STUDIES ON AGRICULTURALLY IMPORTANT PLANT METABOLITES.

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SUMMARY

This thesis deals mainly with the carbohydrate metabolism of plants, and in particular with that of bracken and of potatoes. Four main projects are described:

a) An investigation into the seasonal and other variations in carbohydrate levels in bracken rhizomes, which is relevant to the behaviour of herbicides in the plant and to its ability to withstand herbicide treatment.

b) Examination of the glycolipid and phospholipid composition of bracken, initially to determine whether any glycolipids which could act as intermediates in polysaccharide biosynthesis might be present. None were found, but the results have implications for the composition of plastid membranes and the turnover of carbohydrates in photosynthetic tissue.

c) A parallel investigation on carbohydrates and lipids in potato tubers during low-temperature storage, with a twofold objective: firstly to investigate the mechanism of low-temperature sweetening and the role of membrane lipids therein, and secondly to compare commonly-grown varieties in their tendency to accumulate sugars at low temperatures.

d) The partial characterisation of a complex mucilaginous polysaccharide found in bracken.

The significance of the results for carbohydrate metabolism in bracken and other plants; the storage of potatoes for processing; and bracken eradication techniques is discussed, and some observations on the ecology of bracken and its effects on the soil are presented in two appendices.

CONTENTS

PREFACE

Very broadly speaking, this thesis deals with the metabolism of carbohydrates and carbohydrate-containing substances in plants.[1-6] It also has a bearing on a number of practical matters, amongst which are the growth and eradication of bracken and, to a lesser extent, the storage of potatoes. Since not everyone with an expert knowledge of plant carbohydrate biochemistry is also conversant with the bracken problem (and vice versa), the main part of the Introduction which follows is devoted to explaining some of the background to each of these two subjects. If the treatment of either (or both) is too elementary for the reader's patience, he is respectfully asked to 'skip' a little.

Detailed introductions to the rather diverse practical aspects of the various investigations will be given individually in Chapters 2-5. It must be admitted that the order in which the experimental work was carried out was not as logical as that in which it is described in this thesis - indeed some of the objectives quoted on p. 32 et seq. had not been thought of when work began. Simultaneously with the work on plant metabolites described here, some research on the ecology of bracken and related factors was carried out and some of the results prepared for publication:[7,8] as they have some bearing on the conclusions of this thesis they are attached as appendices.

CHAPTER 1

INTRODUCTION

A. PLANT CARBOHYDRATES

From the point of view of either the chemist or the nutritionist, the most obvious difference between the plant and animal kingdoms is that whereas animals are composed mainly of water and protein with some fat and skeletal material, plants are composed mainly of water and carbohydrate with some protein and fat. The bulk of the carbon fixed by the atmosphere by a photosynthesising leaf appears initially in the form of carbohydrates, which after various transformations may be used to fuel the leaf's metabolic processes or incorporated into its growing tissues: alternatively they may be exported in the form of sucrose to other parts of the plant for respiration, growth, or storage for future use.

After the initial stages of photosynthesis within the chloroplast, the assembled sugars are in general interconverted or polymerised while in the form of sugar phosphates or nucleotide sugars.[9] Some examples of sugar transformations are shown in Figure 1. In general (with possible exceptions which will be discussed later) the enzyme systems which catalyse transformations of the type

$$\text{sugar} - \text{phosphate} + \text{acceptor} \rightleftharpoons \text{sugar} - \text{acceptor} + \text{inorganic phosphate}$$

or

$$\text{sugar} + \text{acceptor} \rightleftharpoons \text{sugar} - \text{acceptor}.$$

tend to be degradative rather than synthetic in plants. In the synthesis of polysaccharides, and of glycosides of all types

including glycolipids, nucleotides now appear to be the main donors of monosaccharide residues, the generalised reaction type being

nucleotide - pyrophosphate - sugar + acceptor →

nucleotide diphosphate + acceptor - sugar.

The monosaccharide is thus attached through its anomeric carbon atom, i.e. is non-reducing.

It will be clear, therefore, that free nucleotides and their sugar derivatives are of central importance in the carbohydrate metabolism of plants. In this laboratory as well as elsewhere they have been used, in a tentative and preliminary way, as indicators of metabolic activity.[10-12] A good deal of information on their occurrence in potatoes and bracken has been amassed, but their extraction and purification from a starting material as intractable as bracken is too laborious to be feasible as a routine method for, e.g., following the effects of herbicide treatment. Having a pyrophosphate bond they are decidedly high-energy compounds, unlikely to be present for long before being metabolised, and readily hydrolysed chemically or enzymically during extraction.

In the work to be reported here it was decided to concentrate on the carbohydrate-containing substances themselves rather than their nucleotide-bound precursors, but in interpreting the results the latter have to be kept in mind.

Energy Carbohydrates

The complex pathways by which atmospheric carbon dioxide is converted to carbohydrate are well known and will not be described here.[13] Whether the Calvin or the Hatch-Slack pathway is followed, the end products (if we ignore the amino acids and other non-carbohydrate substances which are now known to be formed directly in fairly small amounts) are the hexose phosphates fructose-6-phosphate and glucose-6-phosphate. These may be utilised within the photosynthetic tissue; alternatively they may be converted to sucrose by the following route -

Glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate

Glucose-6-phosphate $\rightleftharpoons$ glucose-1-phosphate $\overset{UTP}{\rightleftharpoons}$ UDP-Glucose + P.P

UDP-glucose + fructose-6-phosphate $\rightleftharpoons$ sucrose phosphate + UDP

sucrose phosphate $\longrightarrow$ sucrose + P_i

- and either channelled into the synthesis of starch for temporary storage within the chloroplast or exported to other parts of the plant.

The exported sucrose is translocated through the phloem elements to wherever it is to be used, either for synthesis, for respiration in the mitochondria to provide energy for other metabolic processes, or to be stored for future use. The mechanism of translocation is the subject of some controversy at the moment, the basic dilemma being the very substantial rates of sucrose transfer that can be achieved, exceeding what would be expected from the diffusion coefficient of sucrose in water by more than four orders of magnitude.

The many theories which have been proposed may be divided into two groups; the numerous modifications of the 'activated diffusion' model first proposed by Mason & Maskell[14,15] after their classic investigations during the 1920's, and the 'pressure-flow' hypothesis of Münch,[16] which originally appeared at the same time. The activated diffusion model has the merit of explaining (with certain reservations) the fact that translocation of labelled sucrose often obeys Fick's Law or a rule mathematically equivalent. It requires that all substances being translocated must move independently of one another, each under its own activity gradient. Its main advocate recently has been Canny,[17] who nevertheless allows that a limited amount of mass flow and protoplasmic streaming may take place as well.

The Münch hypothesis has been incorporated into the mass flow concept of Crafts,[18] which supposes that the water in which the sucrose is dissolved, in the phloem, is moving under a pressure gradient set up by osmosis, rather than stationary as in the activated diffusion theory. The mass flow concept has received a boost from the discovery

that the phloem elements are hydrodynamically continuous, the callose plugs at the ends of the sieve elements being a quickly-induced artifact due to injury.[19-22] It very satisfactorily explains the observation that a wide range of natural and artificial substances follow the carbohydrate movements in the phloem very closely.[18] These include a number of herbicides, including the growth regulators amongst others, a fact of great significance for bracken eradication as well as for the technology of translocated herbicides and systemic pesticides generally.

There are undoubtably exceptions to the generalisation that substances of this type are translocated with the flow of sucrose - the plant's own growth hormones are an example - but proponents of the mass flow theory explain these by postulating exchange between xylem and phloem, with any translocated substance having a characteristic degree of preference for one or the other.

The movement of carbohydrates from place to place within a single cell, e.g. between the plastids and the end of the nearest vascular element, may perhaps depend on protoplasmic streaming (circulatory movements of the cytoplasmic fluid) or on surface-energised effects at lipid-aqueous boundaries.[23-26] Both of these mechanisms have been postulated in connection with phloem transport, and other possibilities may be suggested. The whole subject remains speculative.

When the translocated sucrose reaches storage tissue such as seeds, bulbs, or tubers, it cannot be stored as it is, since the resulting concentrated solutions would place too great an osmotic load on the plant cells. In some seeds and fruits such as the olive, oil (mainly triglyceride) is the storage substance. It is much more energy-rich, for a given weight or volume, than carbohydrates. More commonly energy is stored in the form of polysaccharides of one kind or another; $\beta(2\rightarrow1')$- or $\beta(2\rightarrow6')$- linked fructose polymers in the leaves and stems of grasses and the bulbs of some of the Compositae, glucomannans, galactans and a variety of other polysaccharides in the

seeds of various genera.[27] These are mostly amorphous and highly hydrated, so that to store a given amount of energy they require more space than starch, the main reserve substance of the plant kingdom.

Starch[28] is a mixture of two polysaccharides, amylose and amylopectin. The amylose molecule is an unbranched chain of 1000-6000 D-glucopyranose units linked $\alpha(1 \rightarrow 4')$, and although initially soluble in water or alkali tends to crystallise slowly on standing (in the special, linear sense of crystallisation reserved for polymers), the chains intertwining in a helical conformation. The amylopectin molecule is similarly constituted but with $\alpha(1 \rightarrow 6')$ branch points at 18- to 25- unit intervals, the dendritic branching tending to hinder interactions between molecules.

Within the living plant starch is found not in solution, but in the form of dense, compact grains 1-100 μm in diameter, built up of consecutively deposited layers like an onion. In storage tissue these starch grains completely fill the amyloplasts, cytoplasmic organelles closely related to chloroplasts. In some tissues either type of plastid may develop from identical 'proplastids' if the environmental conditions are varied. Plastids have a degree of genetic autonomy; some of the enzymic reactions of which they are capable seem to be genetically determined from within and others by the cell nucleus.[29]

The biosynthesis of starch has been a controversial subject for a long time, and remains so.[30] It is assumed to follow broadly the same routes in chloroplasts and in amyloplasts, but there may be detailed differences such as are becoming apparent between cereal grains[31,32] and underground storage tissues.[33,34] Much of the evidence available so far comes from experiments <u>in vitro</u> with soluble or digitonin-solubilised enzymes, or particulate enzyme preparations of doubtful and complex composition, and with quite artificial substrate concentrations. The incorporation of a substrate into a polymer resembling natural amylose, for example, is no guarantee that it is an important precursor of starch <u>in vivo</u>.

This is a common problem in polysaccharide studies, and is possibly more serious still in investigations on the biosynthesis of cell wall polysaccharides.

It has long been known that polysaccharides similar to amylose can be synthesised *in vitro* by plant phosphorylases, provided that the digest contains a high ratio of glucose-1-phosphate to inorganic phosphate.[35] The possible involvement of phosphorylases in starch synthesis has been repeatedly suggested since then and still cannot be discounted, although it would require a small area of very high glucose-1-phosphate concentration to be compartmented off from the cytoplasm.[36-38] The weight of opinion is now in favour of a purely degradative role for this group of enzymes.

The discovery of a UDP-glucosyltransferase closely associated with starch grains led Leloir,[39] in 1960, to propose that UDP-glucose is the actual precursor of starch *in vivo*. Later experiments showed that ADP-glucose was incorporated much more quickly,[40] and although it was thought for a short time that the slower incorporation of UDP-glucose was offset by the greater amount of it present, later results showed that the nucleotides locked up inside the grain are largely of the adenine series,[10] the UDP-glucose being outside in the cytoplasm and hence readily extractable.

Multiple forms of UDP- and ADP-glucosyltransferases (starch synthetases) have now been obtained from various plant tissues and can frequently be solubilised with digitonin, although particulate forms that can utilise either nucleotide may lose their UDP-glucosyltransferase activity when rendered soluble.[31-33,41-43] The action of these enzymes, unlike the phosphorylases, is essentially irreversible, and they are now generally considered to be responsible for the addition of glucose residues to the amylose molecule. The energy balance in stored potato tubers is consistent with this general picture of synthesis by starch synthetases and degradation by phosphorylases.[34]

The conversion of amylose to amylopectin can proceed more or less concurrently with its synthesis by either starch synthetases or phosphorylases, and is carried out by an enzyme (Q-enzyme) which

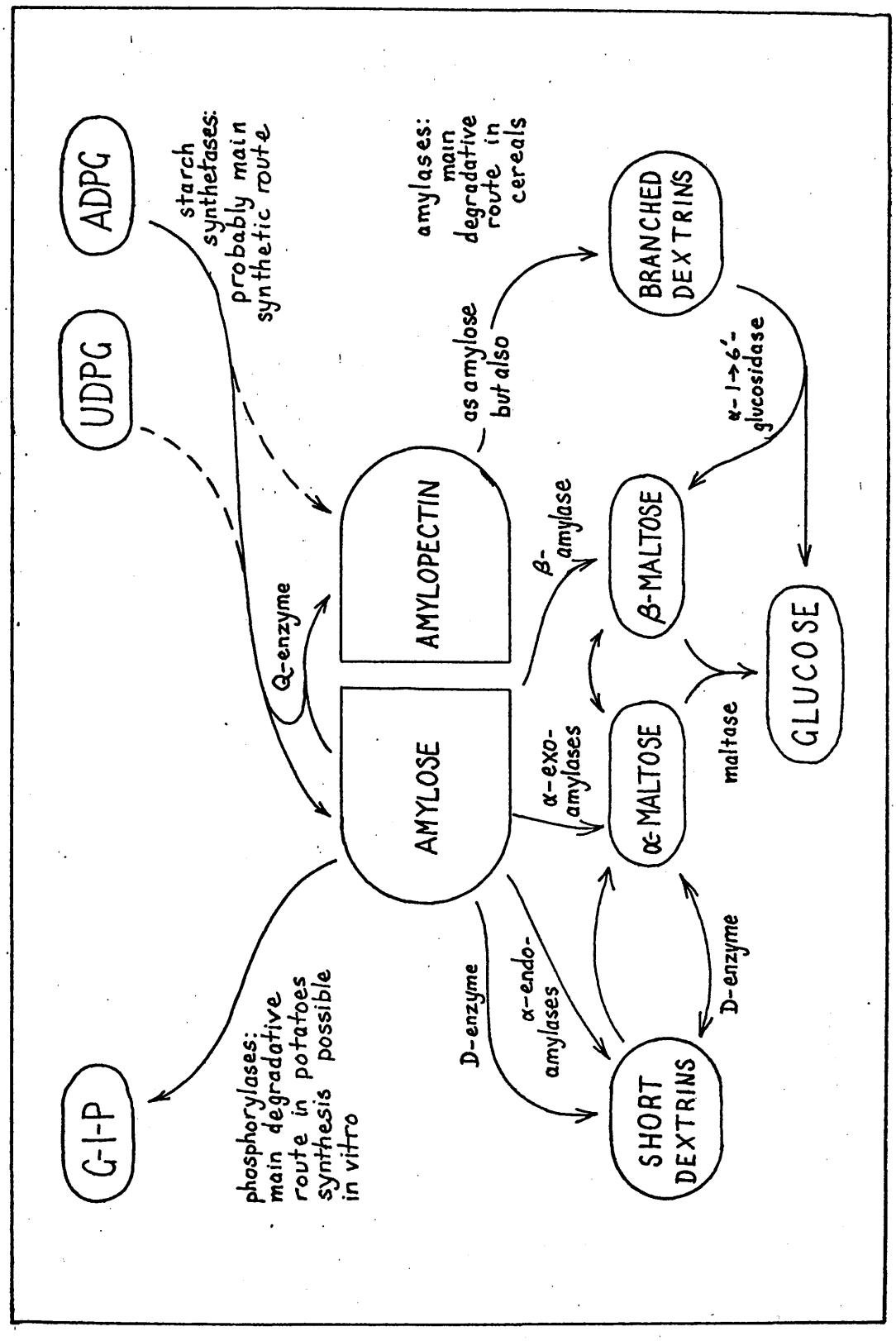


Figure 1.1 - Synthesis and degradation of starch.

disproportionates the polymer by cleaving a glycosidic bond the requisite number of units away from the non-reducing end and simultaneously attaching the fragment to C-6 of a glucose residue in another amylose chain.[44] When Q-enzyme is associated with a primer-less starch synthetase it may in some cases (not always)[31,33,42,43] increase the reaction rate, presumably through the creation of extra growing points. This is possibly the only piece of concrete evidence that starch (or any other plant polysaccharide) is synthesised by attachment to the non-reducing end, in contrast to the situation in bacteria.[9]

Although on present evidence the immediate precursor in continuous starch synthesis is apparently ADP-glucose, the preceding steps from sucrose to this nucleotide are closely linked[32,45-48] and may possibly occur within the amyloplast: there is evidence that the interconversion of the main pools of sucrose, glucose and fructose, which are certainly located outside the plastid, is an independent process.[34,49] It is possible that in cereals, sucrose is hydrolysed into the hexoses before it can pass into the endosperm, but is resynthesised before being converted into starch.[50] It has even been suggested that the transfer into the endosperm is the rate-limiting step in the whole chain of events from photosynthesis in the leaf to synthesis of starch in the grain:[51,52] if proved to be true, this would have far-reaching agricultural consequences at least as far as cereals are concerned. There is no evidence for an analogous phenomenon in plants such as potatoes and bracken.

Starch is unusual, amongst polysaccharides, in being synthesised in a solid form from the start. New starch is added to the grain from the outside, and the concentric skins visible under the polarising microscope probably result from day-to-day changes in the rate of deposition.[53] The molecules seem to be oriented radially across the thickness of the skins, but how the dendritic branching of amylopectin is accommodated, and whether the reducing ends are towards the inside or the outside, remain to be established.

On the whole this evidence points to synthesis by enzymes bound within a membrane surrounding the starch grain. This could be the amyloplast membrane itself, at least in the later stages of synthesis when the grain fills the amyloplast completely; or it could be an unrecognised membrane surrounding the grain from its inception within the stroma.[54] On the other hand bound starch synthetases[37,40-42] and the required nucleotides[10] are certainly present in the heart of the starch grain and may even allow some redistribution of carbohydrate within it over fairly long periods of time. It may be that starch synthesis in the early stages of grain formation does not follow the same pattern as later.[55,38]

Whether the amyloplast membrane has an active role in starch synthesis, or whether its function is to separate the pools of free sugars, possible starch precursors and enzyme systems in the cytoplasm from those within the amyloplast, (with a possible role in sucrose biosynthesis), it is clear that the more information that can be collected on the nature and composition of the membrane itself, the better. It is worth noting that a group in Israel have suggested that the conversion of starch to free sugars on exposure of potato tubers to low temperatures, or at senescence, is due to loss of the compartmenting integrity of the amyloplast membrane.[56] This possibility is examined further in Chapter 4.

Structural Carbohydrates

Cell walls of plants are composed mainly of water, but their strength and resilience comes from an intricate, composite structure of polysaccharides. A network of strong, highly insoluble and stable microfibrils of cellulose fulfils the same role as the steel in reinforced concrete, and is embedded in a matrix composed mainly of gelatinous, highly hydrated polysaccharides, the pectins and hemicelluloses, which hold the cellulose network together and lend plasticity or rigidity, as appropriate, to the whole.[57-60] Some of these contain uronic acid groups, neutralised by a variety of cations amongst which Ca^{2+} usually predominates due to

selective complexing or chelating effects. The metabolism of mineral elements and the composition of the cell wall are thus interconnected.[61-64]

There are broad resemblances in structure between the matrix polysaccharides (particularly the pectic fraction) and the various water-soluble galactans, arabinoxylans, and complex acidic polysaccharides that are grouped together and classed as mucilages and exudate gums.[65] Likewise the structures of the complex reserve polysaccharides that can be isolated from certain seeds, nuts and tubers recall those of the hemicellulosic glucomannans and xylans although they are not similar in detail[66] - e.g. the arabinoxylans from cereal seeds have a $\beta(1 \rightarrow 4')$-linked xylan main chain with single-unit arabinofuranose side-chains, and in these respects are similar to the hemicellulosic xylans; but they differ in containing no glucuronic acid and having so many more arabinose residues as to be water-soluble.[67]

There is nevertheless a decided family resemblance linking all these classes of polysaccharides. It appears that cell wall polysaccharides can be metabolised like reserve substances in certain circumstances,[68,69] and that the Golgi apparatus (vide infra) is responsible for the production of at least some of the mucilaginous group,[70-73] just as it is generally considered to produce the cell wall polysaccharides[58,59] (although this has only been demonstrated for the pectic substances[74] and the xylans,[75] not for the hemicellulosic glucomannans).

The walls of cells in rapidly growing tissue must be flexible and capable of rapid extension. In the primary cell walls, which are formed first, the cellulose network is not closely or regularly interlocked and the matrix consists of polysaccharides of both pectic and hemicellulose groups. The former group predominates in soft tissues such as lemon peel and apples, and in the middle lamella, the cellulose-free area of contact between different cells.[76,77]

In more mature tissues the wall is thickened from the inside, and the cellulose fibrils run parallel to one another making up sheets, the orientation of each sheet being at a slight angle to that of the one below to give strength like that of plywood.[76] In this, the secondary cell wall, the pectic substances disappear from the matrix, and in load-bearing tissue impregnation with lignin[78] and often silica[79-81] follows, giving rigidity and impermeability to the whole structure.

Cellulose, which is ultimately responsible for the structural integrity of the cell wall, is a linear $\beta(1 \rightarrow 4')$-linked glucan with a chain length of 5,000 - 15,000 units. Hydrogen bonds hold about 40 of these long molecules together in a tightly ordered crystalline arrangement within the elementary fibrils, which especially in the secondary wall tend to aggregate into rope-like microfibrils 35-100Å in diameter.[82-84] Less ordered inter-chain hydrogen bonding links the matrix polysaccharides to the surface of the microfibrils, and indeed most cellulose preparations contain small amounts of sugars other than glucose (mainly mannose).[85] It is not certain whether these are incorporated in the cellulose chains or merely held against dissolution by hydrogen bonding.

Much of the earlier work on cellulose biosynthesis involved Acetobacter xylinum, a bacterial species secreting large quantities of extracellular cellulose. A number of theories have appeared, some including glycolipid intermediates.[86-89] The behaviour of this organism is quite different from that of higher plants, however, and it seems better to concentrate on the latter.

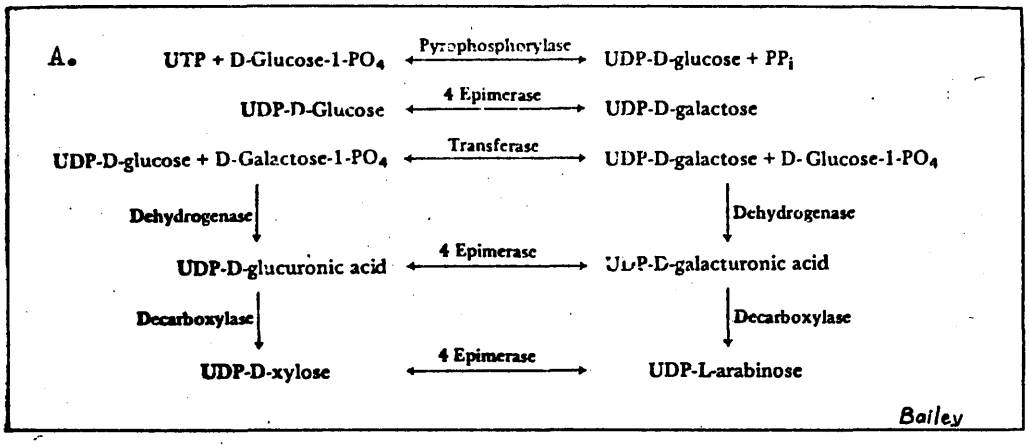
In higher plants the site of synthesis, or at least of assembly into microfibrils,[90] is at the outer surface of the plasmalemma or perhaps within the matrix.[58,91] It has been suggested that the orientation of the microfibrils in the course of synthesis

may be controlled by the interstices in a regular array of small spherical bodies 50-100Å in diameter which have been observed on or above the membrane surface,[92] although the most convincing electron micrographs relate to yeasts rather than to higher plants.[93,94]

There has been some argument about whether the donor molecule in cellulose biosynthesis is GDP-glucose or UDP-glucose. For a long time Hassid's group insisted that in these in vitro experiments the enzyme systems used (the source of which within the living cell is uncertain) were able to synthesise only $\beta(1 \rightarrow 4')$-linked glucans similar to cellulose from GDP-glucose,[95] and only $\beta(1 \rightarrow 3')$-linked glucans similar to callose from UDP-glucose.[96,97] It has since become evident that glucans with both types of linkage can be produced from UDP-glucose, the proportions depending on the reaction conditions and the history of the plant material from which the enzyme system is derived.[98,99] Villemez and Clark claim to have produced a pure $\beta(1 \rightarrow 4')$-linked glucan from UDP-glucose, although they do not state the detection limit for $\beta(1 \rightarrow 3')$ linkages.[100] Either nucleotide, or both, could be the precursor in vivo.[101,102]

There have recently been a number of reports of glucans with mixed $\beta(1 \rightarrow 3')$ and $\beta(1 \rightarrow 4')$ linkages as minor components of cereal and other hemicelluloses.[103-6] In one case the proportions of the two linkages varied with maturity.[107] Since plants are known to synthesise callose at the surface of the endoplasmic reticulum,[91,108] i.e. within the cytoplasm, and cellulose is probably synthesised outside, one can only speculate as to the site from which these mixed glucans originate.

