in a more definite fractionation of the polysaccharides extracted from the holocellulose. The results (Appendix) show however that this was not accomplished satisfactorily, for the composition of the successive extracts did not change greatly. There was, however, an increase in the proportion of xylose to other sugars in the potassium hydroxide and sulphuric acid extracts as compared with the water extract so that some fractionation was achieved.

The composition and amount of the grass fibre from each of the perennial grasses showed similar changes with increasing age and although the values obtained in the analysis of the annual rye-grass, Western Wolths, showed the same general trends there

were certain differences which might be attributable to the differences between the growth habits of the grasses. In particular, the total grass fibre never reached the same values as in the perennial grasses due mainly to a lower level of polysaccharides. At the same time there was more protein and less lignin in the fibre from the annual grass samples, presumably because of the rapid rate of growth of the annual grass. There were greater differences between the composition of the stem and the values calculated for the leaf fractions than in the other grasses. In particular, the stem had greater polysaccharide content than the leaves. There was a greater proportion of true cellulose in the polysaccharides of the Western Wolths rye-grass than in the perennial grasses. Although the hemicellulose content of the Western Wolths was less than the perennial grasses its composition was similar to those from the other grasses. There was also a larger amount of lignin in the samples of the annual rye-grass than in the samples from other grasses with similar polysaccharide contents. The high proportion of cellulose in the Western Wolths was therefore associated with more lignin. This finding suggests that in the annual

grass, which has to complete its life cycle in a shorter time than the perennials the plant synthesises a structural unit of cellulose and lignin to obtain mechanical strength, rather than the hemicelluloses found in other grasses. An examination of rye-grass (S24) in its first year of growth (85) showed that the proportion of cellulose to total polysaccharides was also higher than in established perennial grasses, such as those investigated in the present work. The need for this greater proportion of cellulose in the first than in the later years of growth is most likely to be similar to that suggested above for the annual rye-grass, but the lignin content of the first year perennial rye-grass was less than in other grasses.

The decline in the proportion of non-xylosic components of the cell wall hemicelluloses in all the grasses may be interpreted in at least two ways. One theory would be that they are precursors of some other constituents. The most frequently suggested compound is lignin but this would require that the polysaccharides be rendered soluble if the synthesis of lignin through sedoheptulose and shikimic acid is

to be followed. The polysaccharides which were extracted from the holocellulose with hot water might be used by the plant for this purpose. An alternative theory takes account of the fact that the non-xylosic polysaccharides are likely to be highly branched or have only short chain structures. They may then become interwoven with the larger straight chain molecules of xylan and cellulose. If these branched polymers form a covering to the fibrils or molecular bundles of the other polysaccharides, then as these increase in diameter with age, the proportion of non-xylosic polysaccharide would decrease. The branched polymers cannot be evenly distributed amongst all the long chain molecules otherwise they would be there in constant proportion.

Since the total values for the uronic anhydride and extracted xylan increased similarly, it might appear that they were associated. Most of the uronic acids were extracted from the holocelluloses by hot water but usually only about half the xylan, so there was obviously part of the xylan which was not combined with more than a few uronic acid units. The uronic acids would be expected to be part of highly branched

polysaccharides, as would the galactan, glucosan and araban which were determined in the hemicelluloses. These three polysaccharides, like the xylan, were found in all the extracts so only part of them could be associated with the uronic acids. The simplest mixture of polysaccharides that can be postulated to explain these results is a highly branched polysaccharide which is extractable by water and which contains galactose, glucose, arabinose, xylose and uronic acids and a similar polysaccharide which is not so easily extracted and which has a much smaller amount of uronic acid but more xylose.

The ratio of xylose to glucose in the hydrolysate of the cellulose residue after extraction of the hemicelluloses from the holocellulose, increased with age despite almost all of the lignin having been removed in the earlier stages. On the other hand there was also a tendency for the lignin content of the holocellulose to increase with the age of the grass and this probably affected the extraction of xylan with the subsequent reagents, particularly the hot water which preceded the alkali and acid.

Conclusions

The structural polysaccharides of the five grasses studied have thus been shown to change in a regular manner and to exhibit only slight differences between species. The percentage of the total polysaccharides increased with age but the actual changes between cuts were in certain instances reflections of the change in the other constituents of the samples, especially changes in the amounts of the soluble sugars. For instance, the increase in total polysaccharides between the first and second cuts of the perennial rye-grasses was much less than between similar cuts of the other perennial grasses.

In the hydrolysates of the extracted polysaccharides xylose was the major sugar and this was the only neutral component of these polysaccharides to increase in amount appreciably with age. This increase took place rapidly and at a rate which was slightly greater than the rise in the true cellulose content. The uronic acids also increased with age.

The polysaccharide components of the leaf and stem fractions were similar in amount and composition but the stem had more lignin than the leaf. This

agreed with the suggestion that cellulose and lignin are the main factors imparting mechanical strength to grasses, for the annual rye-grass appeared to produce them in preference to the hemicelluloses.

The work in this part of the thesis was not primarily concerned with the total analysis of the samples by a method which would yield a more comprehensive analysis than the Weende system. It was possible, however, to obtain from the data almost the whole of the composition of the samples. For example, with the first whole sample of rye-grass (S23) over 92% of the dry matter was accounted for from the sum of the ash, crude protein, ether extract, soluble sugars, crude pectin, lignin and polysaccharides.