An acceptor molecule is required to initiate the cellulose chain, and could well be membrane-bound, but its nature remains unknown.[60,109] The work of Marx-Figini and her co-workers at Mainz[110-22] has shown that although the degree of polymerisation of cellulose in the primary cell wall is random and comparatively low, that of the secondary cell wall is higher and, in a single plant, essentially constant. This extraordinary finding implies that the chain length is space - rather than time-determined, i.e. that the cellulose molecule in the secondary wall is synthesised upon some kind of template. On current evidence this appears to be in striking contrast to the biosynthesis of other plant polysaccharides.



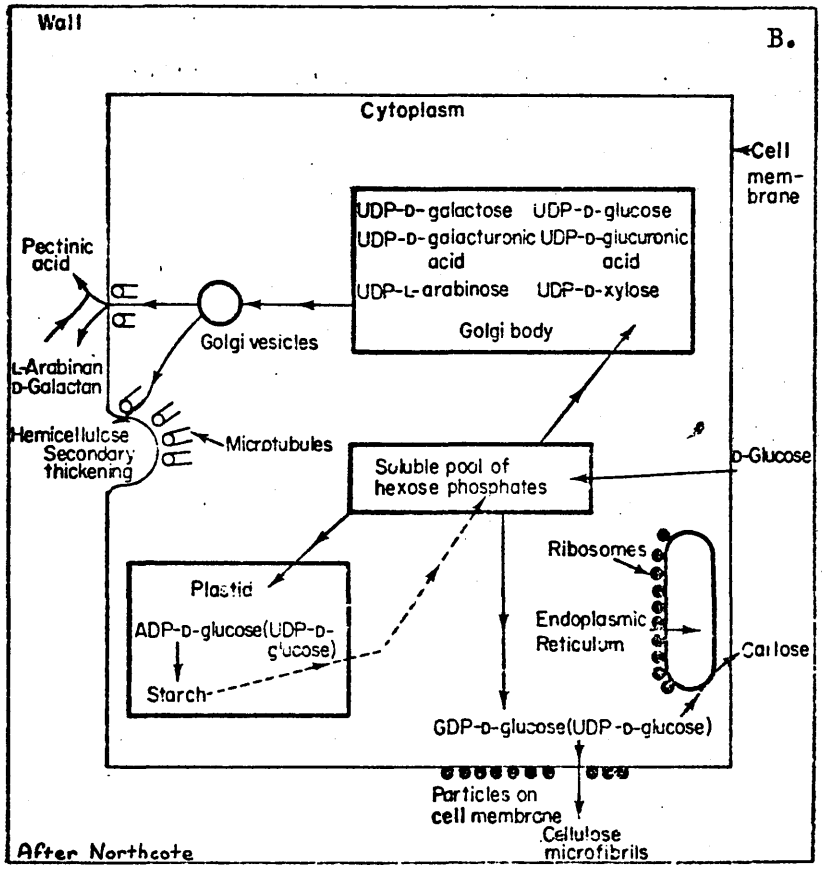


Figure 1.2 - Carbohydrate interconversions (A) and polysaccharide metabolism (B) in plant cells.

The amorphous polysaccharides of the matrix[59,60,65,113] are much more complex than cellulose or starch, and vary from one species to another. In some cases the details of their structure have not yet been elucidated, and not a great deal is known about their biosynthesis. The division into pectic substances and hemicelluloses was originally made on the grounds of solubility, the former being extractable with hot water or calcium-complexing agents like EDTA, and the latter with alkali. In practice the distinction is not as clear-cut as one might wish, but until the present it has been a useful one, reflecting the fact that the hemicellulosic xylans and glucomannans have a much stronger tendency towards crystallinity, like cellulose, than the pectic substances.[114] It is less satisfactory when applied to the diverse and unfamiliar structural polysaccharides now being isolated from leaves.[115-6]

The further solubility-based division of the hemicelluloses into A and B groups[117] has little foundation on structural grounds, and its reproducibility in practice is poor unless unusual precautions are taken.[118] The methods used in animal nutrition for determining 'fibre' and 'soluble carbohydrates' are still more empirical, though in some cases quite sophisticated and introduced only recently. It is unfortunate that there should be such a gap between practices in agricultural science and in plant biochemistry.

The structures of the hemicelluloses show great variety,[122-3] and the differences between genera are often very large. There may also be substantial differences between the hemicelluloses of the primary and the secondary cell wall;[124-5] investigations on those of the primary wall have been infrequent, but there is evidence that in the monocotyledons the main component is an arabinoxylan like that found in larger quantities in the secondary wall.[126-7] A xyloglucan[128] similar to the so-called amyloids (reserve polysaccharides found in seeds and cotyledons) is the main hemicellulosic component of the primary walls of sycamore cells, (and perhaps those of certain other dicotyledonous species),[129] grown in suspension culture, but it is not known if this applies generally to the dicotyledons.

Glucomannans in woody tissues are apparently confined to the secondary wall.[124-5]

For a long time it was in doubt whether the pectic substances contained galacturonic acid, rhamnose, galactose and arabinose in a single molecule, or whether an arabinogalactan was co-extracted with the acidic polymer. It now appears that there are certain plant tissues from which pure galacturonans,[130] arabinans[131] or galactans[132] may be extracted under non-degradative conditions, but that as a general rule highly branched arabinogalactan side-chains are attached directly to the rhamnogalacturonan core[133-4] by linkages from galactose to the C-4 position of rhamnose,[135-6] or to C-3 of galacturonic acid.[135] Blocks of rhamnogalacturonan heavily substituted in this way are separated by lengths of galacturonan chain broken by relatively few rhamnose groups or none at all.[135-6]

Enzyme preparations from various sources have been found, <u>in vitro</u>, to synthesise polysaccharide chains similar to many of the more important types present in pectins and hemicelluloses. The precursors required are nucleotide sugars generally of the uridine series,[9,60,137-9] the main exception being the $\beta(1 \rightarrow 4')$-linked glucomannans which have been synthesised from GDP-mannose and GDP-glucose.[140-1] The same range of nucleotide sugars are involved in the interconversions of monosaccharides,[142] so that starting from UDP-glucose (with the possible addition of myo-inositol which can be a precursor of glucuronic acid)[143] the necessary donor molecules for cell wall biosynthesis can be produced without an excessive number of intermediate steps.

It will be noticed that in the pectic substances all the major constituent monosaccharides have the same configuration on C-4 as galactose, whereas many of those which make up the hemicelluloses have the same configuration as glucose. It has been suggested that the lack of pectic substances in the secondary cell wall may be due to a loss of C-4 epimerase activity, although this seems rather likely to be an over-simplification.[77]

From electron microscopic evidence, it is believed that the pectic substances, the structurally related gums and mucilages, and the hemicelluloses are synthesised by the cytoplasmic organelles known as the Golgi apparatus.[73-7, 144-5] A Golgi body consists of an arrangement of folded membranes like a stack of saucers; during polysaccharide synthesis small membrane-bound vesicles break from its edges and travel across the cytoplasm by some unknown means guided by arrangements of microtubules which have been observed close to the plasmalemma.[146] On arriving, the vesicle membrane is incorporated into the plasmalemma and its contents transferred into the matrix of the cell wall by reverse pinocytosis.

Some workers believe that events at the plasmalemma are of more fundamental significance than is implied in the simplified picture outlined above,[147-8] while others believe that the Golgi apparatus may have a role to play in cellulose biosynthesis as well:[90, 149] it is not known whether the spherical plasmalemma particles synthesise cellulose or merely bundle it into microfibrils, and in yeast they have also been observed on the surface of the Golgi membranes.[58] In addition, the orientation of the microfibrils in the wall seems to be related to that of the microtubules in the cytoplasm.[91-2, 111]

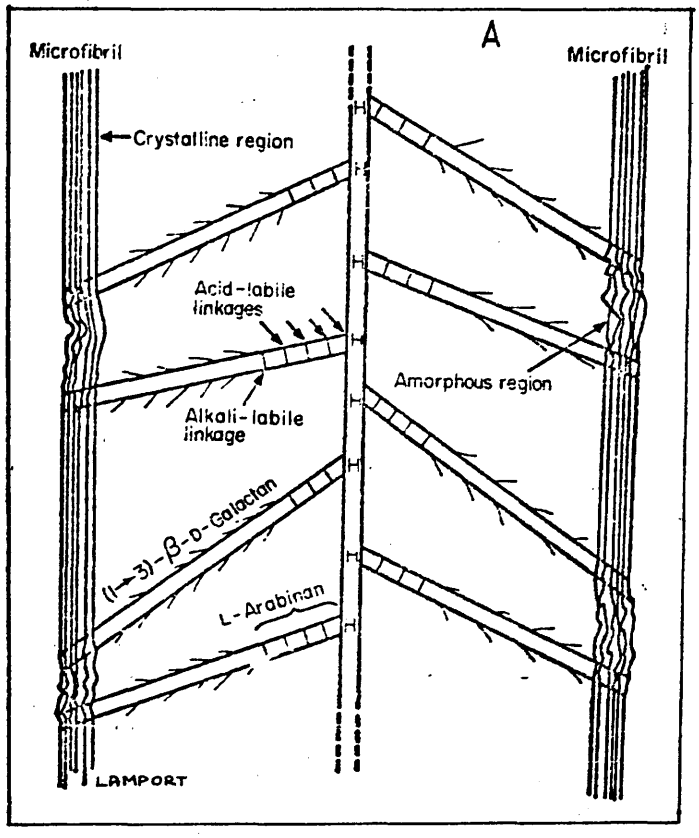
A further possible complicating factor is that although the hemicellulosic xylans are probably synthesised by the Golgi apparatus,[75] this has not been established for the glucomannans; these could just as easily be synthesised, contrary to general opinion, in the same way and in the same place as cellulose.

There have been reports that the polysaccharide content of the cell wall turns over, in certain circumstances.[68-9, 150-1] When this happens the structure of the polysaccharides must reflect the patterns of degradation as well as those of synthesis. Changes in the composition of pectic substances during growth can best be explained by the incorporation of neutral sugars into a galacturonic acid - rich polymer within the cell wall itself, well after these polysaccharides have first been synthesised.[152]

This theory is one of a number that have been proposed to explain the extra flexibility which is presumably required in the walls of rapidly expanding cells, the poor cohesion between the cells of mature fruit[155] and certain varieties of potatoes, and ease of extension which follows treatment with auxin (indole-acetic acid).[156] The pectic substances of ripe apples[165] and of mustard cotyledons[135] contain much higher proportions of neutral sugars than normal, and are less highly charged: this would reduce the opportunities for molecular aggregation of the linear galacturonan portions (as proposed by Rees and Wight)[135] or for bridging between uronic acid carboxyls of neighbouring chains by divalent calcium ions. (an older theory).[156]

The importance of calcium ions is exemplified by the fact that complexing agents such as EDTA are used to extract the pectic substances, and indeed it has occasionally been proposed that natural substances like auxin (indole-acetic acid)[157-8] and phytin,[159] both of which have some complexing ability, might have a loosening effect on the living cell wall in the same way. The amount of auxin available hardly seems comparable with the amount of calcium present, however.[160]

Auxin has controlling effects at the level of gene transcription, and can produce increased activity of synthetic enzymes,[161-3] but newly synthesised polysaccharide material is added to the cell much more slowly (by an order of magnitude) than would be required to explain the onset of auxin-induced elongation.[164-5] Attention has recently been focussed on the hydroxyproline-rich glycoprotein fraction of the cell wall, which clearly has potentialities for structural control.[66] It is apparently attached to the polysaccharide network in some way, but the nature of the linkage has not yet been elucidated. Short arabinogalactan chains are attached through arabinose to many or all of the hydroxy-proline residues, but they do not seem to form a part of the arabinose-rich regions of



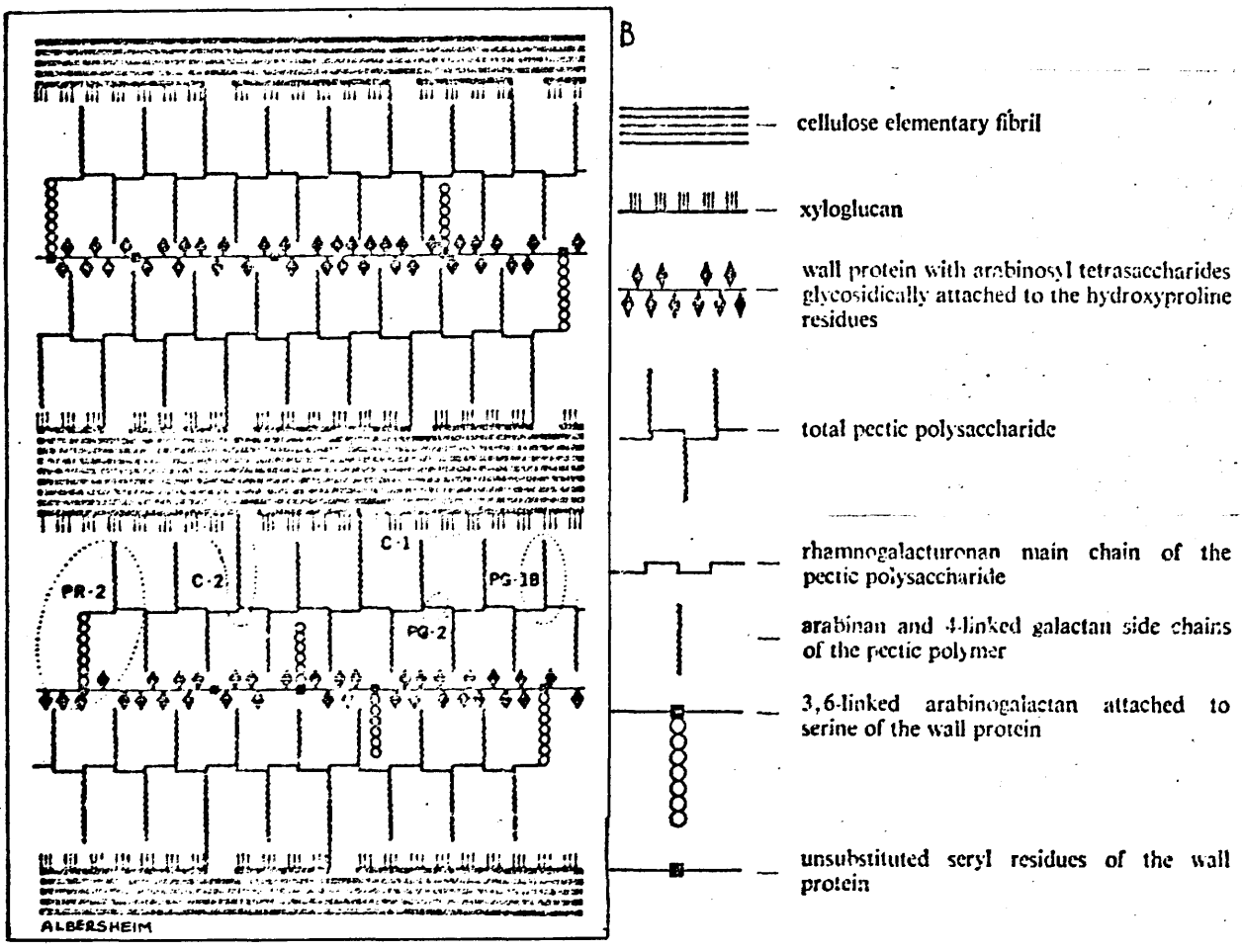


Figure 1.3 - Models of the plant cell wall in relation to its extensibility (A): Lamport, (B): Albersheim.

the pectins or hemicelluloses.[166-9] It has been suggested that the glycoproteins are attached in some other way - possibly through galactose-serine bonds[167] although this has been contested[170] - to the galactan side-chains of the pectic substances in cultured sycamore cells or to a galactose-rich arabinoxylan (hemicellulose B) fraction which is strongly associated with cellulose.[170]

It has been suggested that in cultured sycamore cells the most likely site for rapid auxin-induced extension is the only non-covalent linkage in the wall, the hydrogen-bonded association of the hemicellulosic xyloglucan and the cellulose microfibrils. A suggestion that this was controlled by pH[167] was later retracted.[171] It is however not yet clear whether this tissue, with its possibly exceptional hemicellulose constitution, is representative even of the dicotyledons.

Whatever the mechanism of cell wall extension eventually turns out to be, it is clear that we have a great deal to learn about even the covalent linkages in the matrix - and, for that matter, about the relationship of the complex mucilages to the matrix polysaccharides, when these occur in association. The tertiary structures adopted by cell wall polysaccharides in the living plant, with all their possibilities for controlled hydrogen bonding and inter-chain associations,[172] remain almost unexplored.

B. BRACKEN

Bracken (Pteridium aquilinum (L.) Kuhn) is ubiquitous. It is found on every continent of the world, and in the northern hemisphere its range is from the tropics to north of the Arctic circle.[173-4, 176] In Britain its presence has been recorded almost everywhere except in exclusively agricultural areas and in certain barren parts of the Central and N.W. Highlands of Scotland.[175] On the Continent, it is primarily a woodland plant, typical of the more

open parts of the lowland deciduous forests of Western and Central Europe and the mixed coniferous-deciduous forests between 1000 and 1500m.[177-8] In Scandanavia it is a frequent member of the ground flora of birchwoods.[179]

In Britain most of our corresponding forests have been destroyed at various times between the neolithic revolution five or six thousand years ago and the present day; but the bracken remains, and its distribution is broadly similar to that of the mixed oak and oak-birch forests of former times, as far as this can be determined.[180] Furthermore, the kinds of grass layers found below stands of bracken in the open are not unlike those of modern oakwoods and birchwoods, and the soil types on which bracken is found have a corresponding similarity to woodland soils[181] : in either case there can be a striking contrast with the soils under adjacent heathland.

In woodland, however bracken fronds are in general comparatively sparse, probably for lack of light[182]; the plant 'knows its place' and does not dominate the vegetation as it does over vast areas of open country, sometimes excluding all other species and often rendering the land quite useless for grazing.[183]

As with all ferns there are two generations in the reproductive cycle of bracken.[184-5] The plant as we know it is the sporophyte: when the spores which it carries are dispersed and germinate they grow into the tiny and vulnerable prothallus (the gametophyte) which bears sexual organs. The product of its sexual reproduction is a young sporophyte, which at first is wholly dependent on the prothallus for support and nourishment.[185]

There has been some disagreement on the question of how common this sexual reproduction is in practice. The gametophyte is very sensitive to frost, drought, and soil acidity,[186-7] and the young sporophyte also is vulnerable to frost. Neither is likely to find the Scottish climate at all congenial. The opinion of Braid, Conway and their associates that establishment from spores is at least moderately rare in this country[187] is accepted by many, though not all,[188-9] workers. In wet tropical regions (e.g. Costa Rica),[190]

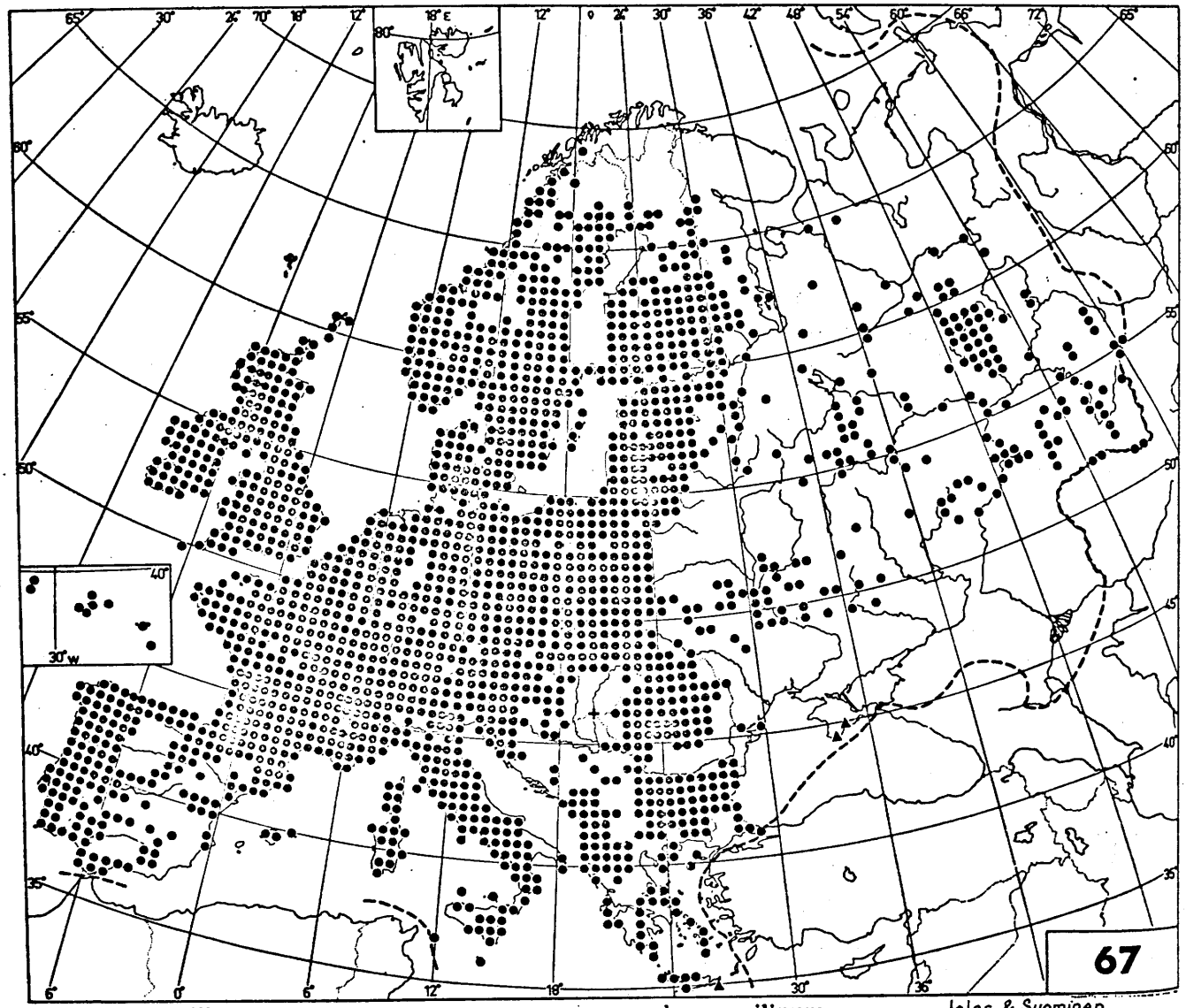


Figure 1.4 - Distribution of bracken in Europe, by 50 km squares.