SUMMARY OF PART II

1. The purpose of the work done in Part II was to examine the structural polysaccharides of four established perennial grasses and one annual grass, each of which was cut at four stages of growth.

2. The examination was made by applying the methods of successive extraction developed in Part IA to dried samples of the aerial parts of the grasses. The grass fibre thus produced and holocellulose prepared from it were examined as described in Part IB.

3. The grass fibre contained ash, lignin, structural polysaccharides and most of the protein originally in the grass.

4. The most important result was that there were only slight differences between the amount and composition of the fibre from different species of perennial grasses cut at the same stage of growth. The analytical values of the fibre of the annual grass showed the same pattern but certain differences (noted below) were observed which might reasonably be taken as characteristic of an annual habit of growth.

5. The analytical results for the corresponding leaf and stem fractions of all the grasses were also quite similar, though the results suggest that there was a rather greater difference between the leaf and stem of the annual grass than between those of the perennials.

6. In all grasses the yield of grass fibre from the whole plant increased with age but in the perennial rye-grasses there was a very rapid increase in soluble sugars between the first and second cuts which was accompanied by a corresponding decrease in the proportion of fibre.

The later cuts of the annual grass had less fibre that the later cuts of the perennials.

7. The polysaccharides in the holocelluloses from all the grass fibres contained fractions which have been called hemicelluloses and cellulosic residues. The former were the more easily extracted polysaccharides and on hydrolysis with acid they yielded galactose, glucose, arabinose, xylose and uronic acids. The cellulosic residues when hydrolysed yielded glucose (from true cellulose) and a small amount of xylose.

8. The amount of hemicellulose and the cellulosic residue increased with the age of the grasses.

9. In all grasses xylan was the major component of the hemicelluloses and the increase in the hemicellulose content with age was due mainly to the increase in the xylan content.

10. The ratio of the true cellulose to the total

polysaccharides was constant for each grass in all cuts. The ratio was similar for all the perennial grasses but was greater in the annual grass.

11. The annual grass, as well as having a lower hemicellulose content, also contained more lignin than the perennials. It is suggested that to establish a firm structure for the rapidly growing plant, cell walls of mainly cellulose and lignin were produced with a smaller proportion of hemicelluloses than were found in the perennial grasses.

12. No definite fractionation of polysaccharides was found to take place when the extractants chosen were employed to study the holocellulose, although almost half the extractable xylan and most of the uronic acids were recovered in the water extract. A possible explanation based on a simple system of polysaccharides has been put forward for this.

13. The cellulosic residues always contained xylan which it is suggested remained because of the presence of lignin in the holocellulose.

14. The total polysaccharides determined in the holocelluloses compared well with the value calculated from the general analysis of the grass fibre and

holocelluloses, except in some samples in which the fibre had contained large quantities of lignin. The presence of alcohol-soluble lignin derivatives which were formed during the preparation of holocellulose was suggested as the cause of the discrepancies.

Table 40. Analysis of the grasses at each cut, by extraction, together with the values for some constituents (as % of the original grass)

Grass	Cut no.	Ether-soluble	Water extract	Ammonium oxalate extract	Grass fibre	Sum	Crude protein	Total soluble sugars	Ash	Crude pectin
Rye-grass S24										
Whole	1	.6.0	36.6	5.2	51.2	99.0	17.8	17.7	9.7	2.3
	2	4.0	46.9	4.4	45.1	100.4	7.5	31.5	7.2	2.3
	3	2.8	31.5	3.8	60.3	98.4	3.7	20.6	4.6	2.3
	4	2.6	11.6	5.6	80.5	100.3	3.7	1.8	4.6	2.1
Leaf	2	4.8	45.2	4.8	43.3	98.1	9.5	27.9	9.7	2.1
	3	4.4	24.2	7.1	66.1	101.8	5.3	8.8	7.0	2.6
	4		Insufficient sample				4.9	1.4	7.0	
Stem	2	2.7	49.7	4.4	42.5	99.3	5.7	37.3	4.8	2.4
	3	1.4	39.6	3.3	55.4	99.7	2.4	28.4	3.3	1.8
	4	1.7	11.0	4.2	83.5	100.4	2.2	2.4	3.4	1.1
Heads	3	3.3	27.4	3.9	63.7	98.3			5.0	2.2
Rye-grass S23										
Whole	1	8.0	31.1	5.3	53.3	97.7	20.9	15.8	10.1	2.5
	2	3.6	40.9	3.7	48.9	97,1	6.1	27.1	5.9	2.5
	3	2.7	29.8	3.6	62.7	98.8	3.4	17.7	4.2	2.2
	4	2.8	14.3	4.7	76.1	97.9	3.1	4.2	3.9	2.9
Leaf	2	5.4	40.8	4.7	49.4	100.3	7.8	22.1	8.5	2.7
	3		Insufficient sample						6.3	
	4	5.0	10.1	7.2	78.4	100.7	4.6	1.1	4.2	2.9
Stem	2	2.2	46.1	3.2	47.2	98.7	4.2	34.5	4.3	1.7
	3	1.7	28.8	2.6	57.9	101.0	1.9	27.0	3.8	1.6
	4	1.8	18.7	3.1	76.7	100.3	2.1	8.2	3.6	1.3
Heads	3	3.4	22.1	4.8	68.8	99.1			4.6	1.7
	4	2.4	6.9	12.2	76.0	97.5			3.5	5.6
Timothy S48										
Whole	1	7.2	36.1	4.9	48.8	97.0	16.4	19.9	8.3	3.5
	2	3.3	37.3	4.7	53.5	98.8	8.6	19.7	6.4	4.2
	3	3.5	27.2	3.7	65.9	100.3	4.6	14.2	3.2	2.1
	4	2.3	21.8	5.7	71.6	101.4	2.4	11.4	3.1	2.0
Leaf	2	5.5	37.6	6.0	50.9	100.0	9.6	20.8	7.0	2.8
	3	6.1	21.0	5.5	67.1	99.7	6.4	5.9	5.2	2.1
	4	4.9	8.4	7.5	78.7	99.5	4.5	0.6	4.3	2.5
Stem	2	3.2	35.1	6.4	55.3	100.0	6.8	20.5	5.5	3.9
	3	1.5	29.5	3.8	64.8	99.6	2.4	19.8	2.6	1.5
	4	1.9	25.9	3.6	69.8	101.2	1.7	14.3	2.4	1.6
Cocksfoot										
Whole	1	7.0	27.0	5.7	56.7	96.4	20.0	12.0	12.5	2.1
	2	5.3	32.3	4.9	58.4	100.9	10.7	14.1	8.9	2.0
	3	3.1	23.1	4.4	68.3	98.9	4.5	11.6	7.3	1.4
	4	2.4	7.7	5.8	86.5	102.4	2.5	0.5	4.9	1.4
Leaf	2	7.2	29.7	5.9	56.3	99.1	14.1	9.8	9.3	1.9
	3		Insufficient sample							
	4	3.8	9.1	8.7	77.4	99.0	4.2	-	9.2	2.7
Stem	2	2.46	33.2	5.5	58.5	99.7	8.5	15.6	7.4	2.8
	3	1.8	24.4	2.4	70.3	98.9	2.7	12.3	5.7	1.0
	4	1.1	7.8	3.3	87.5	99.7	1.3	0.4	4.5	0.7
Heads	4	3.6	5.2	8.0	81.9	98.7			9.6	1.4
Western Wolths										
Whole	1	6.8	36.4	5.1	51.1	99.4	19.8	14.5	10.9	2.2
	2	4.3	32.0	5.1	58.2	99.6	15.3	12.5	9.4	3.3
	3	3.5	35.0	4.5	57.1	100.1	9.4	18.2	6.3	3.8
	4	1.6	18.5	13.9	66.7	100.7	4.3	4.4	5.4	6.5
Stem	3	1.9	34.3	3.3	57.9	97.4	4.3	23.2	5.8	1.8
	4	2.5	17.0	3.8	77.6	100.9	2.1	4.8	4.2	1.4
Heads	3	4.0	31.4	5.8	55.8	97.0			5.8	1.4