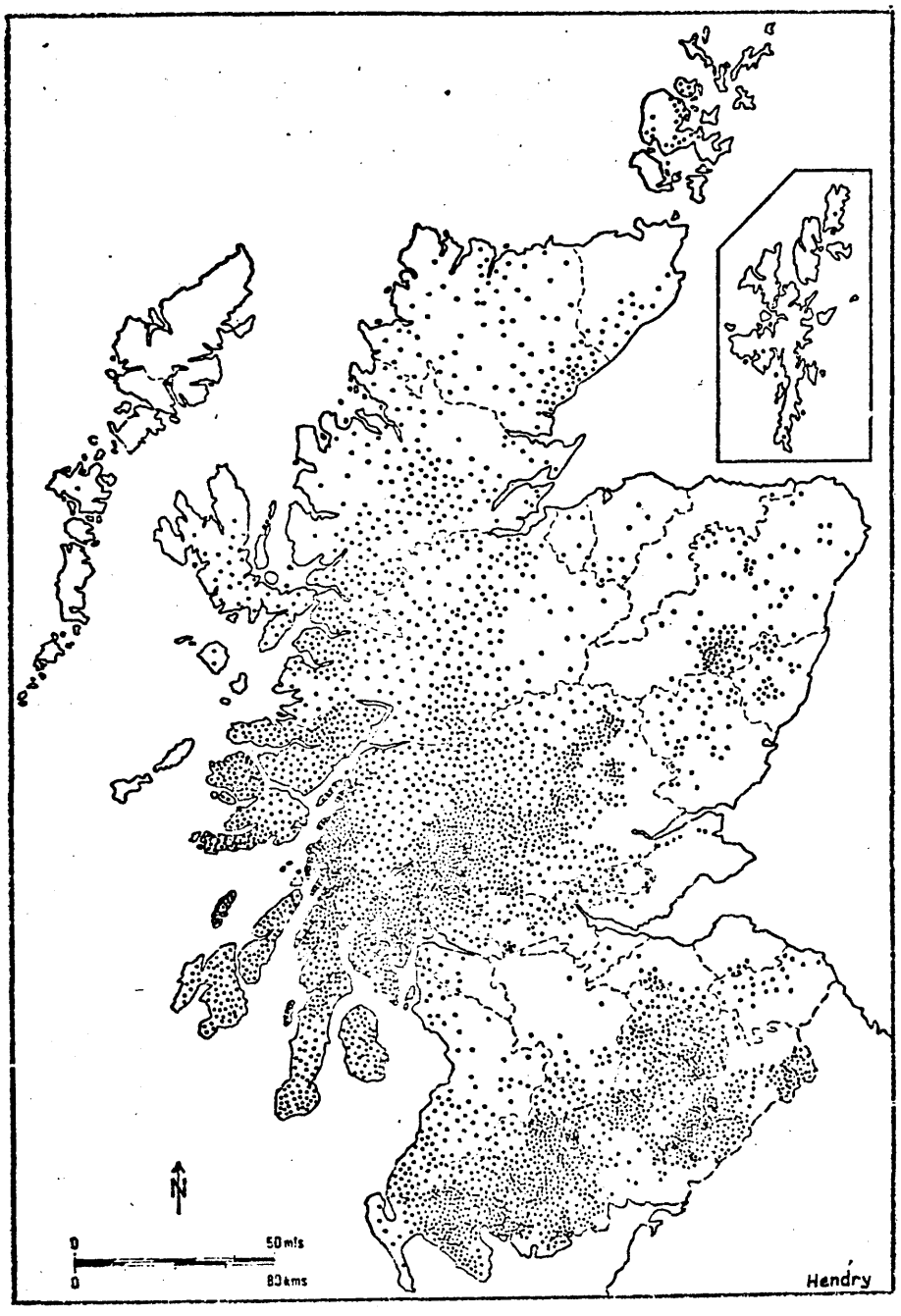


Figure 1.5 - Distribution of bracken in Scotland.
1 dot = 100 acres.

reproduction through spores takes place on a massive scale, while in Continental Europe it occurs to some extent as far north as Finland.[191] Further evidence on this subject is presented in Appendix II. Otherwise this thesis deals solely with the sporophyte.

The normal way in which bracken spreads is by vegetative extension of the underground rhizome system. The main rhizomes of an advancing front of bracken run out parallel to one another, some metres ahead of the first fronds.[192-3] The rate of advance is 1m/year or less, depending on the ground.[191, 194-7]

In Scotland, it has been estimated, about 450,000 acres of pasture are infested with bracken.[198] In agricultural circles it is generally believed to have spread extensively since the period 1700-1850 (in approximate terms) when sheep to a large extent replaced cattle on upland grazings,[199] the practice of muirburn to remove old, unpalatable growth came into regular use,[200] and the cutting of the fronds for bedding, alkali manufacture and other purposes became less common.[201-2] Documentary evidence for such an expansion is sparse, but there seems little doubt that when the intensity of agricultural use in upland areas decreases - and in many upland areas, particularly the north and west highlands, this has been happening almost continuously since the middle of the last century - there is a tendency for bracken to take over the better-drained land. This possibly happens more quickly in areas that have been infested at some time in the past.

Comparatively slight infestation, which does not hinder the growth of the underlying grass, has little effect on the value of pasture. Indeed there is some evidence that the quality of the herbage may in certain cases be improved, for reasons involving the underlying soil (c.f. Mitchell[187, 203] and Appendices I and III). At greater densities, however, the grass layer is composed mainly of shade-tolerant species with little feeding value, or may be suppressed entirely. Such dense bracken communities are quite valueless: they provide no useful grazing for stock, but sheep in poor health often shelter amongst the dense fronds and are then

difficult to locate. Also the fronds harbour sheep-ticks.

Bracken is poisonous to ruminants, and to various other animals as well. It acts in at least two ways, causing both acute thiamine deficiency[204] and carcinogenic effects which resemble those produced by radiation.[205] The incidence of bracken poisoning in cattle is curiously local and sporadic, and its epidemology is not understood at all. One carcinogenic factor has been characterised as shikimic acid, while another remains unidentified[206]: the former at least can pass into the milk of animals eating bracken, and is thus a potential human hazard.[207] There is a correspondence, quite possibly fortuitous but disturbing nevertheless, between the distribution of certain types of cancer in the British Isles and the main bracken areas.[205] Japan, where young bracken fronds are eaten as a delicacy - they are now even being tinned - has one of the highest rates of gastric cancer in the world.[208]

The phases in the annual growth cycle of the plant are shown in Figure 1.6, which is drawn on the basis of one frond emerging per rhizome apex each year. A considerable proportion of frond buds remain dormant, while under some circumstances two adjacent fronds are produced in a single summer.[209] The timing of the phases varies from one place to another and from one year to the next, the complex and delicate balance between the factors involved having been elucidated in formidable detail by Watt.[192-3, 197, 209-12] The main influences are winter and spring frosts, for bracken is not a frost-tolerant plant: even in sheltered or woodland areas it emerges so late that bluebells and creeping soft-grass, in turn, have time to complete their main phases of growth beforehand.[182]

Once the fronds have emerged, however, their growth is extremely rapid, often as much as 0.5m/week. It is this great rapidity of growth that gives the plant its unique ecological advantages, its ability to outstrip and then outshade all competing species under favourable conditions. It is made possible by the large reserves of starch in the storage rhizomes, amounting to 2-3 tons/ha under a dense bracken stand in winter. At some point in the growing season - probably early July on present evidence - the fronds become self-sufficient and thereafter export

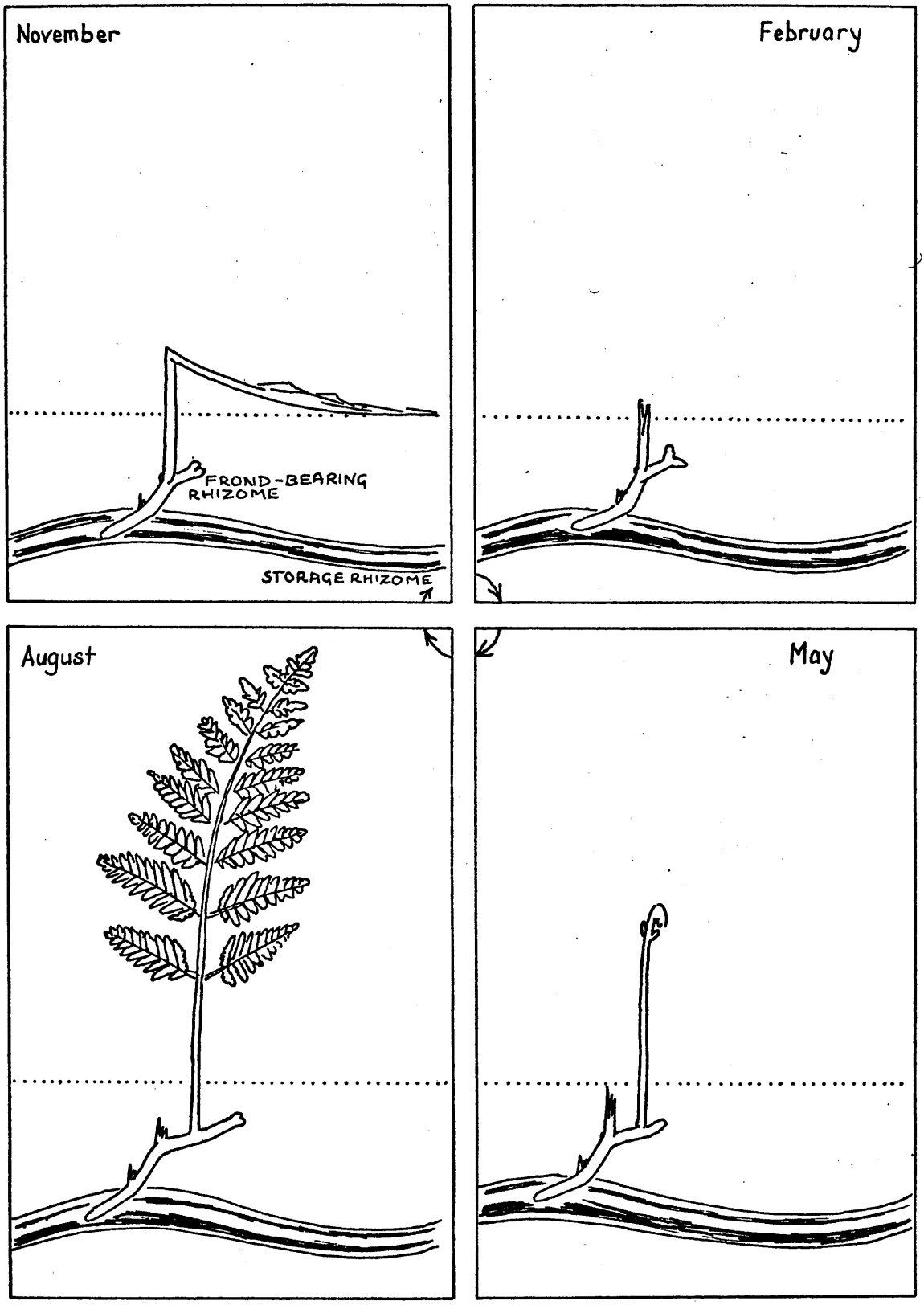


Figure 1.6 - Phases in the annual growth cycle of Scottish bracken.

carbohydrate to replenish the starch reserves in the rhizomes. There is a certain lack of information on the exact timing of these movements in bracken as it grows here, on their dependence on environmental factors at a given site and on possible demands for carbohydrate in the late summer for spore production.

Eradication

A detailed understanding of the sources, flows, and reserves of carbohydrates in natural bracken communities is a prerequisite for any studies on its eradication.[213] Formerly the only eradication method in common use was to destroy the fronds, generally by cutting[214-7] with a scythe although machines for bruising and tearing the fronds had been developed.[218] Cutting when the fronds reach full development, or just before, prevents the starch reserves from being replenished, and if repeated twice a year ought eventually to starve the rhizomes to death.[213] In practice their resilience is extraordinary:[219] after perhaps ten years of regular cutting rather stunted fronds may still be appearing. Cutting bracken is a soul-destroying business, for the plant has more patience than the farmer and does not grow old.

It is obvious that the effectiveness of cutting will depend on the size of the starch reserves, the amounts of carbohydrate used up in new growth, and the distance over which translocation to a growing frond can occur, and these factors presumably lie behind practical observations such as the greater susceptibility to cutting of tall, 'leggy' bracken growing in sheltered areas or in woodland.[220] The detailed quantitative data on the plant's carbohydrate economy necessary to explain these observations are lacking, however.

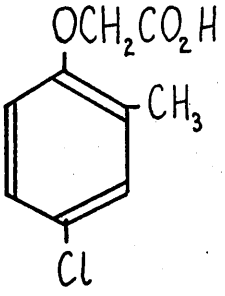
More recently attention has focussed on the use of translocated herbicides for bracken control. Any herbicide which acts only on the growing fronds will naturally be no more effective than cutting;[221-2] nor will it be cheaper as a rule. The problem of finding a herbicide which is translocated to the rhizome and causes sufficient damage there to incapacitate the plant has occupied the attention of numerous workers in the past twenty years, notable amongst whom are

Conway and her successors at this University and at the West of Scotland Agricultural College.[222-8]

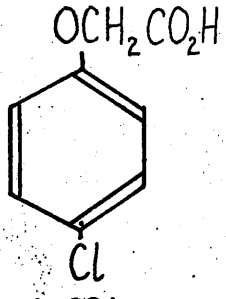
The most successful herbicides used to date have been the growth regulants MCPA,[223,228] 4-CPA,[223,229-30] 2,4-D,[224] picloram,[228,231-2] the carbamate/sulphonamide asulam,[233] amitrole,[234] and glyphosate.[235] All of these are translocated to the rhizome under suitable conditions, and with the exception of glyphosate they produce their effects at the growing points - rhizome apices and developing frond buds. The result is a considerable reduction in frond numbers the following summer, and in some cases a degree of control in subsequent years. Asulam gives the best long-term results at the moment,[235-42] and a rapidly-increasing area is now being sprayed with this chemical, mainly by helicopter.

It is widely agreed that the translocation of this and other herbicides takes place predominantly in the phloem, and that it is closely linked to the movement of sucrose.[18,223,243] The mechanism of this link is not certain: it follows naturally from the mass-flow concept of Crafts,[18] but is not easily reconciled with the activated-diffusion theory[17] or other transport hypotheses. It has important consequences for eradication. A herbicide sprayed onto the fronds in the period of active growth, moves only to the frond apices;[222-3,243] this may give satisfactory control of the fronds for the rest of that summer, and has been found commercially feasible for forest bracken control in Germany (where amitrole is used)[234] and New Zealand (where the emphasis is on asulam).[242] No permanent control results, however.

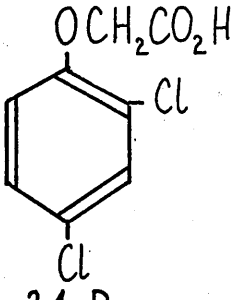
For translocation to the rhizome to be effective the fronds have to have reached full development, and in practice optimum long-term control with most herbicides cannot be achieved before the middle of July.[229-30,236-43] It would seem likely that this is the time when the flow of sucrose becomes predominantly downwards, but until now too little has been known about carbohydrate movements in Scottish bracken for a detailed comparison to be attempted. It remains to be established whether or not there are significant variations in the date when the carbohydrate flow is reversed at different sites. Nor is it known if the less satisfactory control with asulam which has been observed after mid-August[236-41] is caused by a falling-off of downward carbohydrate



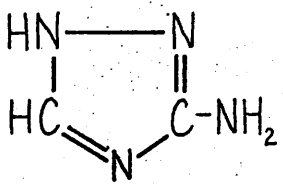
MCPA



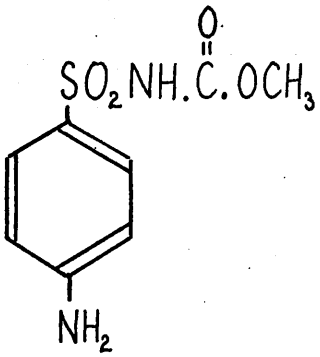
4-CPA



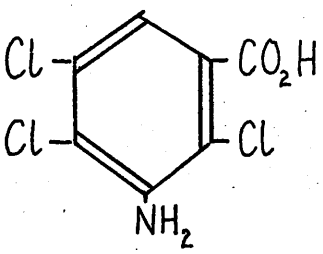
2,4-D



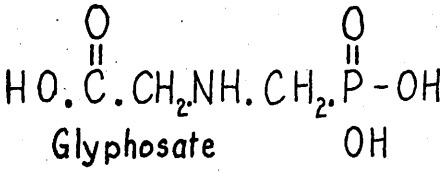
Amitrole



Asulam



Picloram



Glyphosate

Figure 1.7 - Structures of some herbicides that have been used in bracken control.

translocation (due to frond senescence or perhaps the use of carbohydrate for spore production), or by poorer penetration as the cuticle grows thicker and less permeable.[243]

Recent work in this laboratory,[244] indicates that penetration is a slow process and may be prematurely curtailed if a shower of rain, on the day of spraying or even the day after, washes the herbicide from the fronds. The result is that when penetration is the limiting factor control tends to be erratic rather than consistently poor; and this appears to be the case late in the season.[236-41] The incorporation of a surfactant to hasten penetration is thus more desirable than was originally thought,[244] and is probably essential for late-season spraying.

All the above remarks about growth habit, carbohydrate movements and the effect of herbicides apply only to bracken as it grows on the open hill. It cannot be over-emphasised that bracken grown from spores or from rhizome cuttings in the glasshouse or growth chamber, while it may have valuable advantages in providing uniform and reproducible material, behaves in a totally different way from a mature community growing in a relatively exposed site.

Young sporelings reared in the laboratory are in a state of rapid growth. The rhizomes extend rapidly, and up to five or six fronds may be differentiated in a single year.[187] Thus sucrose exported from a mature frond is translocated straight into the growing frond which succeeds it on the same shoot[245]: nothing that can be described as a storage rhizome develops until about the fourth or fifth year of the sporeling's life. As well as this the leaf structure of glasshouse-grown sporelings is much more delicate and the cuticle much thinner; in the field the young plant is very sensitive to frost.[187] As a result of these differences (and perhaps others which are not obvious), most herbicides that have been used have much more pronounced effects on glasshouse plants than can be achieved in the field, particularly at the growing points where most of the visible damage appears.[219,228,243]

To a lesser extent bracken growing naturally in woodland or in sheltered areas shows the same features. The effect of exposure on the structure of the frond was very well described by Boodle more than seventy years ago,[246] and it is likely that woodland bracken also has a smaller proportion of storage rhizome and a greater tendency to produce two fronds on the same shoot in one season, although this has not been proved.

From what has been said above, it follows that much of the work that has been done on the physiology of herbicides in bracken, though in many cases impossible to fault in other respects, is almost valueless from the point of view of understanding the basis of chemical control in the field. In this respect the results of Hamilton and Canny's[245] and Whittle's[247] experiments on sucrose transport are likewise quite misleading, although their value for their intended purpose -elucidating the theory of translocation in higher plants generally - is unquestionable.

Carbohydrate biosynthesis in bracken

The various carbohydrate-containing fractions in bracken are unusually well documented, particularly for a weed species. It has been shown that carbohydrate is translocated solely in the form of sucrose,[245] and that the storage polysaccharide is starch[248] (fructans[249] being absent).[250] Detailed information is available on the cellulose,[251] hemicelluloses,[252-3] free sugars,[248] and phenolic,[254-6] steryl[257] and other[258-9] glycosides in the fronds, and although there is a certain lack of published data on the rhizome the general morphological similarity between the two organs suggests that they should not differ fundamentally in the nature of their carbohydrate constituents. Nothing that was found during the research to be described here contradicted this assumption although naturally there are large quantitative differences in the proportions of different fractions in frond and rhizome.

For a number of years members of this Section have been working on substances thought to be involved in the biosynthesis of sugar-containing substances in bracken. Duncan[12,54] has made a study of the nucleotide sugars present in the rhizomes in a readily extractable form, i.e. excluding those which are locked up inside starch grains. Morton,[260] also in this laboratory, investigated the glycolipid fraction in the rhizomes with the idea that lipid intermediates of the kind found in bacteria might become evident, although because of certain experimental difficulties the results of his work must be interpreted with some reserve. These earlier investigations made it possible to consider any further data on the carbohydrate composition of bracken against its biosynthetic background, and a general comparison of the nucleotide sugar fraction with the monosaccharide residues combined in all the other forms has been attempted.

C. THESIS OBJECTIVES

In the various projects which made up the work for this thesis the only common, unifying objective (if any) was a fundamental rather than an applied one. The general direction taken was towards the elucidation of the overall carbohydrate metabolism of plants, in particular the behaviour of the 'energy' carbohydrates in species which rely for their survival on vegetative regeneration from underground starch containing organs following winter dormancy. (viz., bracken and potatoes). The various practical objectives which were combined with this theoretical one will be outlined individually.

Chapter 2:

Seasonal and other variations in the carbohydrate composition of bracken.

The intention was to build up a background of information on sources, reserves, and patterns of carbohydrate translocation that would make it possible to consider the movement of herbicides within the plant, and its ability to recover after spraying, on a more secure basis. The carbohydrate fractions studied were glucose, fructose and sucrose; starch; and the mucilaginous polysaccharide, Williams and Foley, at the West of Scotland Agricultural College were working simultaneously on the same subject and their results complement those obtained here, with more comprehensive sampling but less detailed analysis.[261-2]

Chapter 3:

Plastid lipids in bracken.

(a) Variations in different parts of the plant.

A necessary preliminary to any work on the subject of hypothetical lipid intermediates in polysaccharide biosynthesis is to find out what ordinary lipids are present as components of cell membranes and organelles. In fact it seems likely that in starch-storing

tissue a considerable part of the lipid content is associated with the amyloplasts, and any circumstantial evidence on the nature of the lipids in these organs (which cannot at present be isolated) is of interest.

(b) Diurnal variation in the fronds.

A trial assessment of this was envisaged as part of the carbohydrate investigations described in Chapter 1, but the results proved interesting enough to justify its completion, and have implications both for the lipid analysis of photosynthetic tissue in general, and for the role of lipids in the chloroplast.

Chapter 4:

The starch-sugar balance and lipid composition of potatoes in low-temperature storage.

Temperatures below about 10°C cause a reversible increase in the free sugar content of potatoes, at the expense of starch. It has been suggested that this is due to breakdown of the amyloplast membranes on chilling, followed by enzymic degradation of the exposed starch grain. If this is the case the membrane damage should be reflected in the lipid composition. The lipids of 4 varieties which 'sweeten' to different extents at low temperatures were therefore examined.

Low free sugar levels are required commercially in potatoes intended for processing. It was therefore of practical interest to find out how other varieties compare in this with Record, the variety generally preferred for processing into crisps. The potatoes were kindly provided by Mr. J. Dalziel of this section, who had grown them as controls in a field experiment.

Chapter 5:

The composition and structure of the mucilaginous polysaccharide in bracken.

Some work on the isolation and composition of this complex polysaccharide had already been carried out in this laboratory. This was confirmed and amplified. After some initial structural studies it was decided that a full-scale structure determination, involving methylation analysis and periodate and lead tetra-acetate oxidation, could not be justified in this agriculturally-oriented laboratory. Examination of the polysaccharide was therefore limited to establishing its homogeneity, attempting to determine its molecular weight, and studying the products of acid fragmentation. Some of the techniques developed for this work, e.g. the handling of oligosaccharides in small quantities and the paper chromatography of monosaccharides, are original and could be generally useful.

The implications of Chapters 2-5 for carbohydrate biosynthesis, and for the eradication of bracken, are discussed in more detail in Chapters 6 and 7.

CHAPTER II

THE 'ENERGY' CARBOHYDRATES IN BRACKEN RHIZOMES

A. THE SEASONAL PATTERN

Introduction

As was explained earlier there is an urgent need for information on the accumulation and movements of carbohydrates in bracken growing on the open hill. The carbohydrate economy of the plant has a twofold significance for its eradication, being the most fundamental guide to its vigour and resilience,[213] and at the same time the primary factor controlling the translocation of herbicides.[213] The most reliable way of studying carbohydrate production and translocation would be to administer $^{14}CO_2$ and follow the radioactive products, but while this is comparatively simple in the glasshouse[243] or growth chamber it would present formidable difficulties with bracken in the field. For this study it was decided to concentrate on extracting as much information as possible from the carbohydrate composition of natural bracken rhizomes sampled periodically. As well as the established 'energy' carbohydrates - starch and free sugars - the mucilaginous polysaccharide was included as its function was unknown.

Experimental

Rhizomes for extraction were collected from Drumclog Muir (site 1) at about 11.00 on each sampling occasion, wet days being avoided. Normally all the rhizomes from a pit of 0.5 m^2 in area were collected, washed, and separated into frond-bearing, intermediate, and storage categories. In some cases the proportions

of these classes were established by weighing and sub-samples for analysis chosen with the same proportions by weight; more often the proportions in the sub-samples were simply estimated.

Two or more 100g sub-samples were extracted either immediately or after 1-2 days storage at 4°C. The moisture content was determined (after 18hr at 110°C) on about 10g of small rhizome segments; on sampling dates after August 27, 1972, each section of rhizome to be extracted had about 10% of its length removed for moisture determination so that the moisture content determined reflected that of the extracted sample as closely as possible. Since bracken rhizomes contain very variable amounts of moisture this procedure improved the reproducibility of the other analytical results, when these were expressed on a dry matter basis.