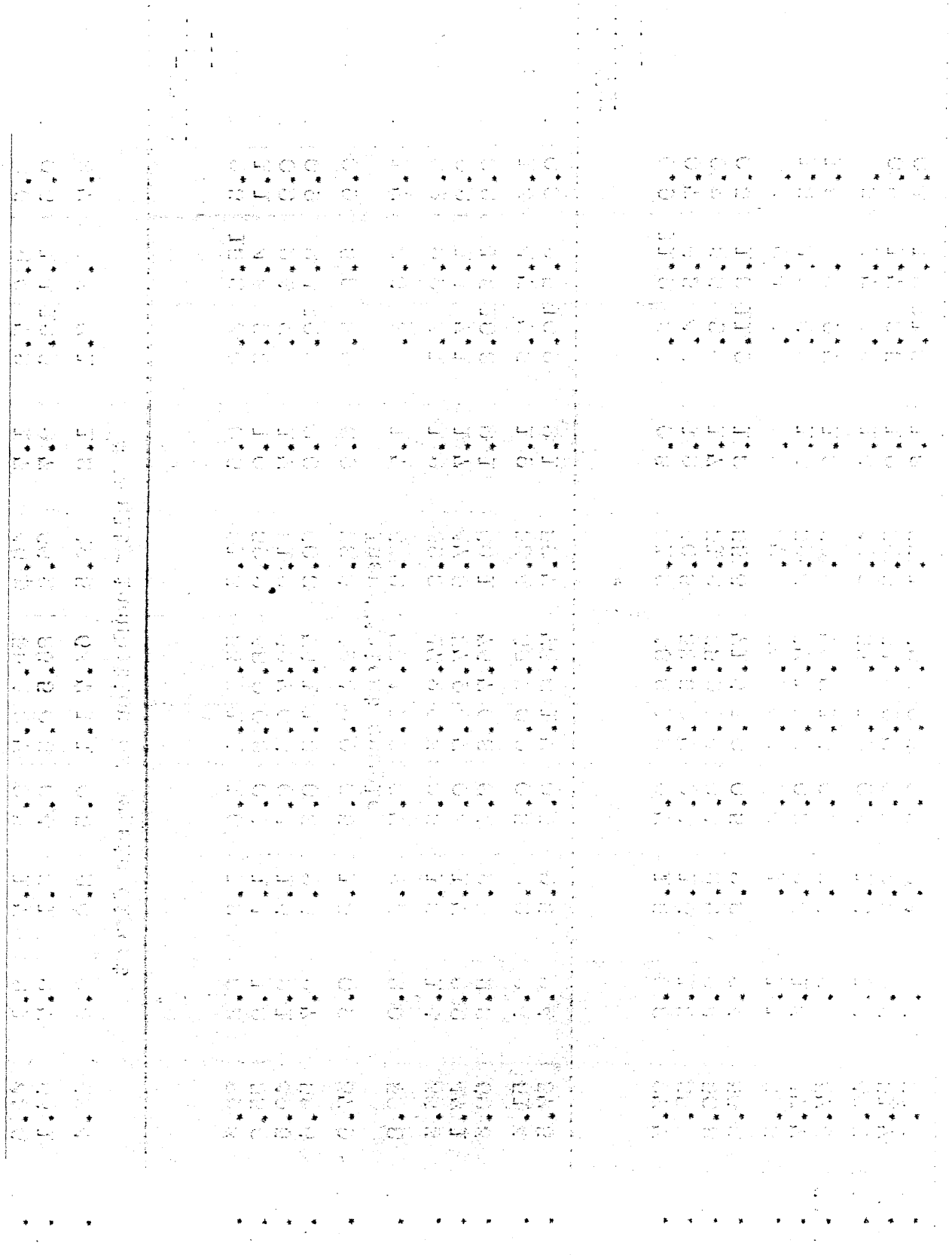


Table 41. General analysis of grass fibre and holocellulose (as % of original grass)

Grass	Cut No.	FIBRE Ash	Crude protein	Lignin	Acetyl groups	Poly-saccharides (by diff.)	Total	HOLOCELLULOSE Ash	Crude protein	Lignin	Acetyl groups	Poly-saccharides (by diff.)	Total
Rye-grass S24													
Whole	1	2.6	13.0	3.6	0.6	31.4	51.2	2.1	2.4*	2.0	0.9	30.4	37.8
	2	1.6	5.9	3.5	0.9	33.2	45.1	1.4	0.3	1.7	0.8	32.5	36.7
	3	0.1	2.7	8.2	1.1	48.2	60.3	0.8	0.4	2.9	1.7	45.6	51.4
	4	1.2	0.9	12.4	1.7	64.3	80.5	1.3	0.5	3.0	2.4	64.1	71.3
Leaf	2	2.1	6.5	2.9	0.9	30.9	43.3	1.7	0.5	1.4	0.3	28.8	32.7
	3	2.0	3.5	6.6	2.0	52.0	66.1	1.8	0.6	2.6	1.7	48.2	54.9
	4	Insufficient sample											
Stem	2	1.2	4.1	5.6	1.0	30.6	42.5	0.5	0.2	1.4	1.0	31.0	34.1
	3	0.6	2.0	7.2	1.7	43.9	55.4	0.7	0.3	1.7	2.1	43.6	48.4
	4	0.6	1.4	12.6	2.7	66.2	83.5	0.3	0.4	3.1	2.7	66.1	72.6
Heads	3	0.7	4.6	7.1	1.8	49.5	63.7	1.1	0.5	2.0	2.3	48.4	54.3
		* Faulty incubation of pepsin digest											
Rye-grass S23													
Whole	1	2.3	15.8	3.0	0.6	31.6	53.3	1.4	1.0	1.8	0.8	29.3	34.3
	2	1.1	4.3	3.3	1.0	39.2	48.9	0.8	0.4	1.4	1.2	35.8	39.6
	3	0.2	2.4	7.2	1.7	51.2	62.7	0.6	0.4	1.6	2.1	49.5	54.2
	4	0.9	2.4	10.0	2.0	60.8	76.1	1.3	0.5	2.6	2.7	58.6	65.7
Leaf	2	0.9	5.8	3.2	0.9	38.6	49.4	2.0	0.5	1.5	0.9	37.9	42.8
	3	Insufficient sample											
	4	1.7	3.6	8.2	1.7	63.2	78.4	1.8	0.7	2.5	2.0	58.8	65.8
Stem	2	0.2	2.9	3.1	1.2	39.8	47.2	0.3	0.3	1.3	1.4	36.3	39.6
	3	0.2	1.4	7.1	1.7	47.5	57.9	0.7	0.3	1.7	2.2	47.1	52.0
	4	0.2	1.5	10.8	2.1	62.1	76.7	0.8	0.4	2.5	2.8	62.3	68.8
Heads	3	1.3	4.7	7.5	1.9	53.4	68.8	0.9	0.5	2.2	2.4	51.4	57.4
	4	0.8	2.5	10.9	2.1	59.7	76.0	1.7	0.5	2.5	2.4	57.5	64.6