The extraction procedure finally decided upon was the result of a number of preliminary trials. The main requirements were:

(1) rapid penetration by an alcoholic solvent to eliminate enzyme activity as quickly as possible after homogenisation began. Invertase activity in bracken rhizomes is particularly high and the enzyme is not readily inactivated, e.g. by heat.

(2) extraction of the mucilage with water, at a temperature high enough to ensure that none remained after 2 extractions, but too low to bring starch into solution. The temperature tended to rise during homogenisation.

The following extraction sequence was found to be satisfactory. Immediately after weighing, the rhizomes were chopped into a blender containing the first batch of methanol. Between homogenisations the slurry was strained through muslin or nylon bolting cloth.

(a) Homogenised with methanol (400ml) for 4 min, starting at room temperature and rising to ca. 40°C.

(b) Homogenised with methanol (200ml) for 1 min at room temperature.

(c) Homogenised with water (200ml) for 3 min, starting at 32°C and rising to 38-40°C.

(d) Homogenised with water (150ml) for 1 min at room temperature.

The combined methanol extracts were left for 30 min to allow the starch grains and cell wall debris to settle, and the supernatant was then poured off and filtered. The sediment was added to the combined aqueous extracts which were then allowed to settle for 3hr at 4°C. The solid material was washed twice with water, with a 30 min settling period after each washing, to remove the fine solid debris (which is less dense than the starch and sediments more slowly), then re-suspended and passed through a 300 mesh sieve to remove coarser cell wall debris. The starch was then dried at 30-40°C and weighed. On examination under the microscope it appeared to contain only a small proportion (a few percent) of contaminating particles.

The supernatant from the aqueous rhizome extracts was acidified at 4°C with 2g of trichloro-acetic acid and centrifuged, still in the cold, for 40 min at 12,000 r.p.m. to remove proteins etc. Leucoanthocyanins and related substances were also largely removed by this treatment. The mucilage was precipitated with 3 volumes of methanol, coagulation being assisted by addition of LiCl to 0.2%, and re-dissolved in water for estimation by the phenol-sulphuric acid method. (G.M.1).

The methanol supernatant and the combined methanol extracts were analysed separately for glucose, fructose, and sucrose (G.M.5). This method was found to be quicker and more accurate than quantitative paper chromatography: Wilson's method gives poor results with sucrose, and determination by the phenol-sulphuric method after leaching from the paper was found to be laborious and comparatively insensitive. Methods relying on glucose oxidase[34] are probably suspect in the presence of the large quantities of phenolic substances which are co-extracted from bracken rhizomes, and which could well reduce the enzyme activity;

this would make for difficulties in calibration. A g.l.c. method has since been developed here, although it would require a preliminary clean-up stage to give good results with these extracts.

Checks on the reliability of the method.

Two of the more obvious criticisms of the method are that storage even for a short time at 4°C prior to analysis might be expected to cause some conversion of starch to free sugars, as with potatoes;[264] and that invertase activity might not have been completely prevented. These two possibilities were therefore investigated further.

a) Storage. Samples were stored for 5 days at 4 different temperatures before analysis. One batch was stored in the deep freeze and should therefore have been unchanged in sugar content. The results are shown in Table 2.1.

Storage temperature	-14°	4°	10°	room temp.
Total free sugars, % D.M.	8.5	8.2	7.9	8.1%

Table 2.1

Free sugar levels after storage at different temperatures.

There was no significant difference in total free sugar content between the deep frozen sample (which should be unchanged by storage) and any of the others. It would appear either that bracken rhizomes do not 'sweeten' at low temperatures in the same way as potatoes, or that they do so more slowly, or that respiration and hence conversion of starch were inhibited by the storage conditions (in sealed polythene bags). At any rate, the sugar levels are apparently unaffected by the storage conditions used.

b) Invertase action. 1g of sucrose was added to the methanol for the first extraction of duplicate 100g rhizome samples, and by comparison with duplicate controls it was estimated that 5% was hydrolysed to glucose and fructose: under the conditions of the experiment this amount is not significantly different from zero.

Enough replicated samples were analysed during the later part of the investigation for the reproducibility of the method to be estimated. The following relative errors are applicable to results within the normal range:

starch	± 20%
mucilage	± 10%
glucose	± 12%
fructose	± 8%
sucrose	± 6%

Low starch values are subject to still greater relative errors. The poor reproducibility in the starch measurement is due to the large variations between different rhizomes, or even between different parts of the same rhizome, rather than to deficiencies in the experimental method. To a lesser extent this applies to the other constituents as well.

Results and Discussion

The variation in dry matter and starch content is shown in Figure 2.1 and the free sugar and mucilaginous polysaccharide levels in Figure 2.2. There are not enough starch determinations during the crucial early summer period for any detailed conclusions to be reached, but the general pattern of a fall during the period of active growth followed by a rise to relatively constant autumn and winter values can be discerned. The dry matter percentage, for which fuller results are available, shows the same seasonal pattern.

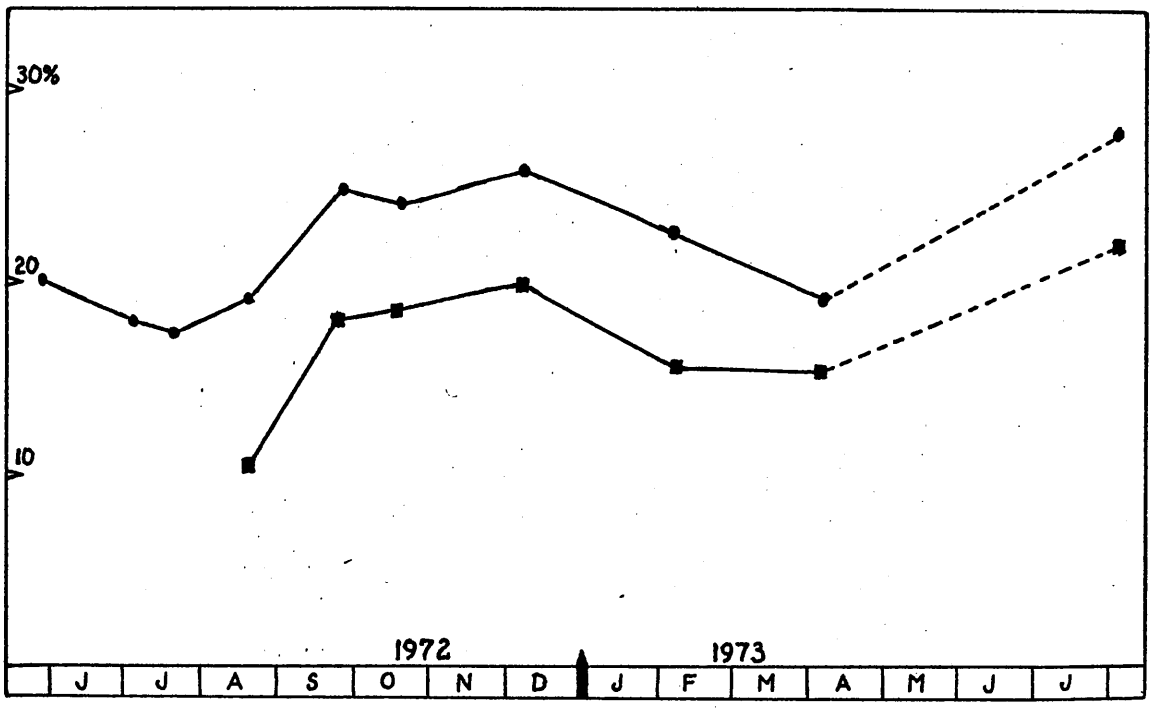


Figure 2.1 - Percentage dry matter (●) and starch content (■) in rhizomes from Drumclog, June 1972 - July 1973.

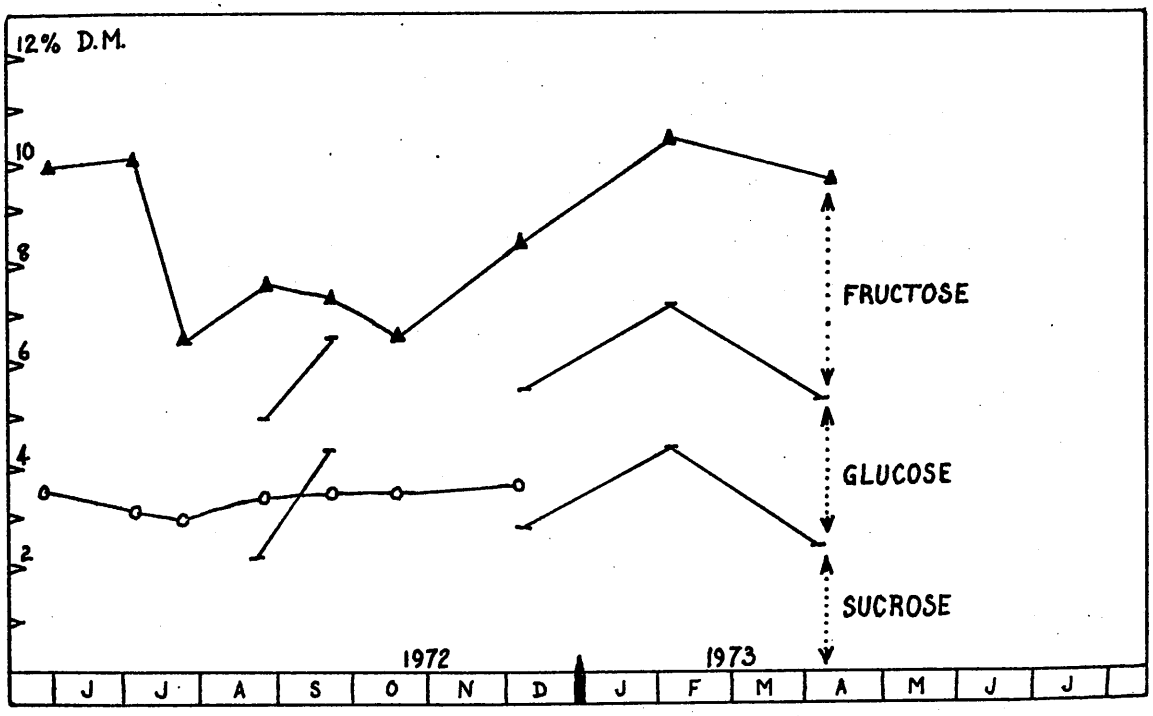


Figure 2.2 - Free sugar (▲) and mucilage content (o) in rhizomes from Drumclog, June 1972 - April 1973.

The provisional results of Williams and Foley,[261] which recently became available, show the minimum in starch content as occurring in mid-July. Reversal of translocation at this date would correspond well with the observation that good control with asulam and other herbicides cannot be achieved until then.[235-42] Both the results in Figure 2.1 and those obtained by Williams and Foley[261] show a rather slow recovery of the starch reserves, continuing well into September.

If this is typical of Scottish sites it means that the less satisfactory results achieved by spraying after mid-August cannot be explained by reduced translocation, and slower penetration is a more likely reason.[243-4] This is supported by the fact that control late in the season is erratic,[236-41] presumably due to rain washing the herbicide from the fronds before uptake is complete, and to increased penetration resistance under weather conditions in which the relative water content of the fronds is low.[244,263] If the rate of penetration at this time of year could be increased by more efficient use of surfactants and perhaps other additives,[263] spraying could possibly be extended into September; this would have advantages for both the farmer and the spraying contractor.

The interpretation of the free sugar results is more difficult, as percentage dry matter is not an altogether realistic basis for expression due to the large variations in starch content. The total sugar levels appear to remain approximately constant during the winter and spring, falling in July and recovering over the rest of the summer and autumn. (Williams and Foley's 'mobile carbohydrates' show similar variations).[261] The proportion of sucrose may be higher in the late summer and low just before frond emergence. The developing fronds at this time had very high reducing sugar levels - 5.3% sucrose and 20.2% reducing sugars.

Williams and Foley went on to draw conclusions about source-sink relationships and carbohydrate translocation from their mobile carbohydrate data. While any reliable information on these subjects is to be welcomed, it could be argued that such speculation,

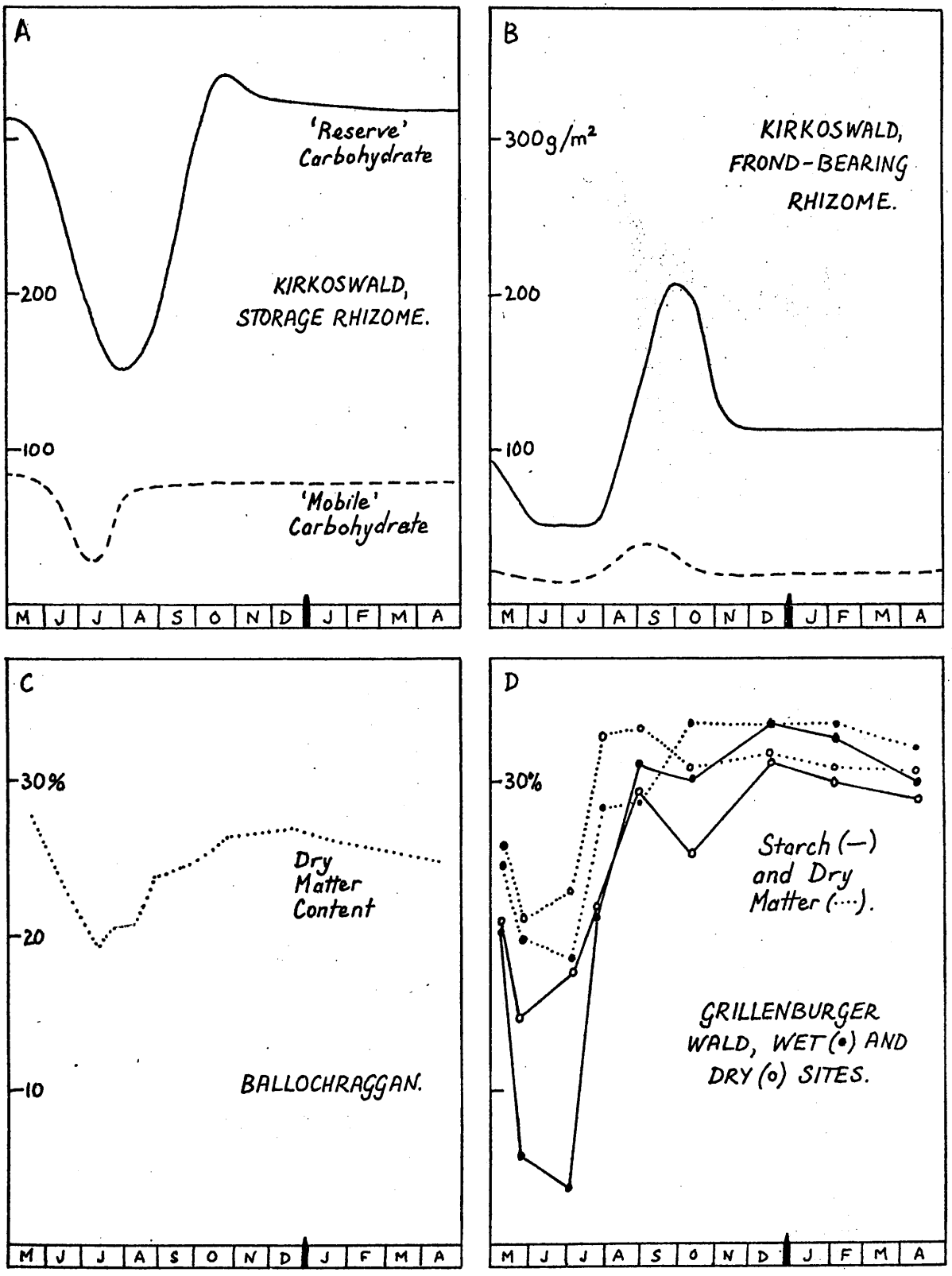


Figure 2.3 - Dry matter and carbohydrate data from other sources. A & B, Williams & Foley (diagrammatic); C, Hunter; D, Muller-Stoll & Michael.

at the present state of knowledge, is somewhat premature. The variations in sucrose and reducing sugar levels in developing potato tubers, for example, can be explained quite satisfactorily on the basis of carbohydrate supply and demand;[264] but the interconversion of sucrose and starch is a complex process and its regulation is not wholly understood,[38,46,49,265-6] nor is there agreement on whether the driving force for translocation[267-8] is located at the source,[269-70] the sink,[271,18,62] or somewhere along the way.[272] Further, in a frost-sensitive plant such as bracken it is likely that free sugar levels will be affected by temperature. (c.f. Chapter V).

The levels of the mucilaginous polysaccharide are fairly constant throughout the year. There is perhaps a slight dip during the period of frond development (May - July), when the levels of non-starch constituents expressed as % D.M. would be expected to rise due to the reduced dry matter content. It is possible, therefore, that a little of the polysaccharide is metabolised for energy at this time, but the amount is hardly significant. A structural or other role for this substance therefore appears more likely.

Dry matter or reserve carbohydrate levels from a number of other sources[248,261,278] are shown in Figure 2.3. Although these are not strictly comparable, it would appear that bracken growing in the open in this country makes smaller demands on its starch reserves, but takes longer to replenish them, than forest bracken in Germany. The difference may be due to either climate or environment. In S. England (and presumably also in Germany) spore production is earlier than in Scotland[182]: on the other hand woodland bracken with its long petioles must require more starch for the growth of the fronds before they become self-sufficient,[273] yet it shows no sign of having proportionately more storage rhizome. The same is true of bracken growing in all sheltered places.

Any such environmental effects on the carbohydrate economy of the plant are clearly relevant to its eradication. It is not known, for example, if at a site where the fronds emerge early, or in a year in which a mild spring encourages early frond emergence,

the reversal of translocation is also significantly earlier. It has been noted[220] that tall, 'leggy' bracken is more readily controlled by cutting than the short, sparse kind growing in exposed situations, but whether or not this applies to chemical control as well is unknown.

B. THE EFFECT OF HERBICIDE TREATMENT

The analysis of the carbohydrate constitution of the rhizome has considerable possibilities for monitoring herbicide-induced effects on the plant. This has been attempted in a rather limited way. Rhizome samples were taken from an Argyllshire site, Bun-an-Loch (site 12) in the autumn following a heavy application of asulam, well in excess of the recommended rate. Their composition was so remarkable that a second series of samples was taken from the same site, and some from another site (Sundaywell, site 5), which had been sprayed at the normal rate of 4.5 kg/ha. The results are shown in Table 2.2.

	% D.M.	Starch, % D.M.	Glucose	Fructose	Sucrose
Bun-an-Loch, 20th September 1972:					
sprayed June	23.1	24.6	3.8	3.0	2.7
sprayed August	21.7	9.8	2.9	2.1	2.5
Bun-an-Loch, 2nd January 1973:					
sprayed June	20.7	22.5	2.5	2.1	2.2
sprayed August	22.3	11.7	2.7	2.3	1.5
Control	26.6	24.8	2.6	2.8	2.3
Sundaywell, 18th November 1973:					
sprayed August	22.2	21.3	not determined		
Control	24.8	22.6			

Table 2.2

Carbohydrate content of rhizomes after asulam treatment.

The starch content of the Bun-an-Loch rhizomes is obviously drastically reduced in the August-sprayed plot, and there is possibly a slight fall in the sucrose level. The rhizomes from the June-sprayed plot and from Sundaywell were quite normal. It seems therefore that the rapid consumption of starch after the August spraying at Bun-an-Loch was an exceptional effect produced by the excessive application rate. There were also visible effects on the rhizomes-sclerotic areas and withering of the frond buds and rhizome apices - which are not observed when asulam is applied at normal rates in the field.

This loss of starch, without any apparent growth or increase in other carbohydrates, implies an unusually high respiration rate in the months following spraying. The opposite result - a significantly decreased respiration rate in the storage rhizome - along with damage to the growing points and eventually to the fronds, was observed during a study of the effect of asulam on Japanese bracken (Pteridium aquilinum var. latiusculum),[274] and there are indications that in rhizomes of Scottish bracken also the respiration rate may be reduced by asulam treatment.[275] The apparent contradiction in results could be due merely to different application rates or different environmental conditions. What is more important is that asulam is capable of producing effects within the storage rhizome as well as at the growing points, and that under suitable conditions these effects can be detected by analysis of the rhizome carbohydrates. The implications will be discussed more fully in Chapter VII.

CHAPTER III

GLYCOLIPIDS AND PHOSPHOLIPIDS IN BRACKEN.

A. THE DISTRIBUTION BETWEEN FRONDS AND RHIZOMES

Introduction

In view of the suggestion that glucose-containing lipids might be intermediates at some stage in the biosynthesis of starch,[54,277] some preliminary investigations of the lipid components of bracken rhizomes were embarked upon. These were intended to follow on from the rather inconclusive work of Morton[260] on the same subject, but at an early stage it became clear that no progress towards the isolation of such hypothetical intermediates - presumably very labile and containing phosphate[278-9] - would be possible until the 'ordinary' glycolipids and phospholipids[279] in the rhizomes had been properly characterised. To do this would be of some value in itself, since not a great deal is known about the lipids associated with amyloplasts within the living plant (as opposed to starch grains which are inevitably subjected to a good deal of physical disruption and enzymic degradation while being isolated).[10,42] Also the lipid membrane surrounding the amyloplast must have an important compartmenting role.[38,56]

Of course, not all the lipid material in the rhizomes is necessarily associated with the amyloplasts. Mitochondria,[280] and the cell membranes themselves,[281-2] are likely to be an important source. Accordingly it was decided to determine the distribution of polar lipids in all parts of the plant, from the pinnae where these lipids are present mainly in the chloroplasts, down through the rachis and petiole which become progressively more etiolated towards the base but contain virtually no starch, into the rhizome. It was hoped that

that this would give an indication of the levels of 'background' lipids not associated with either chloroplasts or amyloplasts, and at the same time the contrast in lipid composition between the two types of plastid was of some interest.

Analysis of intact tissue is the only course possible when it is intended to look at the lipid composition of amyloplasts, due to the present lack of a satisfactory method for isolating these organelles with the outer membrane intact.[56] But it is also to be preferred for photosynthetic tissue[283]; enzymic degradation of chloroplast lipids is extremely rapid,[284] and would be a serious problem with chloroplasts isolated in aqueous media.

Experimental

Materials. Bracken fronds and rhizomes were collected from the woodland at Auchentorlie, Dunbartonshire (site 3), on 29th May 1973, at 11.30 a.m. (Some lipids are known to show diurnal variability[285-6]). The fronds had one pair of pinnae unfolded.

Lipid Analysis. The analytical methods are basically those developed by Roughan and Batt,[285,287] with modifications to improve the sensitivity. After 1½hr of collection, portions (10-20g) of fresh tissue were extracted as described in G.M.10 and analysed for carbohydrate- and phosphate-containing lipids by quantitative TLC. (G.M.11-12). All lipid components were checked for homogeneity by 2-dimensional TLC in CMHOAc followed by $CMNH_3$, and were identified by their chromatographic behaviour on DEAE-cellulose and silica gel; by their reaction with 50% H_2SO_4, α-naphthol-H_2SO_4, Liebermann-Burchard reagent, and ninhydrin; and by their phosphate and carbohydrate contents. In addition, glycolipid bands were scraped from the plate and hydrolysed with 0.5M H_2SO_4 for 1 hr at 100°,[287] without prior removal from the adsorbent. The supernatant was neutralised with ion-exchange resin (AG1-X4 200-400 mesh, carbonate form, and a little AG50-X4, 200-400 mesh, hydrogen form), and evaporated to dryness in vacuo. The sugars released were dissolved in saturated benzoic acid solution and identified by PC in EtOAcPy.

Chlorophyll was determined spectrophotometrically as described by Arnon[288] and Bruinsma[289].

Results and Discussion.

The bracken plants were divided into the following 6 sections:

(1) Pinnae
(2) Upper 15 cm of rachis
(3) Upper 15 cm of petiole
(4) Lower 10 cm of petiole
(5) Upper, frond-bearing rhizome
(6) Lower, storage rhizome.