Timothy S48													
Whole	1	0.9	11.9	3.6	0.6	31.8	48.8	1.2	0.7	1.5	0.8	27.7	31.9
	2	0.7	6.5	4.6	1.2	40.5	53.5	0.7	0.4	1.6	1.3	35.3	39.3
	3	0.2	3.6	9.2	1.7	51.2	65.9	0.4	0.4	2.2	2.4	50.8	56.2
	4	0.5	1.8	11.9	1.8	55.6	71.6	1.0	0.5	2.8	2.4	56.3	63.0
Leaf	2	0.6	7.4	4.2	0.8	37.9	50.9	0.6	0.6	1.4	1.1	33.5	37.2
	3	1.5	4.8	6.8	1.4	52.6	67.1	1.9	0.8	2.3	1.6	47.7	54.3
	4	1.8	3.2	9.7	1.8	62.2	78.7	1.6	0.9	2.6	2.2	57.3	64.6
Stem	2	0.5	4.7	4.9	1.4	43.8	55.3	1.0	0.4	1.6	1.6	40.3	44.9
	3	0.2	1.7	8.5	1.9	52.5	64.8	0.7	0.3	2.5	2.6	51.7	57.8
	4	0.4	1.2	11.3	1.8	55.1	69.8	0.6	0.3	2.4	2.6	56.4	62.3
Cocksfoot													
Whole	1	5.8	15.9	4.8	0.9	29.3	56.7	4.3	1.0	2.5	0.5	28.6	36.9
	2	1.2	8.4	5.1	1.1	42.6	58.4	1.2	0.6	2.7	1.0	39.1	44.6
	3	0.9	3.0	8.9	1.8	53.7	68.3	1.7	0.4	3.0	2.4	52.1	59.6
	4	1.9	1.6	14.6	2.2	66.2	86.5	1.5	0.5	4.2	2.6	67.1	75.9
Leaf	2	1.6	10.0	5.8	0.7	38.2	56.3	1.6	1.1	2.1	0.9	35.8	41.5
	3	Insufficient sample											
	4	5.0	2.5	9.2	1.9	58.8	77.4	4.3	0.8	3.0	2.4	56.5	67.0
Stem	2	0.7	5.9	8.8	1.5	41.6	58.5	1.1	0.4	2.2	1.5	40.4	45.6
	3	0.2	1.9	9.5	2.3	56.4	70.3	0.7	0.3	2.2	3.0	56.4	62.6
	4	1.0	1.0	14.4	2.5	68.6	87.5	0.8	0.5	2.4	2.7	71.4	77.8
Heads	4	6.6	3.4	13.2	2.5	56.2	81.9	6.2	0.9	3.6	3.2	56.9	70.8
Western Wolths													
Whole	1	2.2	13.9	4.1	1.1	29.8	51.1	1.1	0.6	1.7	0.9	29.8	34.1
	2	0.6	10.4	7.0	1.3	38.9	58.2	0.7	0.8	1.6	1.5	37.3	41.9
	3	1.0	6.4	7.9	1.1	40.7	57.1	0.9	0.5	1.7	1.8	40.2	45.1
	4	1.3	2.5	10.6	1.7	50.6	66.7	1.0	0.4	2.3	2.9	50.4	57.0
Stem	3	0.3	2.8	8.2	1.6	45.0	57.9	0.4	0.4	1.1	2.5	45.6	50.0
	4	0.5	1.3	11.2	2.3	62.3	77.6	0.4	0.4	1.7	2.9	63.0	68.4
Heads	3	1.4	7.0	7.1	1.4	38.9	55.8	1.6	0.5	2.1	1.8	37.3	43.3

Table 42. Complete analysis of carbohydrates in holocellulose (as % of the original grass)

Grass	Cut no.	HEMICELLULOSE				CELLULOSE RESIDUE			Total poly-saccharides determined	Total poly-saccharides calculated as in Table 41 col.13
		Galactan	Glucosan	Araban	Xylan	Uronic anhydride	Glucosan	Xylan		
Rye-grass S24										
Whole	1	0.8	2.1	2.2	6.0	2.3	16.0	1.6	31.0	30.4
	2	0.5	2.0	1.4	7.5	1.7	18.0	2.2	33.3	32.5
	3	0.9	1.6	2.5	12.0	2.4	23.2	3.5	46.1	45.6
	4	0.9	1.8	2.6	14.3	3.2	32.8	5.0	60.6	64.1
Leaf	2	0.7	1.5	1.6	5.4	1.6	16.2	1.8	28.8	28.8
	3	1.5	2.1	2.4	10.6	2.7	24.6	4.9	48.8	48.2
Stem	2	0.9	3.0	1.7	6.7	1.4	15.9	2.3	31.9	31.0
	3	0.6	1.2	1.7	9.7	2.5	22.0	2.7	40.4	43.6
	4	0.5	1.4	2.0	12.3	3.5	32.5	5.1	57.3	66.1
Heads	3	0.6	1.1	1.7	11.4	2.4	20.4	7.2	44.8	48.4
Rye-grass S23										
Whole	1	0.5	1.6	1.8	5.4	2.0	15.5	1.5	28.3	29.3
	2	0.7	1.7	1.7	6.7	2.2	19.2	2.5	34.7	35.8
	3	0.5	1.3	2.1	11.5	2.9	24.7	3.7	46.7	49.5
	4	0.7	1.5	2.3	13.2	3.3	29.0	4.1	54.1	58.6
Leaf	2	1.3	2.2	1.5	7.2	2.4	19.5	1.7	35.8	37.9
	3	Insufficient sample								
	4	0.9	1.8	2.6	10.9	3.2	32.6	3.0	55.0	58.8
Stem	2	0.5	1.9	1.6	7.1	2.1	18.4	2.3	33.9	36.3
	3	0.9	1.6	1.7	8.8	2.9	24.8	2.8	43.5	47.1
	4	0.5	1.4	2.0	13.8	3.6	30.8	5.3	57.4	62.3
He Heads	3	0.6	1.1	2.9	11.2	2.8	26.0	3.6	48.2	51.4
	4	0.9	2.2	2.2	13.9	3.4	29.4	2.9	54.9	57.5
Timothy S48										
Whole	1	0.9	1.3	2.0	5.0	1.5	16.4	0.9	28.0	27.7
	2	0.7	1.6	2.4	7.1	2.2	19.0	2.9	35.9	35.3
	3	1.3	2.2	2.1	12.0	3.0	23.5	6.0	50.1	50.8
	4	1.2	1.7	2.1	10.7	3.5	25.7	5.6	50.5	56.3
Leaf	2	1.1	1.7	2.4	6.6	1.9	18.3	2.0	34.0	33.5
	3	0.9	1.1	3.0	10.1	2.8	24.6	4.5	47.0	47.7
	4	1.1	1.1	2.8	10.7	3.3	30.3	4.7	54.0	57.3
Stem	2	0.7	2.3	2.5	9.6	2.4	20.7	2.4	40.6	40.3
	3	0.5	1.4	1.8	11.7	3.2	26.2	4.5	49.3	51.7
	4	0.4	1.1	2.0	10.7	3.5	26.7	5.2	49.6	56.4
Cocksfoot										
Whole	1	1.0	1.4	1.7	4.9	1.8	16.2	1.4	28.4	28.6
	2	0.7	2.0	2.1	7.8	2.2	20.8	2.8	38.4	39.1
	3	1.2	1.8	2.5	13.6	2.8	27.3	2.2	51.4	52.1
	4	0.9	2.1	2.8	15.1	4.3	36.8	2.1	64.1	67.1
Leaf	2	0.8	1.7	2.1	6.7	2.0	19.7	2.2	35.2	35.8
	3	Insufficient sample								
	4	1.6	1.5	2.7	11.6	3.2	29.1	3.4	53.1	56.5
Stem	2	0.9	2.3	2.2	8.4	2.1	20.9	3.1	39.9	40.4
	3	0.5	1.0	1.9	11.7	3.5	30.0	2.8	51.4	56.4
	4	0.5	0.7	2.2	14.6	4.2	35.0	5.1	62.3	71.4
Heads	4	0.7	0.6	2.4	13.8	3.1	26.5	4.5	51.6	56.9
Western Wolths										
Whole	1	0.2	1.1	1.1	5.3	1.6	18.7	2.3	30.3	29.8
	2	0.6	1.3	1.0	7.4	1.8	21.4	2.1	35.6	37.3
	3	0.4	1.0	0.9	7.4	2.0	20.8	2.2	34.7	40.2
	4	0.3	0.9	1.5	10.3	3.0	26.2	4.1	46.3	50.4
Stem	3	0.3	0.6	1.0	7.7	2.3	22.1	3.1	37.1	45.6
	4	0.5	0.9	1.8	12.0	3.1	32.7	4.7	55.7	63.0
Heads	3	0.8	1.7	1.7	9.5	2.0	17.5	3.2	36.4	37.3

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APPENDIX

Table 43. The composition of the polysaccharide extracts of holocelluloses

Grass	Cut no.	Extract	Galactan	Glucosan	Araban	Xylan	Sum
Rye-grass S24							
Whole	1	Water	0.4	0.8	1.5	1.9	4.6
		1% potassium hydroxide	0.3	0.6	0.5	1.5	2.9
		N sulphuric acid	0.1	0.7	0.2	0.9	1.9
		Cellulose residue		16.0		1.6	17.6
	2	Water	0.2	0.8	0.6	1.5	3.1
		1% potassium hydroxide	0.2	0.6	0.6	3.4	4.8
		N sulphuric acid	0.1	0.6	0.2	1.3	2.2
		Cellulose residue		18.0		2.2	20.2
	3	Water	0.4	0.6	1.1	3.8	5.9
		1% potassium hydroxide	0.4	0.8	0.9	4.8	6.9
		N sulphuric acid	0.1	0.2	0.5	1.6	2.4
		Cellulose residue		23.2		3.5	26.7
	4	Water	0.6	0.6	1.7	5.8	8.7
		1% potassium hydroxide	0.2	0.6	0.5	4.0	5.3
		N sulphuric acid	0.1	0.6	0.4	2.2	3.3
		Cellulose residue		32.8		5.0	37.8
Leaf	2	Water	0.3	0.6	0.8	1.7	3.4
		1% potassium hydroxide	0.2	0.4	0.5	1.6	2.7
		N sulphuric acid	0.2	0.5	0.3	0.9	1.9
		Cellulose residue		16.2		1.8	18.0
	3	Water	1.1	1.1	1.7	4.5	8.4
		1% potassium hydroxide	0.3	0.4	0.4	2.0	3.1
		N sulphuric acid	0.1	0.6	0.3	2.1	3.1
		Cellulose residue		24.6		4.9	29.5
Stem	2	Water	0.4	1.6	1.2	2.3	5.5
		1% potassium hydroxide	0.1	0.6	0.3	2.1	3.1
		N sulphuric acid	0.4	0.8	0.2	1.2	2.6
		Cellulose residue		15.9		2.3	18.2
	3	Water	0.4	0.6	1.3	4.7	7.0
		1% potassium hydroxide	0.1	0.2	0.3	1.5	2.1
		N sulphuric acid	0.1	0.4	0.1	1.6	2.2
		Cellulose residue		22.0		2.7	24.7
	4	Water	0.3	0.4	1.3	4.3	6.3
		1% potassium hydroxide	0.1	0.4	0.5	2.8	3.8
		N sulphuric acid	0.1	0.6	0.2	2.6	3.5
		Cellulose residue		32.5		5.1	37.6