The distribution of glycolipids and phospholipids in the fronds and rhizomes is shown in Table 3.1. In addition, the rhizomes contained two unidentified glycolipids which released glucose on acid hydrolysis, and ran respectively just behind, and just ahead of, digalactosyl diglyceride. Neither contained any phosphate. Only traces of phosphatidyl ethanolamine were detected, and phosphatidic acid, a common product of enzymic degradation,[290] was absent.

The figures in Table 3.1 are averages from between two and five determinations: typical relative errors vary between ± 4% (e.g. MGD in pinnae, 4.8 μmoles/g) and ± 30% (e.g. MGD in frond-bearing rhizome, 0.03 μmoles/g).

The overall lipid composition of the pinnae is qualitatively similar to that of Dryopteris fronds,[291-2] and quantitatively not unlike that of Blechnum fronds,[285] though with lower proportions of PG, DPG and PE. The molar ratio MGD:DGD increases from 1.0 in the etiolated lower petiole to 1.9 in the pinnae, the same trend as has been noted when etiolated tissues of higher plants turn green[283,286,293].

If we take the lipid content of the lower petiole and the frond-bearing rhizome as representing the 'background' of lipids not

associated with chloroplasts or amyloplasts, this is comparatively small and would not make a great deal of difference if subtracted from the amounts in the frond and the storage rhizome. It is normal, however, to find more mitochondria in photosynthetic and storage tissues than in those whose main purpose is structural or concerned with transport , and the mitochondrial contribution to the overall lipid composition of the fronds and rhizomes cannot be neglected. Mitochondrial membranes contain a high proportion of phospholipids, the main components being PC, PE, PI and DPC[280]. There may also be somewhat larger numbers of other lipid-containing organelles in the storage rhizome than in the relatively woody tissues of the lower petiole and the frond-bearing rhizome, but the contrast between the very small amounts of the recognised chloroplast lipids - the galactolipids, phosphatidyl glycerol and the sulpholipid - and the much larger quantities in both the frond and the storage rhizome, does on the whole suggest that these lipids are also constituents of the amyloplast membranes. A low monogalactosyl:digalactosyl diglyceride ratio has been observed in other storage tissues[290, 294-6, 299-301] and would appear to be a typical feature of the amyloplast lipid assemblage.

One of the two unidentified glucose-containing lipids from the rhizomes gave a most unusual yellow-green colour with the sulphuric acid spray; its nature is quite unknown and would merit further investigation. As neither of these glycolipids contained any phosphate they are unlikely candidates for the role of an intermediate in starch biosynthesis. No other lipids that seemed suitable for this function were detected, either with or without the separation on DEAE cellulose. With the exception of this stage the procedure followed was relatively gentle, and the pH was never far from neutrality (6-7.5) during extraction. If any polyisoprenoid sugars or analogous substances are present, they must therefore be at such low concentrations as to be undetectable amongst the other glycolipids. The detection limit is well under 0.01 μmoles/g, although smaller quantities than this could admittedly be active intermediates in polysaccharide biosynthesis[9, 297].

	DGD	MGD	SL	GC	SG	PI	PC	PG	DPG	Chlorophyll
Pinnae	2·54	4·8	0·23	0·25	0·16	0·22	1·14	0·15	0·07	1·33
Upper rachis	0·32	0·48	0·05	0·15	0·09	0·09	0·37	0·09	0·06	0·13
Upper petiole	0·13	0·17	0·07	0·09	0·08	0·03	0·14	0·05	0·03	0·07
Lower petiole	0·10	0·10	0·04	0·06	0·06	0·03	0·18	Trace	Trace	Trace
Frond-bearing rhizome	0·04	0·03	—	0·03	Trace	Trace	0·01	Trace	0·01	—
Storage rhizome	0·21	0·07	Trace	0·20	0·09	0·01	0·07	0·02	0·02	—

Abbreviations: DGD—digalactosyl diglyceride; MGD—monogalactosyl diglyceride: SL—sulpholipid: GC—glucocerebroside; PI—phosphatidyl inositol; PC—phosphatidyl choline; PG—phosphatidyl glycerol; DPG—diphosphatidyl glycerol.

Table 3.1 - Quantities of glycolipids and phospholipids (moles/g fresh weight) and of chlorophyll (mg/g fresh weight) in different parts of the bracken plant.

B. THE DIURNAL VARIATIONS IN THE FROND.

Introduction.

In the previous section it was stated that all the samples were collected at the same time in case their lipid composition varied during the day.[286] Since there is a moderately rapid synthesis of some lipid classes and of chlorophyll when dark-grown tissues are transferred to the light,[296,299-301] corresponding to the growth of the thylakoid membrane systems within the chloroplast,[302] such diurnal variations might be anticipated; and in fact Roughan and Batt, in a passing reference to unpublished work in New Zealand[285], mention that sulpholipid levels in clover are highest during the early afternoon. Accordingly it was decided to follow the levels of the major 'chloroplast' lipids during the day. It proved necessary to work at a different site from that used for the previous study, and the bracken fronds were by this time fully mature, in fact only two weeks from the onset of senescence.

Experimental

Plant Material. Samples were collected at Drumclog Moor, Dunbartonshire (site 1), at approximately 3 hr intervals on 14th August 1973. Throughout the sampling period and for two days beforehand, the weather was warm and dry with some haze but virtually no cloud. The top 12 cm of the lamina (6 - 7 pinnae) was removed from each of 12 healthy fronds on each occasion. The samples were immediately frozen on solid CO_2, brought back to the laboratory in a vacuum flask, and stored at $-15^{\circ}C$.

Lipid Analysis. Analytical methods were as described in the previous section.

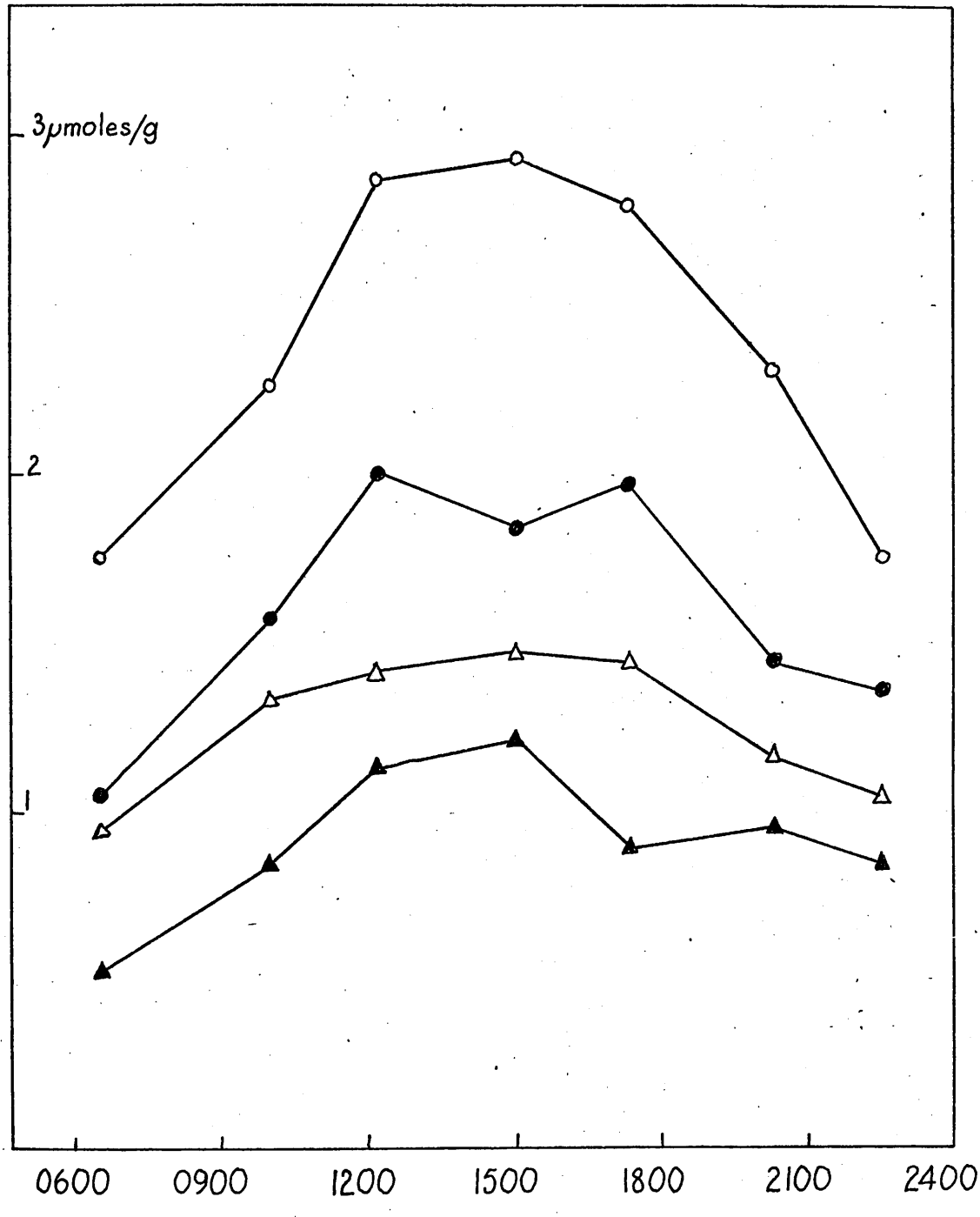


Figure 3.1 - Amounts of monogalactosyl diglyceride (o), digalactosyl diglyceride (•), sulpholipid (△), and phosphatidyl glycerol (▲), in moles/g fresh tissue, at different times during the day.

Results and Discussion.

The amounts of MGD, DGD, SL, and PG, expressed in μmoles/g fresh weight, are shown in Figure 3.1. The objection to expressing the results on a dry weight basis is that, as with seasonal variations in rhizome composition, the dry matter content will be influenced by any accumulation of photosynthesised material if translocation is not immediate. With the analytical method used it was not possible to determine the dry matter content of the fronds being analysed, but some parallel determinations showed that variations were only of the order of ± 5%. This is much smaller than the variations in lipid content actually found, and Figure 3.1 would look very similar if the results were expressed on a dry matter basis, though the peak in the early afternoon might be slightly flattened due to lowering of the relative water content during photosynthesis in the sunlight.[303]

The levels of all the lipids studied increased by 50-100% during the morning and fell during the evening, the fall beginning before sunset (Figure 3.1). During the night there may have been a slower decrease to the original levels, but this is not certain as the fine weather might have caused some net synthesis of lipid material.

The increase during the morning is more rapid than is usually observed when etiolated tissues of higher plants turn green in the light[296,299-301], and unlike the greening process it is not accompanied by any clear change in the ratio of monogalactosyl to digalactosyl diglyceride[296,301,304]. The decrease in lipid levels during the evening is considerably faster than that due to senescence,[305] for example, and its mechanism remains obscure. In particular, it is not clear whether the fatty acids and glycerol are degraded at night or merely transferred to another site.[300]

The size of these diurnal variations, and the equally large differences between the previous results (quoted in part A of this Chapter) and the present series of data taken as a whole, imply that great caution is necessary when results of different investigations

are compared. (The earlier results applied to a different part of the frond, and to woodland bracken at a different stage of maturity). Within a single investigation the histories of leaf samples for lipid analysis should clearly be as similar as possible.

The daily turnover of carbohydrate in the glycolipids, as they are synthesised and then disappear, is about 3.5 μmoles of monosaccharide/g fresh weight. It is of interest to compare this figure with the amount of carbohydrate synthesised in a day: for bracken this is not available, of course, but for pasture grasses the necessary data can be assembled from such studies as those by de Wit[306] and Anslow and Green[307]. A ryegrass sward is able to photosynthesise a maximum amount of carbohydrate equal to about 5% of its dry weight per day[306], but more normal figures are much less than this[307], around 1-2% of the dry weight or approximately 15-20 μmoles of monosaccharide/g/day. The comparison is extremely crude and approximate, but it would appear that the diurnal turnover of lipid-bound carbohydrate is by no means insignificant when compared with the net rate of photosynthetic production.

This would not in itself mean that the chloroplast lipids are necessarily involved in the biosynthesis, interconversion or transport of carbohydrates, and in fact there is no evidence that this is the case. Roughan[283] found that under continuous illumination there was very little turnover of chloroplast glycolipids, and concluded that the common suggestion that the galactolipids are involved in sugar transport across the plastid membrane is erroneous. While the diurnal turnover described here would allow such transport to take place to a certain extent, it does not seem to be necessary.

In a situation such as this there are in general two possibilities, which are shown in diagrammatic form, for this example, in Figure 3.2.

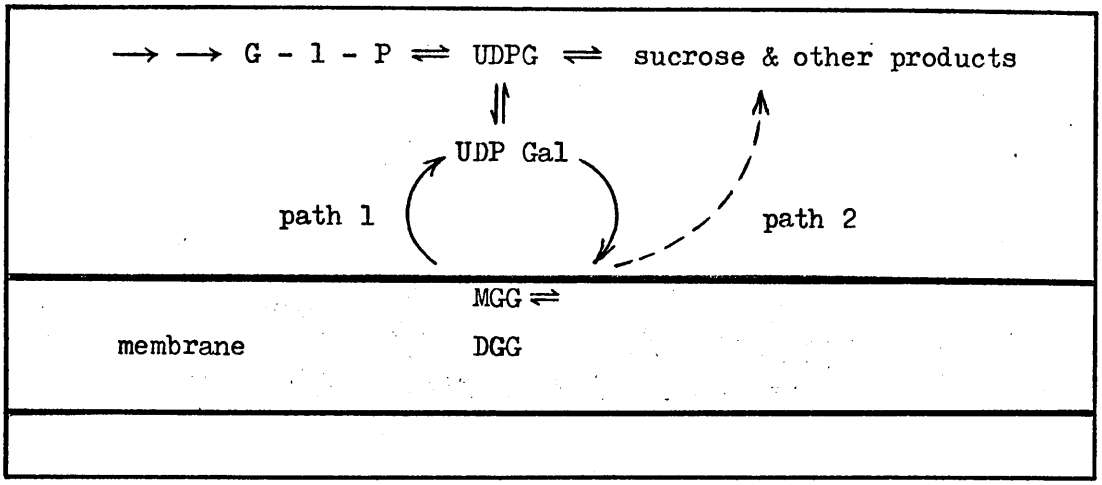


Figure 3.2

Biosynthesis and degradation of galactolipids: alternative pathways.

Here all the evidence favours the closed-loop pathway, path 1, but if the synthetic routes to galactose and to sucrose both draw on the same pool of UDPG, the kinetics of one will be affected by the other. The two types of pathway cannot always be distinguished in kinetic (especially radiochemical) experiments; for example failure to recognise a closed-loop pathway led to the proposal of a lipid intermediate in the biosynthesis of hemicellulosic glucomannans[309], which was later shown to be unfounded[310]. These considerations would have to be borne in mind during any future investigation of possible roles for the amyloplast glycolipids in starch biosynthesis or in sugar transport and compartmentation.

CHAPTER IV

FREE SUGARS AND LIPIDS IN STORED POTATOES

Introduction

The free sugar content of potatoes is a major factor in determining their suitability for frying and for different kinds of processing (dehydration, crisp manufacture and canning). The changes in reducing sugar and sucrose levels which occur during storage have been described by Burton[311,264] and will be only briefly summarised here.

When potatoes are lifted at maturity, left for a 'hardening' period of a few days at 10-15°C and then stored at various temperatures, the free sugar content adjusts itself within 1-2 weeks, by interconversion with starch, to an equilibrium value which is low and independent of temperature above 10°C, and progressively higher at lower temperatures. If the potatoes are lifted when immature the sucrose content at lifting is high, and the reducing sugar content relatively low. These adjust to more normal values, depending on the temperature, within two weeks of lifting: however at a given temperature the equilibrium reducing sugar content is higher than it would be with mature potatoes, especially during the first two months of storage.

As long as the tubers are dormant and respiring freely, at a low level, this equilibrium will be maintained. Eventually, if the potatoes are kept in store for 5-7 months, the free sugar content begins to rise and may reach very high levels. This 'senescent' sweetening, unlike the sweetening due to low temperatures, is irreversible and is much more serious at higher temperatures. It is apparently connected with sprout growth. Some intervarietal differences are known.

Potatoes grown in the more northerly parts of the British Isles tend to have a higher free sugar content at harvest than those grown further south[312]. This renders the tubers less suitable for crisp manufacture or dehydration, since at high temperatures there occurs a series of reactions which involve α-amino compounds and carbohydrates, particularly reducing sugars, and give an unacceptable dark brown colour to the product.[313] A reducing sugar content less than 0.1% is now regarded as necessary for crisp production in England,[312] although the only Scottish factory finds it necessary to accept levels up to 0.25% and adjust the frying conditions accordingly.

Even this level is difficult to achieve consistently in Scotland, not only due to the effect of latitude but also because of low-temperature sweetening during storage, particularly on the farm, during the colder Scottish winter. Some degree of artificial control over the storage temperature can be achieved, but higher storage temperatures mean poorer long-term keeping quality and encourage sprout growth with its attendant difficulties.[314] The potatoes can be de-sweetened at the processing plant by holding them at up to 20°C for a short period, but this is only satisfactory if the reducing sugar levels are not excessively high beforehand and is avoided wherever possible.

The biochemical factors linking sugar content to temperature are not well understood. The rise in the proportion of reducing sugars to sucrose may be explained by increased invertase activity at low temperatures.[315] Other factors as well as changing enzyme activity are likely to be responsible for the increased sugar levels, however. It has been suggested, for example, that the plastid membrane normally isolates the starch grain from degradative enzymes (presumably soluble phosphorylases) in the cytoplasm; but that during storage at low temperatures this membrane disintegrates.[56]

This Chapter describes the effect of low-temperature storage on sugar levels in 11 commonly-grown potato varieties. Considerable variation was observed, and four varieties with high, low, and medium tendencies to accumulate sugars were selected for lipid analysis, to explore the possibility of membrane degradation and its effects.

Experimental

Materials. The potatoes for this investigation were grown under the following conditions by Mr. J. Dalziel of this Section. They were in fact control samples from an experiment on the long-term effects of chemical sprout suppressants on seed potatoes.

Seed potatoes of 11 varieties were obtained from the Department of Agriculture and Fisheries for Scotland, Scientific Services, East Craigs, Midlothian. They were sprouted on chitting trays at 10°C under constant illumination, and planted on 8th - 9th May 1973 at Garscube Estate, Glasgow (Grid Ref. NS 550705). For each variety duplicate rows, each of 21 plants, were laid out, randomised within the plot. At lifting on 14th - 15th October, the tubers of all varieties except Golden Wonder were at or very near maximum weight, although in some cases the haulms were not completely dried out. The Golden Wonders were immature, and the free sugar data for this variety is therefore of little significance.

After 4 weeks in store at 16-18°C, 12-15 healthy tubers (> 42 mm) of each variety were selected and divided into two lots, which were held at 18°C and 4°C respectively for a further 26 days before analysis.

Analysis. Transverse cores 8 mm in diameter were removed for extraction. A total weight of 20-26g (one core from each of 6-8 potatoes) was extracted for carbohydrate analysis by homogenising in 150 ml of methanol and re-extracting the residue with 100 ml of the same solvent. For lipid analysis half cores (10-13g) were homogenised with 100 ml of chloroform - methanol (2:1 v/v), filtered, and washed with 100 ml of the same solvent. Lipid and carbohydrate analysis were as described in G.M.11-12 and G.M. 5.

Results and Discussion

a) Carbohydrate Content.

There was differences between varieties both in the amount of free sugar present at 18°C and in the increase which occurred at 4°C (Figure 4.1). Both reducing sugar and sucrose levels generally rose at the lower temperature, but the proportion of sucrose to reducing sugars decreased. The varieties Pentland Ivory, Record, Maris Peer, Desiree, and King Edward had reducing sugar contents below 0.1% when stored at 18°C, and less total sugar than the others. The ratio of the total sugar content at 4°C to that at 18°C averaged 2.23. In the variety Desiree, however, the ratio was 4.43, while that of the variety Pentland Ivory was only 1.02.

The small scale of this experiment permits only tentative conclusions. However, the results indicate that while a number of varieties, when grown in Scotland, can have reducing sugar levels as low as or lower than that of Record, at present widely used for crisp manufacture, they accumulate more sugar than is desirable for processing if stored under the cold winter conditions normal in the Scottish maincrop potato-growing areas. The variety Pentland Ivory is an exception, having a low sugar content that seems relatively little affected by storage at 4°C. In most other respects this variety is very suitable for crisp manufacture; it has a high dry matter content and thus requires less oil, the most expensive raw material in the process. Its extremely pale flesh could be a slight disadvantage, as yellow-fleshed varieties such as Record and Bintje give a brighter product if low in reducing sugars.[313]

b) Lipid Composition.

Only the major phospholipids and glycolipids, which are usually regarded as membrane components, were examined. The results are shown in Table 4.1. The differences between the 18°C and the 4°C samples are not large, in general smaller than the differences between varieties, but two trends are apparent.

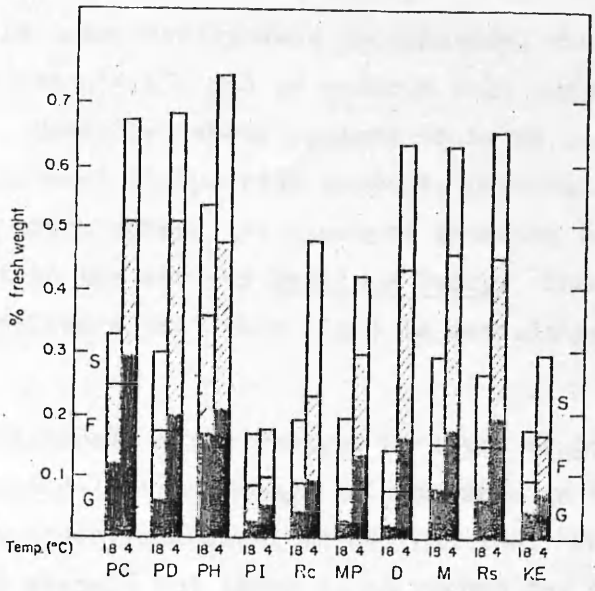


Figure 4.1 Percentages of glucose (G), fructose (F) and sucrose (S) in fresh tissue of potatoes stored at 18 and 4 °C. Varieties: PI Pentland Ivory; M Majestic; PC Pentland Crown; Rc Record; Rs Redskin; PD Pentland Dell; MP Maris Peer; KE King Edward; PH Pentland Hawk; D Desiree.

Variety	Desiree		Record		Majestic		Pentland Ivory	
Temperature (°C)	18	4	18	4	18	4	18	4
Phosphatidyl choline	0.260	0.256	0.233	0.205	0.250	0.214	0.232	0.202
Phosphatidyl ethanolamine	0.151	0.145	0.103	0.122	0.103	0.116	0.136	0.108
Phosphatidyl glycerol	0.024	0.030	0.017	0.025	0.034	0.020	0.021	0.026
Phosphatidyl inositol	0.075	0.095	0.065	0.070	0.071	0.062	0.088	0.080
Digalactosyl diglyceride	0.048	0.072	0.056	0.073	0.046	0.063	0.048	0.053
Monogalactosyl diglyceride	0.045	0.063	0.051	0.068	0.051	0.053	0.053	0.052
Sulpholipid	0.031	0.037	0.020	0.030	0.025	0.030	0.023	0.019

TABLE 4.1 Phospholipid and glycolipid levels (μmol/g fresh weight) in potato tubers of different varieties, after 26 days storage at 18 °C and 4 °C

Firstly, there is consistently less phosphatidyl choline, a component of mitochondria[280] and of various cell membranes,[282] after storage at 4°C. Secondly, there appears to be an increase in the amount of digalactosyl diglyceride present, particularly in the variety Desiree which showed the greatest increase in free sugar content, but not in the variety Pentland Ivory. There is circumstantial evidence that this lipid is associated with amyloplasts.

These relatively minor changes in lipid composition imply that at 4°C the cold-induced damage to membranes in the tuber does not include widespread chemical degradation. Physical disruption may take place,[56] but there is no reason for this to be irreversible if the membrane components remain intact. Changes that follow senescence are of course another matter.[264]

It is noteworthy that high levels of galactosyl diglycerides, and of polyunsaturated fatty acids which are particularly common in these lipids,[294] are observed when plants are frost-hardened by exposure to moderately low temperatures,[316-7] and have been associated with the resistance of membranes to both chilling[318] and freezing damage.[320] Phase transitions in artificial lipid membrane systems rich in polyunsaturated fatty acids tend to occur at lower temperatures.[319] The presence of high concentrations of soluble sugars is likewise thought to render many plants more resistant to injury in freezing conditions.[320-1]

CHAPTER V

THE MUCILAGINOUS POLYSACCHARIDE FROM BRACKEN

Introduction

The viscous fluid which exudes freely from the storage rhizomes or young fronds of bracken when these are cut does not seem to have been studied chemically outside this laboratory. Kwasniewski[254] makes a passing reference to 'viel schleim' but it was not realised that the mucilaginous properties were due to a polysaccharide until Rees and his co-workers began work on the subject here. Morton[260] established that the constituent sugars included galactose, xylose, arabinose, mannose, L-fucose, and an acidic sugar, the identity of the fucose being confirmed by optical rotation measurements and the preparation of a crystalline osazone.

The polysaccharide is thus a complex example of the mucilaginous category, which are usually assumed to be related to the matrix polysaccharides of the cell wall[65], and synthesised like them by the Golgi apparatus[145]. Because of its complexity a full structure determination by established methods - methylation analysis and periodate and lead tetra-acetate oxidation - would require much labour even for a laboratory working solely on polysaccharide structures, and organised accordingly. It was decided, therefore, to concentrate on the following more limited objectives:

a) Establishing the homogeneity of the polysaccharide.

b) Confirming the identity of its constituent sugars and determining their proportions.

c) Making an estimate of the molecular weight.

d) Obtaining as much structural information as possible by graded acid hydrolysis and examination of the products.

Isolation

The standard extraction series used for the free sugar and starch determinations described in Chapter II was originally developed for isolating the mucilaginous polysaccharide. In some subsequent work it was scaled up to deal with 1-2 kg of rhizome material in a single batch. Almost any aqueous extraction system can be used, however, and in fact the sap from cut storage rhizomes yields a useful amount of the polysaccharide when poured into alcohol.

For preparative work the polysaccharide was normally purified as follows. Proteins and phenolic compounds were removed from the crude aqueous extract by precipitation with trichloro-acetic acid at 4°C and centrifuging for 30 min at 12,000 r.p.m., still at this temperature. The polysaccharide was then precipitated with three volumes of methanol, redissolved in water and reprecipitated, with the addition of LiCl to aid flocculation. The fibrous, sticky precipitate was air-dried at 30°C or freeze-dried from a little water, giving either the free acid or the lithium salt (depending on the pH before the second methanol precipitation) as a friable or lumpy white powder.

When dried or merely dehydrated with methanol the polysaccharide could be redissolved in water only with some difficulty, and the solutions were noticeably turbid in comparison with the centrifuged crude extracts or the untreated rhizome sap.

Homogeneity

Morton[260] attempted to fractionate the polysaccharide by partial precipitation with cetyl trimethyl ammonium bromide (CETAB), but the precipitated material was indistinguishable in composition from what was left in solution. Precipitation of a 0.2% solution with alcohol (1.7 volumes) or with 0.2M Al^{3+} ions during a progressive raising of the pH, did not succeed in fractionating the polysaccharide, and unlike some mannose-containing polymers[122] it was not precipitated on addition of $Ba(OH)_2$, to a concentration of 0.05 or 0.15M. Gel permeation

chromatography (see below) did not reveal any fractions differing in molecular size.

During high-voltage electrophoresis on glass-fibre paper (G.M.8) in 0.05M borate buffer (pH9.2) for 1½ hr at a potential gradient of 11.5 V/cm, the polysaccharide moved as a single band towards the cathode by endosmosis, with slight tailing towards the origin (Figure 5.1).

The polysaccharide as isolated was therefore taken as being substantially homogeneous.

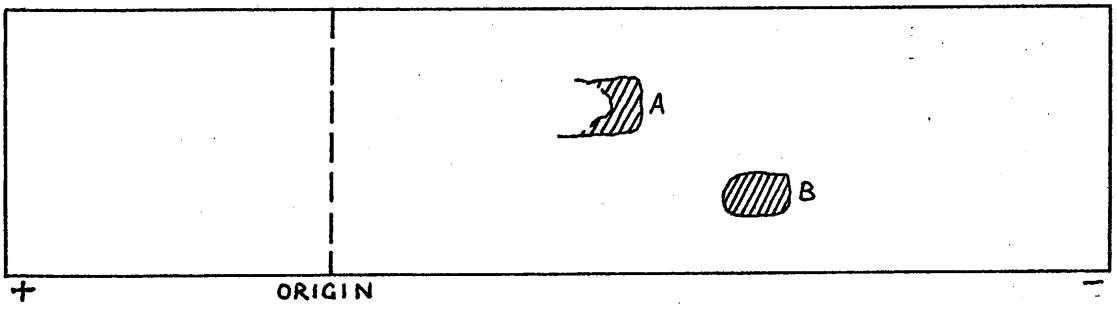


Figure 5.1 - Electrophoretogram in borate buffer (pH 9.2).
A: bracken polysaccharide
B: dextran

Molecular Weight Determination

Of the various methods of molecular weight determination which are suitable for polysaccharides, gel permeation chromatography was chosen as giving the fullest information.[322-3] Unlike other techniques which provide either a weight-average or a number-average figure for the molecular weight, it gives the distribution of molecular size; it can thus distinguish between polyhomogeneous and polymolecular macromolecules. Disadvantages are that the resolution is not all that could be desired, and calibration in terms of molecular weight depends on the shape of the macromolecules in question: thus a linear dextran of M.W.10,000, with a random-coil configuration, behaves similarly to a globular protein of M.W.30,000.[324]

The main kinds of column-packing material available for permeation chromatography are highly hydrated polyacrylamide, modified polystyrene, or cross-linked dextran or galactan gels, and porous glass beads.[322] The latter are prone to erratic behaviour due to adsorption onto the glass : this can be eliminated by coating with polyethylene glycol[325], but difficulties of calibration are introduced. Polysaccharide gels were used exclusively here.

As well as reducing the work involved, it improves the resolution available if a continuous method of monitoring the column effluent is used. Continuous, automated adaptations of e.g. the orcinol-sulphuric acid method are available[326], but a simpler method is to bind one of the 'Procion' range of dyes (I.C.I. Dyestuffs Ltd)to the polysaccharide[327] and pass the effluent through a continuously recording colorimeter[328]. During initial investigations with the bracken polysaccharide it was found that 'Procion Blue M2XB' had a stronger adsorption at 260 nm than in the visible (see Figure 5.2), and a commercial UV-monitoring system designed for nucleotide analysis and equipped with a fraction collector and a purpose-built amplifier was adapted for this application. The stability, sensitivity, and low dead volume of this apparatus made it ideal for polysaccharide work.

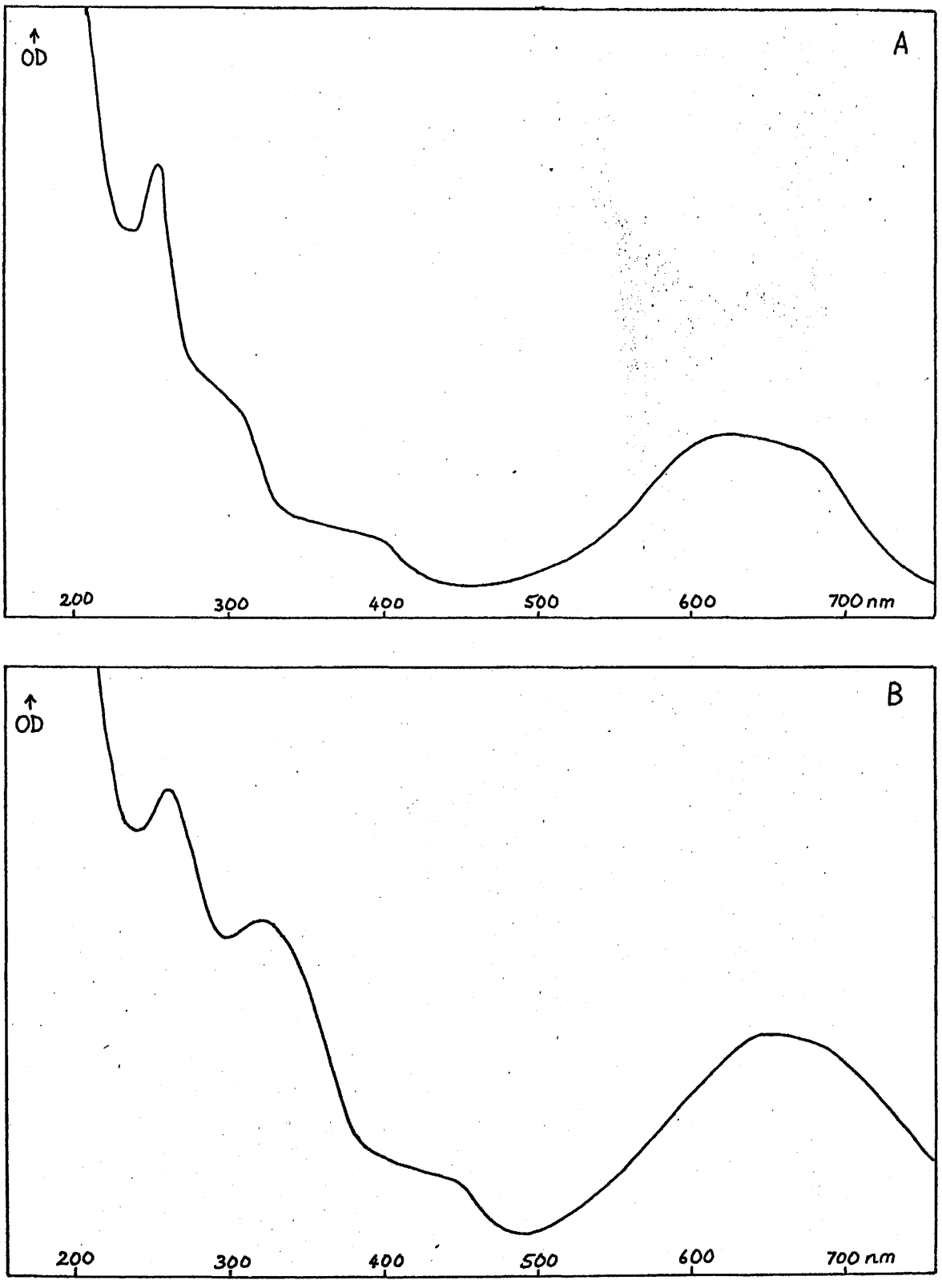


Figure 5.2 - Absorption spectra of Procion Blue MX2B (A) and Dextran 20 dyed with this reagent (B).

These dyes were originally developed for cellulose, and probably bind through the C-6 oxygen of neutral aldohexoses[327]. The mildly alkaline conditions required have not been found to cause any degradation of other acidic polysaccharides.[328] For steric reasons the dye may however help to prevent aggregation: solutions of the dyed bracken polysaccharide were clear, whereas before dyeing there was always slight cloudiness even after centrifuging for 1 hr at 15,000 r.p.m.

In view of the possibility that the polysaccharide might form part of a glycoprotein of the type that have been isolated from plant cell walls[66], with polysaccharide chains attached to one another via the protein or peptide moeity, an aliquot was subjected to hydrazinolysis[169] to break up the polypeptide fraction, if any, and chromatographed thereafter.

Experimental

The methods used for binding the dye and preparing the columns are described in the General Methods Section (G.M. 9). Anderson & al state that optimum column dimensions should be established for each polysaccharide,[328] but it was found that the preparation of a column with good resolving qualities involved a certain element of luck, and when a good column of a particular gel was achieved it was used exclusively thereafter. The columns used generally were of the following dimensions; Sephadex G-50, 50 cm x 1.5 cm; G-75, 45 cm x 1.5 cm; G-200, 30 cm x 2.5 cm; Sepharose 4B, 45 cm x 1.5 cm.

Dextran fractions of known molecular weight were dyed in the same way and used for calibration (Pharmacia T 20-500). Exclusion and inclusion limits were established with Pharmacia blue dextran 2000 and either adenosine monophosphate or acetone, respectively. They were reproducible to $\pm$ 2 - 5 ml from one run to another.

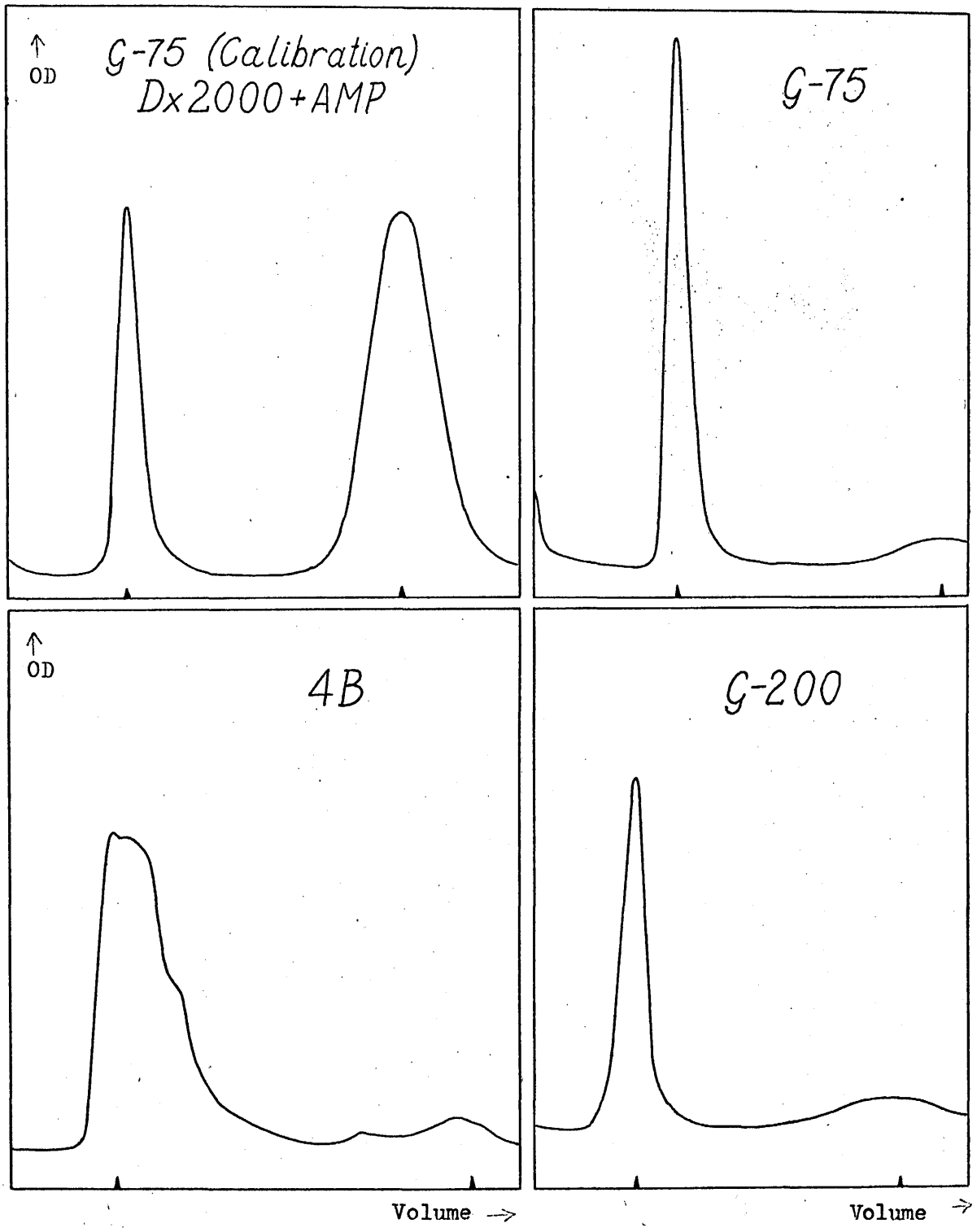


Figure 5.3 - Gel chromatograms of the dyed mucilaginous polysaccharide on Saphadex G-75 and G-200 and on Sepharose 4B. Also a calbration chromatogram on Sephadex G-75.

Elution was generally with water: 1M NaCl gave precisely the same results. The use of 6M urea as an eluting solvent was tried briefly but had to be abandoned due to clogging of the apparatus with urea crystals whenever it was not running.

For hydrazinolysis, 10 mg of polysaccharide was heated for 18 hr at 100° in a sealed tube with 1 ml of glass-distilled anhydrous hydrazine[158]. 4 ml of water was added and the polysaccharide dyed in the normal way. Even on addition of the water the solution remained brilliantly clear, although coloured light brown: it would seem that this method of dissolution effectively avoids aggregation. Hydrazine is not considered to degrade polysaccharides under the conditions used[158].

Results

The intact, dyed bracken polysaccharide was found to be remarkably large. It was sharply excluded from all grades of Sephadex gel up to G-200, which for soluble dextrans has an approximate exclusion limit of M.W.200,000[324]. A considerable proportion was also excluded from the galactan gel Sepharose 4B, and it would appear that much of the polysaccharide has a molecular weight of several millions. As analysed it was certainly polymolecular although partial depolymerisation during the extraction process cannot be ruled out entirely. (see Figures 5.2-5.3). Hydrazinolysis of any peptide linkages present[158] had no apparent effect on the chromatographic behaviour of the polysaccharide, so it does not seem to be a glycoprotein of the kind described by Lamport[66].

Monosaccharide Composition

To determine the monosaccharide composition of a neutral polysaccharide is a comparatively simple matter, and hydrolysis with, say, 1M mineral acid followed by chromatographic analysis of the hydrolysate will usually give quite satisfactory results.

Glycosidic bonds vary greatly, however, in their susceptibility to acid hydrolysis. Polymers of uronic acids and of N-acetyl glucosamine are highly resistant, while residues attached in the furanose form (L-arabinose generally) are removed very easily - even, with acidic polysaccharides, by autohydrolysis[65].

Neutral aldoses are fairly resistant to acid degradation, but the severe hydrolysis conditions required for acidic polysaccharides cause some decomposition of their component neutral sugars, to furfuraldehyde and its derivatives in the first instance[329]. The uronic acids themselves are more severely affected, undergoing decarboxylation followed by other degradative reactions. At one time decarboxylation with strong acid and manometric estimation of the CO_2 evolved was the preferred method for estimating uronic acids in polymers[330], but the results it gives are consistently too high and it was eventually realised that CO_2 can arise from other sources under these conditions[331].

Direct titration of the polysaccharide in the free acid form is more satisfactory provided its preparation has been carefully carried out[332]: oxidising conditions at high pH must be avoided during extraction as free carboxylic acid groups can be artificially produced. This technique was used by Morton[260], but has now been largely supplanted by various modifications of the carbazole method, the most effective being that of Bitter and Muir[333]. (G.M.2.)

A common procedure for quantitative analysis of a complex acidic polysaccharide is to determine the uronic acid <u>in situ</u> by the carbazole method, and to estimate the neutral sugars separately by a relatively mild acid hydrolysis, e.g. 0.5M HCl/100°/1 hr, followed by chromatography. Such gentle conditions will not, however, release any neutral sugars to which uronic acids are attached, and the estimation of these can be a serious problem: aldobiuronic acids such as galacturonosyl rhamnose from pectic substances and glucuronosyl mannose from the bracken polysaccharide (<u>vide infra</u>) are degraded almost as quickly as they are hydrolysed.

The approach adopted to circumvent this difficulty made use of a graded series of acid hydrolyses. The build-up and subsequent decrease in the levels of the free monosaccharide as it was released and degraded were analysed mathematically, and a method of calculating the original amount present in the polymer was worked out.

Experimental

1 ml aliquots of a 0.2% solution of the polysaccharide were hydrolysed with 0.005M or 0.5M H_2SO_4 in sealed tubes at 100°C, and the rate of release of the neutral sugars followed by quantitative paper chromatography (G.M. 6). The 0.5M series were neutralised to pH 4 with $Ba(OH)_2$ and chromatographed in both EtAcAcF and EtAcPy, but neutralisation with AG1-X4 resin (carbonate form) followed by chromatography in EtAcAcPy were adopted for 0.005M series which were done at a later date. For comparison of polysaccharide samples from different sources standard hydrolysis conditions of 0.5M H_2SO_4/100°/8 hr were used.

The acidic monosaccharide was identified as glucuronic acid by chromatography in EtAcPy and EtAcAcPy for 72 hr, although the possibility that a small amount of galacturonic acid was also present could not be wholly excluded. The carbazole method of Bitter and Muir was used to determine the uronic acid in the intact polysaccharide, with appropriate corrections for the neutral sugars present.[333]

Results

The rates of release and subsequent degradation of the monosaccharides under the two sets of hydrolysis conditions used are shown in Figures 5.4 and 5.5. In general the hydrolyses were followed only for 24 hr, but the release of mannose by 0.5M H_2SO_4 was followed for an additional 48 hr in a later experiment under the same conditions.

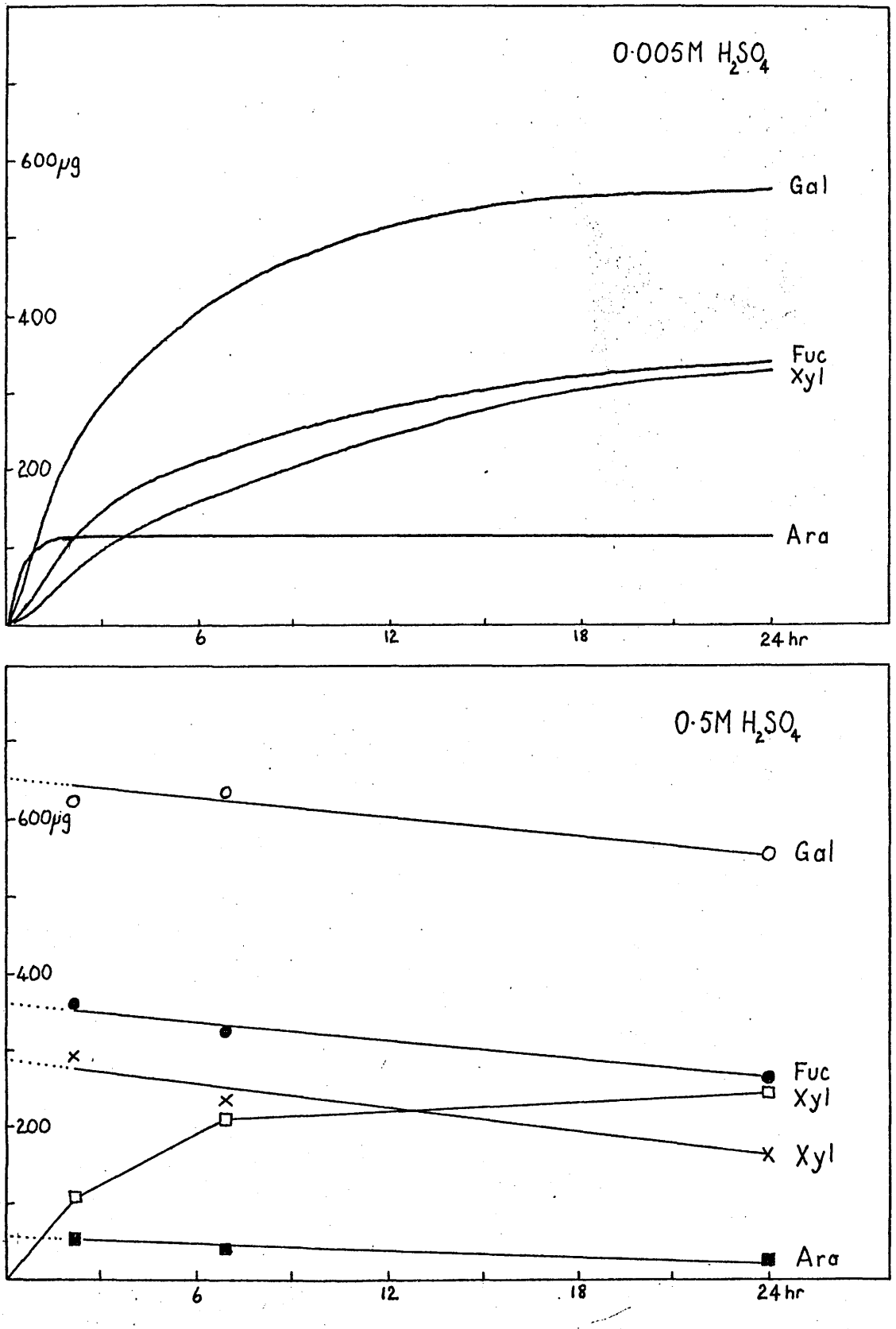


Figure 5.4 - Release of monosaccharides during acid hydrolysis.