Grass	Cut no.	Extract	Galactan	Glucosan	Araban	Xylan	Sum
Rye-grass S23							
Whole	1	Water	0.2	0.4	0.7	0.9	2.2
		1% potassium hydroxide	0.2	0.5	0.7	1.9	3.3
		N sulphuric acid	0.1	0.7	0.4	1.1	2.3
		Cellulose residue		15.5		1.5	17.0
	2	Water	0.3	0.6	0.7	1.2	2.8
		1% potassium hydroxide	0.3	0.4	0.7	2.4	3.8
		N sulphuric acid	0.1	0.7	0.3	1.4	2.5
		Cellulose residue		19.2		2.5	21.7
	3	Water	0.1	0.3	0.6	1.2	2.2
		1% potassium hydroxide	0.3	0.3	1.0	5.8	7.4
		N sulphuric acid	0.1	0.7	0.5	2.3	3.6
		Cellulose residue		24.7		3.7	28.4
	4	Water	0.2	0.4	1.0	2.7	4.3
		1% potassium hydroxide	0.4	0.5	0.9	6.4	8.2
		N sulphuric acid	0.1	0.6	0.4	1.6	2.7
		Cellulose residue		29.0		4.1	33.1
Leaf	2	Water	0.2	0.4	0.3	0.8	1.7
		1% potassium hydroxide	0.9	1.2	0.8	3.3	6.2
		N sulphuric acid	0.2	0.6	0.4	1.3	2.5
		Cellulose residue		19.5		1.7	21.2
	3	Insufficient sample					
	4	Water	0.4	0.5	1.3	2.0	4.2
		1% potassium hydroxide	0.4	0.5	0.9	4.7	6.5
		N sulphuric acid	0.1	0.8	0.4	1.8	3.1
		Cellulose residue		32.6		3.0	35.6
Stem	2	Water	0.1	0.6	0.5	0.9	2.1
		1% potassium hydroxide	0.3	0.5	0.7	2.7	4.2
		N sulphuric acid	0.1	0.8	0.4	1.9	3.2
		Cellulose residue		18.4		2.3	20.7
	3	Water	0.1	0.3	0.7	1.4	2.5
		1% potassium hydroxide	0.7	0.7	0.6	3.1	5.1
		N sulphuric acid	0.1	0.6	0.4	2.1	3.2
		Cellulose residue		24.8		2.8	27.6
	4	Water	0.2	0.3	1.0	2.7	4.2
		1% potassium hydroxide	0.2	0.5	0.6	6.0	7.3
		N sulphuric acid	0.1	0.6	0.4	2.4	3.5
		Cellulose residue		30.8		5.3	36.1

Grass	Cut no.	Extract	Galactan	Glucosan	Araban	Xylan	Sum
Timothy							
Whole	1	Water	0.4	0.5	0.6	0.9	2.4
		1% potassium hydroxide	0.3	0.5	0.9	1.7	3.4
		N sulphuric acid	0.2	0.3	0.5	1.3	2.3
		Cellulose residue		16.4		0.9	17.3
	2	Water	0.3	0.5	0.8	1.0	2.6
		1% potassium hydroxide	0.3	0.5	0.8	3.0	4.6
		N sulphuric acid	0.1	0.6	0.8	1.4	2.9
		Cellulose residue		19.0		2.9	21.9
	3	Water	0.8	1.1	1.1	2.7	5.7
		1% potassium hydroxide	0.4	0.5	0.7	4.4	6.0
		N sulphuric acid	0.1	0.6	0.3	2.6	3.6
		Cellulose residue		23.5		6.0	29.5
	4	Water	1.0	1.1	1.5	3.5	7.1
		1% potassium hydroxide	0.1	0.1	0.2	2.0	2.4
		N sulphuric acid	0.1	0.5	0.4	2.6	3.6
		Cellulose residue		25.7		5.6	31.3
Leaf	2	Water	0.5	0.6	1.2	1.5	3.8
		1% potassium hydroxide	0.4	0.6	0.9	2.3	4.2
		N sulphuric acid	0.2	0.5	0.3	1.4	2.4
		Cellulose residue		18.3		2.0	20.3
	3	Water	0.3	0.4	1.0	1.6	3.3
		1% potassium hydroxide	0.4	0.3	1.4	4.0	6.1
		N sulphuric acid	0.2	0.4	0.6	2.4	3.6
		Cellulose residue		24.6		4.5	29.1
	4	Water	0.6	0.4	1.6	3.3	5.9
		1% potassium hydroxide	0.4	0.4	0.8	3.3	4.9
		N sulphuric acid	0.1	0.3	0.4	1.6	2.4
		Cellulose residue		30.3		4.7	35.0
Stem	2	Water	0.5	1.3	1.3	2.7	5.8
		1% potassium hydroxide	0.1	0.4	0.9	3.5	4.9
		N sulphuric acid	0.1	0.6	0.3	1.6	2.6
		Cellulose residue		20.7		2.4	23.1
	3	Water	0.2	0.5	1.0	2.6	4.3
		1% potassium hydroxide	0.2	0.3	0.5	4.2	5.2
		N sulphuric acid	0.1	0.6	0.3	2.5	3.5
		Cellulose residue		26.2		4.5	30.7
	4	Water	0.1	0.3	1.1	2.3	3.8
		1% potassium hydroxide	0.2	0.3	0.6	3.4	4.5
		N sulphuric acid	0.1	0.5	0.3	2.4	3.3
		Cellulose residue					