The amount of each monosaccharide in the original polysaccharide was found by extrapolating the graph of its concentration in the 0.5M acid hydrolysates to zero time, thus excluding the effect of degradation. This over-estimates the actual value slightly, but is a good approximation. In fact the ratios of the different sugars were used in calculating the percentage composition of the polysaccharide (making due allowance for the uronic acid which was determined separately), rather than the absolute amounts released per aliquot of polysaccharide solution: this gives an improvement in precision as the largest errors are involved in the preparation of a polysaccharide solution of known concentration, and in applying a precise volume of each hydrolysate to the chromatogram.

The percentage of each sugar lost by degradation during the 8 hr hydrolysis period used for comparison of samples from various sources was found by interpolation and applied as a correction to the figures obtained. The comparison is shown in Table 5.1.

	Galactose	Xylose	Fucose	Arabinose	Mannose	Uronic acid
Rhizomes, 31-4-72.	36	16	20	3	14	12
Rhizomes, 3-7-72.	31	19	21	5	12	12
Rhizomes, 27-8-72.	31	21	20	5	14	10
Fronds	37	17	19	9	7	12
Rhizome Average	33	19	20	4	13	11

TABLE 5.1

Percentage composition of bracken polysaccharide from different sources.

Since degradation proceeds concurrently with the release of mannose from the aldobiuronic acid the maximum concentration reached in the hydrolysates is substantially less than the amount of mannose present in the polysaccharide originally. If it is assumed, inter alia, that both hydrolysis of the aldobiuronic acid and degradation follow first-order or pseudo-first-order kinetics, and that the hydrolysis of the aldobiuronic acid is very slow compared to its release from the polysaccharide under constant, acid conditions, then it can be shown that the rise and subsequent fall in the free mannose concentration are described by a function of the form:

$$M = \frac{k_H}{k_D - k_H} P \left(e^{-k_H t} - e^{-k_D t} \right) \quad \ldots (1)$$

where M is the amount of free mannose present after time t,

k_H and k_D are the rate constants for hydrolysis and degradation respectively, and P is the amount of mannose in the original polysaccharide.

When M is plotted on a logarithmic scale this function rises to a maximum A and thereafter falls, soon approximating to a straight line with gradient $-k_D$ and intercept B when extrapolated to the M axis (see Figure 5.5). The linearity of the later part of the curve is a satisfactory test of first-order (or pseudo-first-order) kinetics for degradation. It is simple to show that:

$$A = \frac{k_H}{k_H + k_D} P \quad \text{and} \quad B = \frac{k_H}{k_H - k_D} P \quad \ldots (2) \ \& \ (3)$$

It follows that:

$$P = \frac{2A}{1 + \frac{A}{B}} \quad \ldots (4)$$

so that the amount of mannose in the original polysaccharide can be calculated very easily.

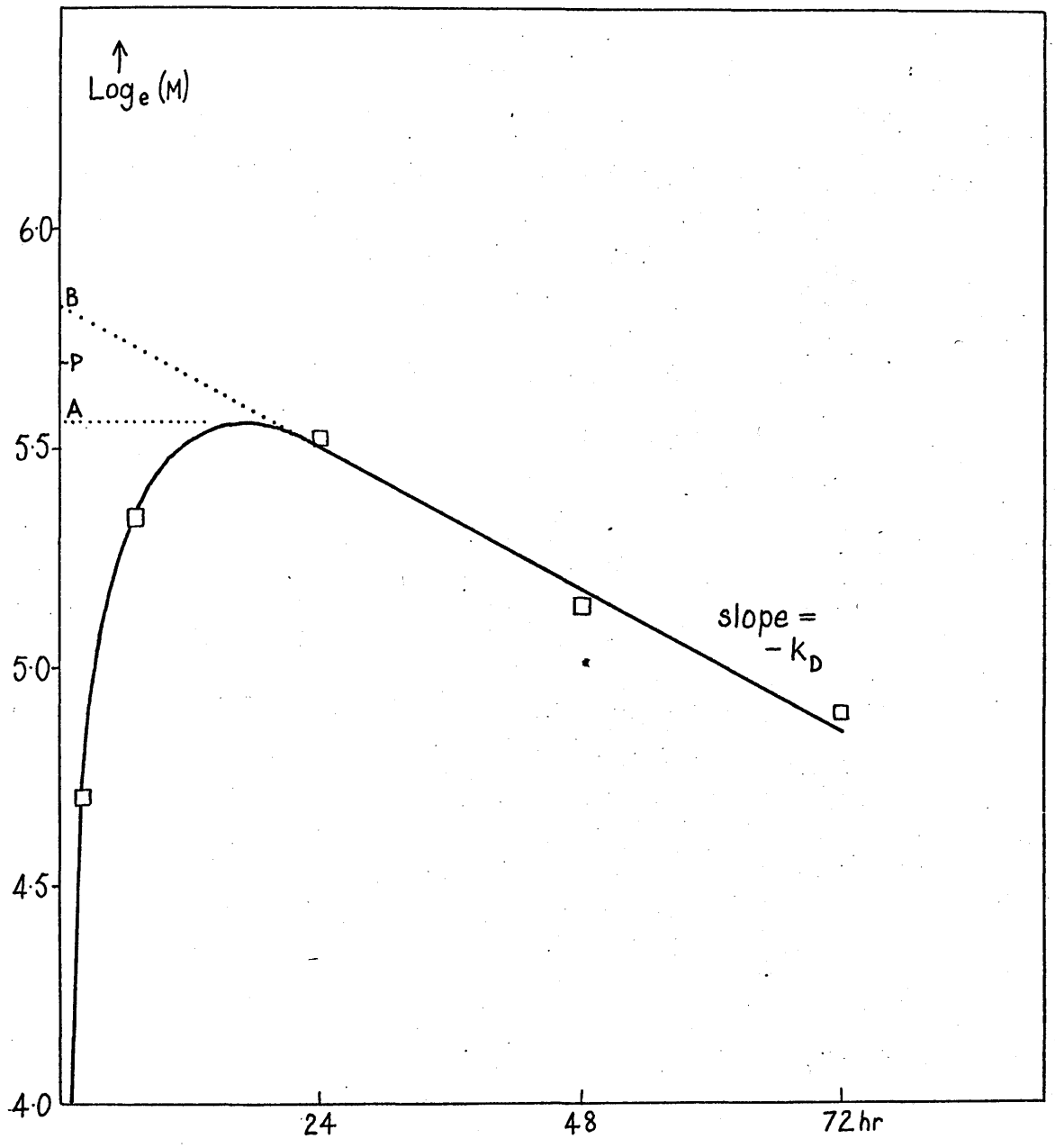


Figure 5.5 - Release and decomposition of mannose. Natural logarithm of free mannose present (Log_e (M)) plotted against time.

The above analysis only holds good if $k_H > k_D$, as appears to be the case here. In the special situation where the rate constants for hydrolysis are equal Equation (1) becomes meaningless. If $k_D > k_H$ a curve of the same shape results, approximating to a straight line of gradient $-k_H$ at large values of t and with:

$$B = \frac{k_H}{k_D + k_H} P \text{ as before but } A = \frac{k_H}{k_D - k_H} P \quad \ldots (5)$$

A priori it is not in general possible to distinguish between these two situations when confronted with an experimental curve of this shape, but if there is any doubt an approximate value of k_H may be found by plotting the curve on a linear scale and estimating its gradient at zero time. This approximation is satisfactory unless k_H is very small compared with k_D.

Other workers have complained about the lack of a suitable method for estimating sugars bonded to uronic acids within a polysaccharide[334], and it is hoped that this method will provide a satisfactory solution to the problem. It might be expected that hydrolysis of highly resistant polymers such as chitin and alginic acid could be handled in the same way; but in fact the random scission of a long polysaccharide does not release monosaccharide units at a steady rate, even if all the glycosidic bonds are of equal stability[335-6]; the apparent first-order rate constant for production of free monosaccharide generally increases as the reaction proceeds[337]. A rather simplistic partial explanation is that hydrolysis of the disaccharide in the late stages of the reaction produces two monosaccharide units although only one bond is broken.

A full mathematical treatment of the hydrolysis of polysaccharides in this way is therefore more complex than the disaccharide case; a solution is not available at the time of writing but work is still going on on the subject.

Fragmentation Analysis

A second consequence of the different rates at which different glycosidic bonds are hydrolysed by acid is that at certain stages of hydrolysis, the more stable disaccharides and higher oligomers may build up to levels at which they can be isolated and studied. In the case of the bracken polysaccharide the rates of production and subsequent hydrolysis of individual oligosaccharides were not determined quantitatively, but the optimum hydrolysis conditions for isolating particular oligosaccharides were determined empirically.

The milder hydrolysis conditions used for this purpose (grade 1; 0.01M HCl/1 hr/100°C) released small amounts of a wide range of oligosaccharides, examination of which was limited to determining their monosaccharide composition. More severe conditions (grade 2; 0.01M HCl/ 6 hr/100°C) allowed two of the most stable of these oligosaccharides to be isolated in larger amounts sufficient for partial characterisation, while the acidic components were obtained by a small-scale ion-exchange separation.

Hydrolysis

For the grade 1 and grade 2 hydrolyses 250 ml of 0.2% polysaccharide solution was acidified to 0.01M with HCl and refluxed for 1 hr and 5 hr respectively. After a number of preliminary trials a one-step method of isolating and desalting the neutral oligosaccharides was developed. The hydrolysate was neutralised to ca.pH5 with KOH and adsorbed onto a 8 cm x 1 cm thick pad of BDH activated charcoal/celite (1:1 w/w) sandwiched between layers of pure celite on a sintered glass filter (this arrangement cuts the amount of charcoal fines escaping to a minimum). Thorough washing with water removed the residual polymeric material, the salt and the monosaccharides with the exception of fucose. The oligosaccharides (and fucose) were then isolated by elution with 40% ethanol and the eluate brought to neutrality and evaporated carefully to 1-2 ml volume at $< 30°C$.

The acidic fraction of the grade 2 hydrolysate was separated by retention on a small column of anion-exchange resin (AG1X4 200-400 mesh, acetate form; 4 x 10 mm column made up in a Pasteur pipette), and elution with 10% acetic acid.

Separation of Oligosaccharides

Initial attempts to fractionate the oligosaccharide mixture by gradient elution from charcoal/celite columns with aqueous ethanol were largely unsuccessful. Flow rates were extremely low (2-3 days being required for complete elution), considerable hydrolysis of oligosaccharides occurred on the column, and the resolution achieved was unsatisfactory. Much better results were obtained by preparative paper chromatography (G.M. 6) in the solvent EtAcPy which offers suitable $R_{galactose}$ values and discourages hydrolysis.

1-2 mg of oligosaccharide mixture from a grade 1 hydrolysis was resolved into 6 identifiable fractions after running for 48 hr in this solvent on Whatman No.1 paper. Their chromatographic properties are shown in Table 5.2. The oligomeric products of grade 2 hydrolysis, on the other hand, included only components N1 and N2, and some slower-moving fractions which included the acidic sugars when these had not previously been removed by ion exchange.

	$R_{galactose}$(EtAcPy)	$R_{galactose}$(EtAcAcF)
Component N1	90	77
N2	75	50
N3	40	42
N4	33	30
N5	not	20
N6	determined	at origin

TABLE 5.2

Chromatographic properties of neutral oligosaccharide components on Whatman No. 1 paper.

Identification

The monosaccharide composition of the 6 oligosaccharide fractions from the grade 1 hydrolysis was established by the following micro-scale technique. One end of a melting-point tube was sealed and approximately 100 λ of 1-2 mg/ml oligosaccharide solution and 10 λ of 0.25M HCl were added using a Pasteur pipette drawn out to a long, fine point. The other end was sealed and the oligosaccharide hydrolysed for 16 hr at 100°. After breaking open the tube and adding a drop of pyridine for neutralisation the products were applied directly to two chromatograms and separated in EtAcAcF and EtAcPy (the universal solvent EtAcAcPy had not then been developed). The dilute acid used made desalting unnecessary. The results are shown in Table 5.3.

Component	N1	Galactose, fucose.
Component	N2	Galactose only.
Component	N3	Galactose, xylose.
Component	N4	Galactose, xylose, fucose.
Slower-moving material: complex.		

Table 5.3
Monosaccharide composition
of oligosaccharide fractions.

The larger quantities of components N1 and N2 available from the grade 2 hydrolysis made it possible to examine these two oligosaccharides in more detail. N1 contains galactose and fucose in equal proportions (found: gal/fuc = 0.96:1). Its high mobility in EtAcPy (and other solvents) implies that it is a disaccharide. Reduction with sodium borohydride (1 mg/ml, 3 hr at room temperature) followed by acid hydrolysis and paper chromatography yielded fucose and galactitol rather than fucitol and galactose.

Refluxing the crude oligosaccharide mixture with 0.025M Na_2CO_3 for 20 min resulted in widespread degradation but left component N1 intact. Since after grade 2 acid hydrolysis it was isolated in higher yield than any other oligosaccharide it would appear to contain a particularly acid-stable glycosidic linkage. This is surprising in view of the fact that fucose in structural plant polysaccharides is generally bound in the α-configuration , but would be consistent with a (1 → 2') link to galactose. Authentic α-L-fucopyranosyl (1 → 2') D-galactose has been isolated by alkaline degradation of the corresponding fucosyl lactose (present in human milk) under the same conditions as were described above,[338] the anomeric carbon being sterically protected. None could be obtained for comparison, however, and the amount of component N1 available was insufficient for the preparation of a crystalline derivative. The identification of this component as α-L-fucosyl (1 → 2') galactose, a disaccharide not previously known in plants, therefore remains conjectural.

From its mobility in EtAcPy and EtAcAc, component N2 is apparently a galactose disaccharide, but the nature of the linkage was not established. Its stability to acid probably rules out an α-link. Authentic β-galactosyl (1 → 4') galactose was prepared under grade 1 conditions from citrus pectin, and the β (1 → 3') and β (1 → 6') disaccharides as described by Aspinall et al.[339] from acid hydrolysates of arabic acid or of an acidic galactan (Cal-Biochem Inc.), prepared by removal of arabinose side-chains from the latter polysaccharide .

Due to the small amounts of all these disaccharides and some rather poor chromatographic resolution it was not possible to identify component N2 unambiguously when this was briefly attempted. Approximate $R_{galactose}$ values are shown in Table 5.4.

Linkage	source	$R_{galactose}$ (EtAcPy)
$\beta(1\rightarrow3')$	arabic acid	55
$\beta(1\rightarrow4')$	citrus pectin	50
$\beta(1\rightarrow6')$	arabic acid	30
?	bracken	50

Table 5.4.
Approximate mobilities of
galactose disaccharides in EtAcPy.

In a small-scale spot test with the aniline/diphenylamine phosphate reagent originally developed as a specific spray in chromatography,[340-1] component N2 gave a yellow-brown colour whereas the galactosyl-galactose from citrus pectin gave the bright blue typical of $(1\rightarrow4')$-linked disaccharides.[341] As component N2 did not show any sign of the stability to alkali expected of a $(1\rightarrow2')$-linked disaccharide, the evidence favours the $(1\rightarrow3')$ linkage by process of elimination. Definite proof is lacking, however.

Only one acidic oligosaccharide was isolated, and it appeared to be homogeneous in paper chromatography in EtAcAcF, EtAcAcPy and EtAcPy although its mobility in the second two solvents was very low. It was chromatographically identical with authentic glucuronosyl (1-2') mannose isolated under the same conditions from Anogeissus leiocarpus[342] gum, kindly provided by Dr. D.M.W. Anderson of Edinburgh University and purified by reprecipitation with ethanol.

Examination of the polymeric fraction.

For this study another graded hydrolysis series was used. 250 mg of the polysaccharide was suspended in 50 ml of 0.1M HCl and refluxed, dissolution being complete after 10-15 min. 10 ml aliquots

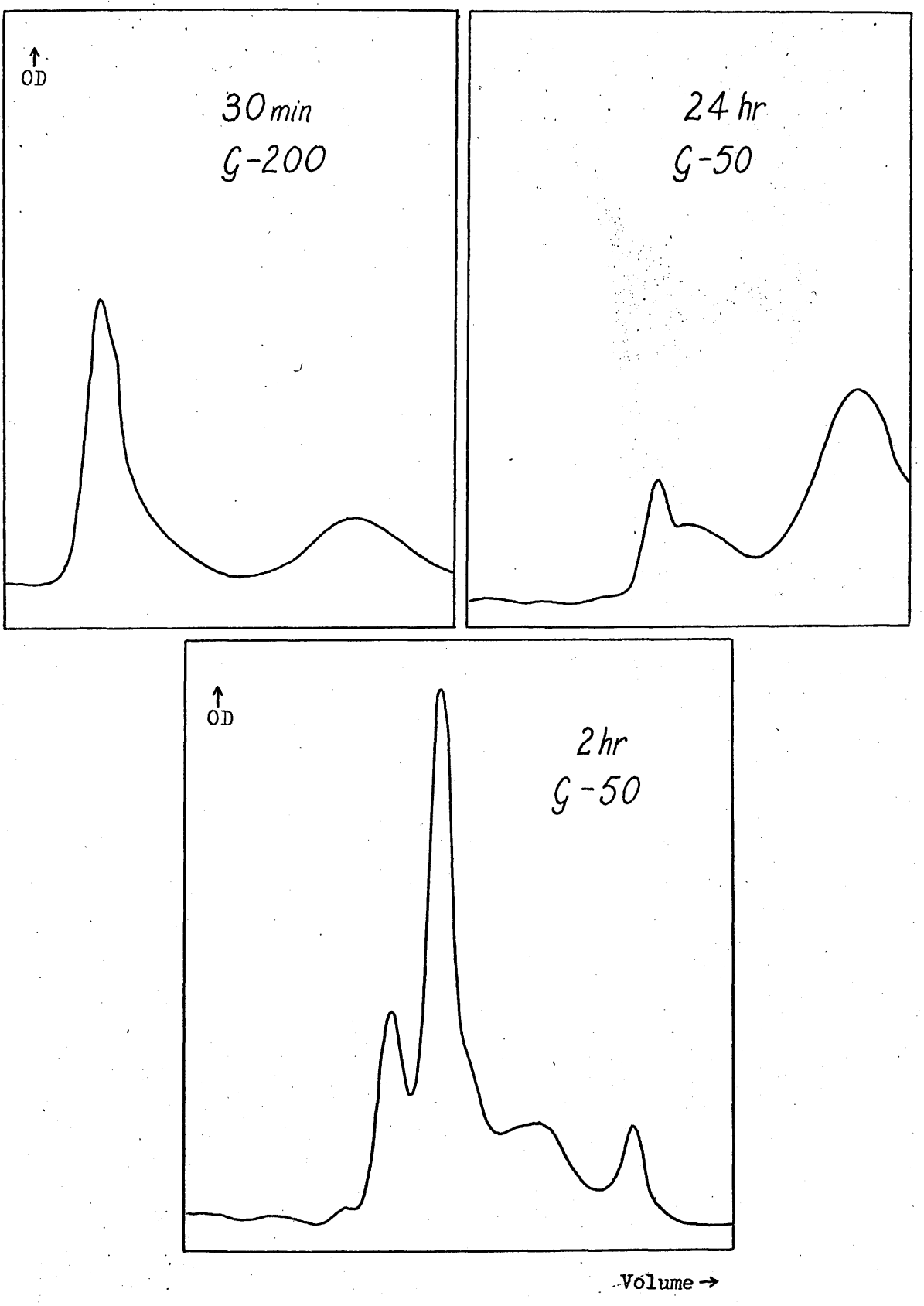


Figure 5.6 - Gel chromatograms of the polymeric fraction remaining after hydrolysis.

were withdrawn after 30 min, 2 hr, 6 hr, and 24 hr, cooled, and brought to about pH 8 with 1.1 ml of 0.5M Na_2CO_3. The residual polysaccharides were then dyed with Procion MX2B (see G.M. 9), salts and carbohydrate fractions of low molecular weight being removed at the stage of preparative gel chromatography.[327] The dyed polysaccharides were then applied to 30 cm x 2 cm column of Sephadex G-200 or a 45 cm x 1.5 cm column of G-50 for examination of their molecular size distribution.

The results are shown in Figure 5.10. Not surprisingly the size of the greater proportion of the polysaccharide becomes less as hydrolysis proceeds, but there is still some polymeric material left after the 24 hr hydrolysis. The distribution profile of molecular size remains a smooth curve throughout: the peaks at either end of the chromatograms correspond to complete exclusion and inclusion respectively, and there is no sign of intermediate peaks corresponding to fragments of discrete size, such as those found by Churms and Stephen[337,343] and by Anderson[344]. From the sharpness of the peaks at the inclusion and exclusion limits, it can be estimated that intermediate peaks of reasonable height would probably be evident if they were separated by an amount corresponding to 1000-1500 units in molecular weight: this places a probable upper limit on the size of any hypothetical sub-units.

Discussion

The monosaccharide composition of the mucilaginous polysaccharide is unusually complex. Like some of the other mucilages and exudate gums it contains sugars of both the glucose and the galactose series, so that if the assumption that C-4 epimerase activity is lost at the transition from primary to secondary wall formation has any foundation (see p.15), then the biosynthesis of the polysaccharide should be more likely to be associated with that of the primary wall matrix.

There is quite a close parallel with the mucilaginous polysaccharides which are exuded from the peripheral root cells of various species,[345-7,70-1] but these have not been characterised beyond their monosaccharide composition and it is not even known whether they are single homogeneous substances or complex mixtures. With the exception of L-fucose, it is not known whether the sugars are present in the D- or the L-form. However it is unlikely that bracken differs from the rest of the plant kingdom in this respect, and the forms present are probably L-arabinose and the D-form of the others, with some doubt about galactose.[348] L-fucose is not an uncommon sugar in plants,[349] but is rarely found in more than trace quantities. The amount present in the bracken polysaccharide accounts for no less than 0.5% of the dry weight of the rhizomes, which should therefore be a convenient source of fucose for laboratory purposes. The following suggested method of isolation would probably be satisfactory on a preparative scale: bracken rhizomes or young fronds are extracted with 1-1.5 times their weight of water, and the liquid pressed out, filtered, acidified to 1M with H_2SO_4 and refluxed for 8-12 hours. The crude hydrolysate is then passed through activated charcoal and the fucose eluted with 10% alcohol. After treatment with a mixed-bed ion-exchange resin it should be relatively pure.

Certain deductions can be made from the comparative rates of release of the different monosaccharides. The arabinose is released much more quickly than any of the others, and so is probably located in the peripheral regions of the molecule and bound in the furanose form.[27] None of the galactose is released particularly rapidly, and

α-linked galactopyranose end-groups are therefore unlikely.[253] Since mannose is released only very slowly even under the most severe hydrolysis conditions, it is likely to be linked to a uronic acid; and since the molar proportions of mannose and glucuronic acid are the same, within experimental uncertainty, it would appear that these two sugars are present solely as constituents of an aldobiuronic acid. This was later isolated.

The amount of unambiguous structural information obtained from the fragmentation analysis was rather limited, but by analogy with other plant polysaccharides it is possible to outline some of the likely features of its construction. It is normal for arabinose to be attached on the periphery,[27,65] and this would seem to be the case here as all the arabinose can be removed by acid without releasing an equivalent amount of any other monosaccharide. From the range of neutral oligosaccharides isolated it seems likely that a galactan, perhaps containing $\beta(1 \rightarrow 3')$ links although others may be present as well, forms the backbone of the neutral part of the polysaccharide as in arabic acid and some of the other exudate gums.[27] If other sugars do not form part of the main chain they must be at least partly linked in the form of long side-chains rather than single-unit stubs, as the combined percentage of xylose, fucose and arabinose exceeds that of galactose.

It is not certain whether the aldobiuronic acid is attached directly to the neutral core as a two-unit side-chain, or forms the repeating unit in a chain of alternating glucose and mannose units. Both of these situations can be paralleled in other plant polysaccharides.[350-1; 342] The linkage between the neutral and acidic portions of a polysaccharide is always difficult to establish.