Grass	Cut no.	Extract	Galactan	Glucosan	Araban	Xylan	Sum
Cocksfoot							
Whole	1	Water	0.7	0.4	0.9	1.4	3.4
		1% potassium hydroxide	0.2	0.4	0.5	1.4	2.5
		N sulphuric acid	0.1	0.6	0.3	0.8	1.8
		Cellulose residue		16.2		1.4	17.6
	2	Water	0.4	0.8	1.0	1.9	4.1
		1% potassium hydroxide	0.2	0.5	0.7	2.8	4.2
		N sulphuric acid	0.1	0.7	0.4	1.5	2.7
		Cellulose residue		20.8		2.8	23.6
	3	Water	0.4	0.5	1.4	5.4	7.7
		1% potassium hydroxide	0.2	0.4	0.6	3.8	5.0
		N sulphuric acid	0.6	0.9	0.5	2.3	4.3
		Cellulose residue		27.3		2.2	29.5
	4	Water	0.5	1.1	1.7	5.2	8.5
		1% potassium hydroxide	0.3	0.6	0.8	4.5	6.2
		N sulphuric acid	0.1	0.4	0.3	2.2	3.0
		Cellulose residue		36.8		2.1	38.9
Leaf	2	Water	0.5	0.7	1.3	2.3	4.8
		1% potassium hydroxide	0.3	0.5	0.6	2.0	3.4
		N sulphuric acid	0	0.5	0.2	1.0	1.7
		Cellulose residue		19.7		2.2	21.9
	3	Insufficient sample					
	4	Water	1.3	0.6	1.8	4.4	8.1
		1% potassium hydroxide	0.2	0.4	0.6	2.8	4.0
		N sulphuric acid	0.1	0.5	0.3	2.0	2.9
		Cellulose residue		29.1		3.4	32.5
Stem	2	Water	0.6	1.3	1.3	3.3	6.5
		1% potassium hydroxide	0.2	0.5	0.7	2.2	3.6
		N sulphuric acid	0.1	0.5	0.2	1.4	2.2
		Cellulose residue		20.9		3.1	24.0
	3	Water	0.3	0.5	1.3	4.2	6.3
		1% potassium hydroxide	0.1	0.2	0.4	2.9	3.6
		N sulphuric acid	0.1	0.3	0.2	2.0	2.6
		Cellulose residue		30.0		2.8	32.8
	4	Water	0.4	0.3	1.3	4.0	6.0
		1% potassium hydroxide			0.6	4.4	5.0
		N sulphuric acid	0.1	0.4	0.3	3.1	3.9
		Cellulose residue		35.0		5.1	40.1

Grass	Cut no.	Extract	Galactan	Glucosan	Araban	Xylan	Sum
Western Wolths							
Whole	1	Water	0.1	0.5	0.6	1.8	3.0
		1% potassium hydroxide	0.1	0.2	0.3	1.3	1.9
		N sulphuric acid	±	0.4	0.2	1.0	1.6
		Cellulose residue		18.7		2.3	21.0
	2	Water	0.5	0.8	0.5	2.4	4.2
		1% potassium hydroxide	0.1	0.1	0.3	2.0	2.5
		N sulphuric acid	±	0.4	0.2	1.6	2.2
		Cellulose residue		21.4		2.1	23.5
	3	Water	0.2	0.4	0.5	2.0	3.1
		1% potassium hydroxide	0.1	0.2	0.2	2.6	3.1
		N sulphuric acid	0.1	0.4	0.2	1.3	2.0
		Cellulose residue		20.8		2.2	23.0
	4	Water	0.1	0.3	0.9	3.0	4.3
		1% potassium hydroxide	0.1	0.3	0.4	3.4	4.2
		N sulphuric acid	0.1	0.3	0.2	1.6	2.2
		Cellulose residue		26.2		4.1	30.3
Leaf	3 & 4 Cuts - Insufficient samples						
Stem	3	Water	0.1	0.1	0.5	1.9	2.6
		1% potassium hydroxide	0.1	0.2	0.3	2.6	3.2
		N sulphuric acid	0.1	0.3	0.2	1.6	2.2
		Cellulose residue		22.1		3.1	25.2
	4	Water	0.3	0.3	1.3	4.3	6.2
		1% potassium hydroxide	0.1	0.3	0.3	3.1	3.8
		N sulphuric acid	0.1	0.3	0.2	2.3	2.9
		Cellulose residue		32.7		4.7	37.4