The function of the polysaccharide and its cellular location in the rhizome are uncertain. It does not seem to be metabolised as a reserve substance (see p.43), nor could it be expected to lend rigidity to any structure of which it formed a part. From the way in which it is exuded freely from cut ends of storage rhizome it is most likely to be located between the cells or

in the peripheral regions of the cell wall, lining the intercellar air spaces which are a conspicuous feature of the storage rhizome and may provide aeration for the roots in waterlogged soils. If this is the case its water-holding capacity and ability to prevent desiccation could be an important factor in the water relations of bracken. The rhizome system's buffering capacity against drought has attracted comment from an ecological point of view.[303] Other roles for the mucilaginous polysaccharide could of course be suggested.

CHAPTER VI

BIOSYNTHESIS OF CARBOHYDRATE-CONTAINING SUBSTANCES IN BRACKEN

In attempting to produce an overall picture of the carbohydrate composition of the bracken plant it has been necessary to include information relating to both the frond and the rhizome, but there is no evidence that discrepancies between the two organs are serious. The mucilaginous polysaccharide for example is present in both, and their qualitative glycolipid composition is the same although there are some obvious quantitative differences (see Table 4.1).

It is convenient to divide the bracken carbohydrates into groups according to their structure and the probable manner and site of their biosynthesis in so far as these are known by analogy with other plants. The first group includes sucrose and starch, which in bracken are the sole forms in which energy is transported around the plant[245] (Hamilton and Canny, 1960) and stored[248,250] (Muller-Stoll and Michael, 1949). The second group comprises the cell wall polysaccharides: these are all considered together since although in the past cellulose and the matrix polysaccharides were thought to differ fundamentally in the site and manner of their biosynthesis,[145] there is now some uncertainty and this cannot be taken for granted.[90,146-9]

Bracken cellulose is similar to that from other plants,[251] while the two main hemicellulose components, a galactoglucomannan[253] and an acidic xylan,[252] have been characterised in detail and shown to be intermediate in structure between those of the gymnosperms and the angiosperms.[252-3] During the work for this thesis pectic substances were extracted from both fronds and rhizomes with 2% EDTA after the standard extraction series, and there was no reason to believe that their composition was widely different from pectic material from other sources: they were readily precipitated by Ca^{2+} ions and have been reported to have satisfactory gelling properties.[352]

The mucilaginous polysaccharide is also included in this group.

The other groups of carbohydrate-containing substances are the phenolic[254-6] and the cyanogenic glycosides.[258-9] The various steryl glycosides[257] are included with the glycolipids.

Comparison of the carbohydrate-containing fractions and the nucleotide sugars.

There is a fairly close correspondence between the range of monosaccharides combined in various forms in the plant (Table 6.1) and those present in the nucleotide fraction extracted from the rhizomes (Table 6.2). Rhamnose and sulphoquinovose are absent from the latter, although the conversion of UDP-glucose to UDP-rhamnose has been recorded in other plants.[353-4] Sulphoquinovose is thought to be synthesised separately rather than by conversion from another sugar[355]: the sulphur atom is probably donated to a 3-carbon moiety by PAPS, which is in fact available in bracken rhizomes,[12] and is likely also to be the sulphur donor in the synthesis of the sulphated sugars which have recently been found in thephenolic fraction from the fronds.[256] It is noteworthy that fucose is present only as the GDP-sugar: enzyme systems from micro-organisms are able to catalyse the conversion GDP-mannose $\rightleftharpoons$ GDP-fucose,[142] although it has not previously been recorded in plants.

Most of the nucleotide sugars required as donors for the construction of oligo- and polysaccharides, glycolipids and glycosides by accepted pathways[9] are present in the rhizomes, but there are some interesting exceptions. ADP-glucose is absent from the extracts, and is presumably to be found in the interior of the starch grains as is the case with potatoes.[10] ADP-glucose is held to be involved in starch synthesis and UDP-glucose in the synthesis of sucrose by the sucrose phosphate route, but it is possible that the latter could be an intermediate in the sucrose-starch conversion if sucrose is broken down by sucrose synthetase (see Figure 6.1).

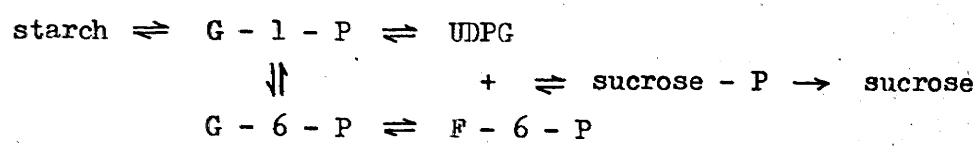
SUGAR	NUCLEOTIDE	SOURCES
Glucose	UDP-glucose	starch, sucrose, cellulose galactoglucomannan steryl glycosides, glucocerebroside phenolic glycosides.
Galactose	UDP-galactose	galactoglucomannan, mucilage, pectic substances, galactosyl and digalactosyl diglycerides.
Mannose	GDP-mannose	galactoglucomannan, mucilage.
Glucuronic acid	UDP-glucuronic acid	xylan, mucilage.
Galacturonic acid	-(?)	pectic substances.
Rhamnose	-	pectic substances, rutin.
Fucose	GDP-fucose	mucilage.
Xylose	UDP-xylose	xylan, mucilage.
Arabinose	UDP-arabinose	xylan, pectic substances, mucilage.
Sulphoquinovose	-	sulphoquinovosyl diglyceride.

Table 6.2

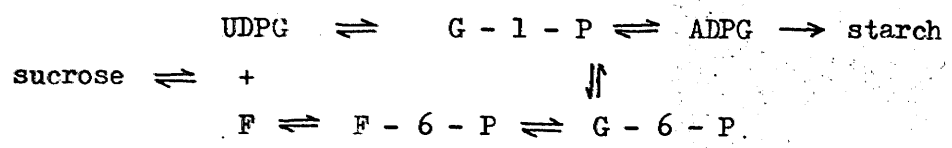
Comparison of sugars in nucleotide fraction and in other fractions from bracken.

Contrary to earlier opinions[32,256] it is now generally considered that the main synthetic route to sucrose is via sucrose phosphate, and the action of sucrose synthetase is primarily degradative.[37,45,48] However from the energy balance of stored potatoes during changes of temperature it seems more likely that invertase is the more important hydrolytic enzyme during the conversion of sucrose to starch.[34]

1. Sucrose phosphate synthetase (EC 2.4.1.14)



2. Sucrose synthetase (EC 2.4.1.13)



3. Invertase (EC 3.2.1.26)

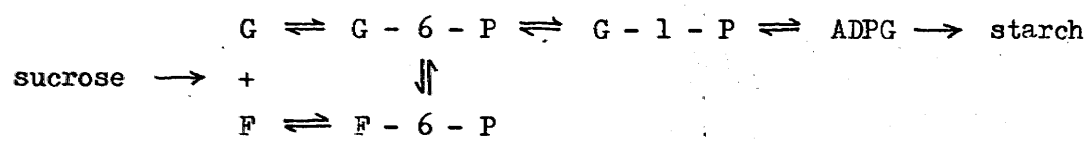


Figure 6.1

Synthesis and breakdown of sucrose during interconversion with starch.

It is not certain which of the intermediate reactions occur in the cytoplasm and which within the plastid. Since the ADP-glucose is inside[10], however, and both the UDP-glucose[12] and the main pools of free sugars are outside (though the latter may be divided in a complex way between cytoplasm and vacuole)[49,353], the implication is that it is the sugar phosphates which are transferred across the amyloplast membrane during sugar-starch interconversions. This would agree with other observations on carbohydrate transfer across plant cell membranes[49,357] (the tonoplast excepted)[358] although agreement on this question has not been reached.[359] It is of course quite possible that preceding or succeeding reaction stages in the sequence occur at the membrane surface concomitantly with transfer.[49]

Isherwood suggested that the conversion of sucrose to starch is as outlined above, but that all the stages on the degradative route take place within the amyloplast and the end product, sucrose, is exported into the cytoplasm.[34] In fact the case for starch degradation being divided between plastid and cytoplasm is stronger than that for starch synthesis, as UDP-glucose seems to be involved only on the degradative route (see Figure 6.1). It is not unlikely that sugar phosphates are the transported species in both cases.

If this is true, or even if it is only true for starch synthesis, a mechanism for the low-temperature sweetening reaction can be suggested. It is now known that the net conversion of sucrose to starch is caused by a small shift in the balance between the synthetic and degradative reactions, both of which are going on continuously at a relatively high rate.[34] The rate of starch production is controlled at the level of ADPG synthesis[265] so that if the supply of sugar phosphates flowing into the amyloplast for incorporation into this nucleotide is curtailed by phase changes in the amyloplast membrane at low temperature, the synthesis of starch will fall behind its degradation and the free sugar content will build up until the balance is restored.

It is of course quite possible that degradation rather than synthesis is under temperature control; and the sweetening reaction that occurs in potato tubers at senescence[264] is an altogether different phenomenon. If the latter is caused by the destruction of the amyloplast membrane as Ohad et al. suggest,[56] then the high levels of UDP-glucose in the cytoplasm might serve to keep the corrosion of the denuded starch grains by degradative enzymes under control to some degree.[38]

The other nucleotide sugar conspicuously absent is GDP-glucose for the synthesis of cellulose.[9] It could of course be present at a level below the detection limit, or could just possibly be locked up in a particulate fraction and not released during homogenisation, but on the whole its absence can be taken as circumstantial evidence that UDP-glucose is capable of acting as a precursor in cellulose biosynthesis in bracken, as has been suggested for other plants.[98-101]

It is not clear whether this would have any bearing on the question of where the cellulose chains are constructed.

It is also interesting that mannose is apparently available only as the GDP-sugar. This is the form in which mannose is first produced,[142] and has also been proposed as the mannose donor for the synthesis of the hemicellulosic glucomannans both *in vitro*[140,309-10,360] and *in vivo*.[141] The GDP-glucose which is also required does not seem to be available in bracken, and in its absence UDP-glucose would presumably have to serve for the synthesis of the galactoglucomannan. As was mentioned in the Introduction the possibility remains open that this polysaccharide is more closely related to cellulose than to the other hemicelluloses in the manner of its biosynthesis. The evidence is as follows -

a) G-series nucleotide sugars can act as donors.

b) Mannose is often found even in purified cellulose fibrils, particularly in the peripheral regions.[85] It is not impossible, however, that this could arise from very strongly adsorbed contaminating glucomannan material.

c) The same particulate enzyme preparations from leguminous species are capable of both cellulose and glucomannan synthesis.[95-6,140,360]

d) The linkage is the same $(\beta(1 \rightarrow 4))$ in both polysaccharides.

e) It has been observed that the pectic substances, the hemicellulosic arabinoxylans, and some of the gums and mucilages are produced by the Golgi apparatus,[70-5] but this does not seem to have been shown for the glucomannans.

A suitable range of nucleotide sugars are available for the synthesis of the mucilaginous polysaccharide. Unless another mannose nucleotide is available in very small amounts, the presence of GDP-mannose implies that it is the donor to whatever the aldobiuronic acid is attached to. The presence of GDP-fucose implies, with the usual reservations, that this is the fucose donor *in vivo*. The arabinose of the polysaccharide is in the furanose form, as is generally found in plant polysaccharides. It is bound in the pyranose form to nucleotides, however,[361] and presumably the transfer to an acceptor allows a simultaneous configurational change.

With the exception of fucose it was not established whether the components of the mucilage were of the D or the L form, but by analogy with other polysaccharides it seems possible that the terminal galactose residues in the bracken galactoglucomannan are of the L-form, and in that case their biosynthesis and transfer to the polysaccharide would differ from those normal for D-galactose.[348] If the chain is indeed synthesised from the reducing end the availability of the donor for these galactose units could limit the chain length (in a statistical fashion), unless a more specific control mechanism is available.

Order and randomness in plant polysaccharides

In the previous Chapter it was noted that during controlled acid hydrolysis of the mucilaginous polysaccharide there was no sign of any discrete fractions, detectable by gel chromatography, with molecular sizes which were multiples of a common factor. When Churms and Stephen observed regularly-spaced multiple peaks in gel chromatograms of hydrolysed *Cussonia spicata* gum, they concluded that the galactan core of the polysaccharide contained a repeating unit of 7 monosaccharide residues.[343] In other cases where such multiple peaks have been observed they have been put down to successive hydrolysis of bonds of increasing stability,[337] or to loss of protein sub-units.[344]

Now the general opinion held in the last twenty years, particularly by the Edinburgh school of carbohydrate chemists,[27,65,362] has been that the matrix polysaccharides of plant cell walls are continuously variable in both composition and structure, and certainly contain no regular repeating units. (Formerly a heteropolysaccharide containing, say, xylose and arabinose in the approximate molar proportions 2:1 was sometimes stated to have a triaccharide repeating unit of this composition without any other evidence being put forward,[eg 363] but this practice has fortunately been rare for a long time). Recently, however, other plant polysaccharides as well as the Cussonia spicata gum have been suggested as being composed of repeating units, although in no case is the experimental evidence wholly conclusive.[128,136,342]

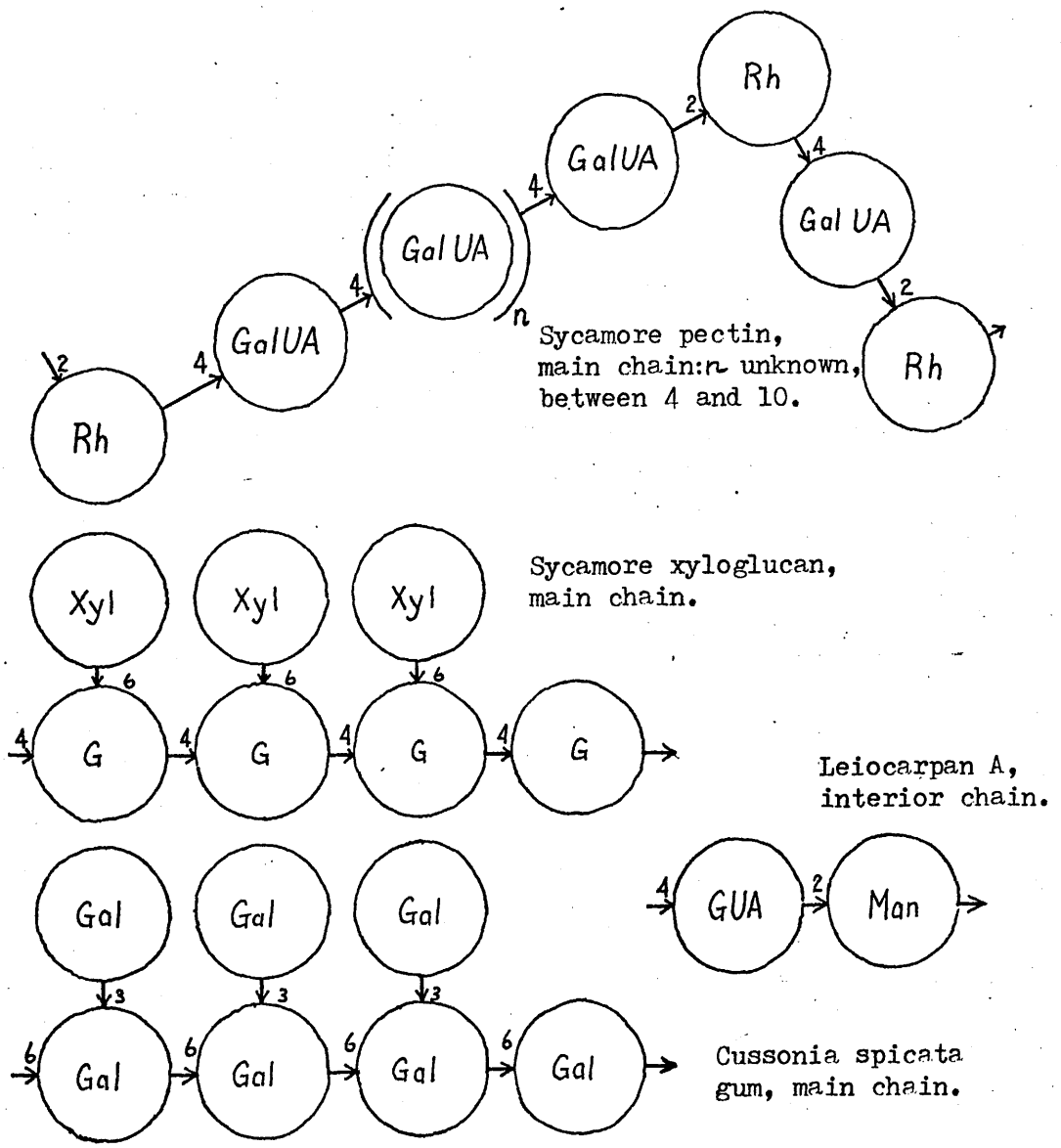


Figure 6.2 - Proposed repeating units in plant polysaccharides.

It is possible that the bracken polysaccharide contains the same repeating unit as has been proposed for the main chains of leiocarpan A[342] (see the previous Chapter). If this is the case then the nucleotide sugars most likely to be involved in its formation are UDP-glucuronic acid and GDP-mannose[12], although other suitable nucleotide sugars may be available in quantities to small to be detected.

The proposal[136] that the acid main chain of a pectic polysaccharide contains a repeating unit is of particular interest, but when the published data are examined it is not possible to ascertain whether the number of uronic acid residues in the 'repeating unit' varies around a mean, or is constant but not accurately known. If it is constant, the resulting polymeric structure would have considerable possibilities for non-covalent intermolecular association, provided it was not heavily substituted with neutral side-chains.[135] Microcrystalline bundles of the type envisaged by Rees[172] could gain additional stability from an ordered disposition of monosaccharide units along the chain if conformations suitable for hydrogen bonding were thereby encouraged, and particularly if there was a place for Ca^{2+} ions at regular intervals holding the chains together by interactions with both carboxyl and hydroxyl groups. There is evidence from the theory of polyelectrolytes that an ordered multiplicity of weak interactions can be as effective in binding large molecules together as a smaller number of strong covalent bonds.[364] A model such as the one just suggested could explain the rapid effect of pH[365-6] and complexing agents[156-9] on the structural integrity of the cell wall, and would be compatible with its dynamic behaviour under mechanical stress,[60] assuming that the suggested repeating unit in sycamore pectin is in fact genuine.

These proposed repeating units in plant polysaccharides are also of crucial importance to our understanding of their biosynthesis. Mention has been made of the long history of attempts to show that polysaccharide biosynthesis in plants proceeds through a lipid intermediate and starts from the non-reducing end of the chain,[87-8, 109, 309-10, 360, 367-8] as is the case in the bacterial cell wall. Lipid intermediates in the synthesis of a linear homopolysaccharide would leave no trace discernible in the completed

structure, and their involvement is almost impossible to disprove unless by demonstrating from which end chain construction begins. Where there is a complex repeating unit, on the other hand, it is attractive to assume that it is prefabricated while attached to a high-energy lipid as in bacteria.

There are other possibilities, however. The conditions for synthesis of a polysaccharide of random, polydisperse structure as described by Aspinall,[65] for example, can be put in precise terms:

a) When a linear chain contains more than one kind of monosaccharide unit, the transfer of each kind to the end of the chain must be unaffected by the nature of the present terminal sugar. Thus the order of the monosaccharide units will be random.

b) The branching of a chain, or the addition of branches to one already formed, is independent of the presence or absence of branch chains on the monosaccharide residues to either side of the acceptor sugar. Thus the disposition of side-chains along the main chain will be random.

In a number of cell wall polysaccharides which have been examined in detail the structure is such as to imply that condition (b), at least, has been largely fulfilled during biosynthesis. For example the arabinoxylans from cereal flour have arabinose-rich and arabinose-poor regions along the main chain, but within these regions the distribution of single-unit arabinose side-chains is apparently random.[369]

The hemicellulosic glucomannans[140] possibly conform to condition (a), but the interior chains of leiocarpan A with their aldobiuronic acid repeating unit do not.[342] In this case it would seem entirely possible that glucuronic acid is transferred specifically to mannose, and mannose to glucuronic acid, on the non-reducing end of the growing chain. If the same situation occurs in the biosynthesis of the bracken polysaccharide, then the fact that mannose is possibly donated by a G-series nucleotide and glucuronic acid by a U-series one may be relevant.

It is easier to envisage this kind of specificity when one sugar is charged and the other is neutral.

If such a mechanism were responsible for synthesising a polysaccharide with a larger repeating unit such as the one proposed for sycamore pectin[136] (see Figure 6.2) the number of highly specific enzymes required would be greatly increased and some means of 'counting' the galacturonic acid residues as they were added to the uninterrupted part of the chain would have to be introduced. A system with these capabilities would necessarily be highly complicated, and the same criticism applies if the repeating unit is prefabricated in this way on a lipid acceptor.

A simpler alternative, more economical in its requirements for multiple enzymes but possibly less precise in its control of the structure of the end product, may be suggested: the specificity of the sugar-donating system could depend on the general physico-chemical properties of the portion of the chain already synthesised, rather than merely on the nature of the last sugar unit. The properties which might be involved include charge, ability to associate with other polymeric molecules or with membrane surfaces, and in particular the coiling tendencies of the end part of the chain, which must affect the way in which the acceptor sugar is presented to the active site on the enzyme.

Thus the uninterrupted galacturonan portion of sycamore pectin, according to the proposed model (see Figure 6.2) would build up to 2-4 three-unit helical turns (perhaps held in place by one or more cations) before the chain end could fit into the right position to accept another rhamnose molecule. Only two or perhaps three separate enzymes would then be required, rather than 12 or 15.

In a similar way the synthesis of the repeating units proposed for Cussonia spicata gum[343] and the sycamore xyloglucan[128] could be explained if the main chain adopted a helical conformation with, say, four monosaccharide units to each turn, the addition of a side-chain to one unit only in each turn being sterically prevented. This model would avoid the need for multi-enzyme systems able to 'count' up to seven consecutive sugar transfers.

Of course the models for polysaccharide biosynthesis suggested above are entirely speculative. It is hoped, however, that they will draw attention to the possibilities of investigating the relationship between the detailed structure of plant polysaccharides and their biosynthesis. This interesting field has received some attention in the past,[30,370] but hardly as much as it deserves.

Note: Dr. H.J. Duncan is responsible for some of the ideas mentioned in the first part of this Chapter. See 'Studies on nucleotides and related compounds in plants: part V'.[12]

CHAPTER VII

DISCUSSION ON BRACKEN CONTROL

It was mentioned in the Introduction that for bracken control on hill pastures in Britain the herbicide asulam is now being used almost exclusively, and this forms the main subject of the present Chapter. Abroad, save in New Zealand and Japan, bracken is of more importance to forest management than to grassland farming, and it is of some concern to foresters in this country as well. The techniques in use for controlling bracken in forests are however rather different and more varied[371,234]: for this reason they will not be considered in detail, but as moorland bracken presents the most difficult problems in control and subsequent management it is hoped that some of the conclusions drawn may be applicable, in modified form, to bracken control elsewhere.

The effect of asulam on bracken

Penetration:

Penetration into the frond, the first stage in the progress of the herbicide through the plant, is at present under investigation in this laboratory.[244,263] With a highly water-soluble herbicide such as asulam (when formulated as the sodium salt), the Scottish climate renders speed of penetration essential, and the extended length of time for which the chemical was allowed to lie on the fronds during some of the earlier greenhouse experiments with bracken[243] is not at all representative of the field situation. Spraying at the high volume rates (up to 550 l/ha) originally recommended for tractor-mounted or knapsack sprayers results in up to two-thirds of the herbicide running off the fronds immediately,[372] but this is not likely to be a problem with the much lower rates (11-44 l/ha) used in helicopter spraying.

In general penetration is slower late in the season,[244] and with the more mature pinnae on a particular frond,[373] due to the increasing thickness of the cuticle.[243] The amount of asulam which is able to penetrate depends not only on this and on the time which elapses before the next shower of rain, but also on the relative water content of the fronds, which controls the continuity of aqueous connections across the cuticle[244] and is itself greatly affected by weather conditions[303] and perhaps by the time of the day.[234] The buffeting caused by the helicopter's downwash is likely to cause some damage to the cuticle and hence cut down its resistance to penetration: also a larger area of the grass below the fronds will be exposed as they are blown about.

It is impossible, however, to give typical figures for the amount of herbicide that finds its way into the fronds after aerial spraying, the amount that is retained by the grass layer, and the amount that passes into the soil. The variation is undoubtably very wide.

Translocation

The pattern of translocation in the frond and its seasonal dependence seem relatively straightforward (see the discussion in Chapter 1). It remains only to find out how the timing of the carbohydrate movements varies from one site to another, and to what extent translocation continues into the late August and September period.

Current ideas on translocation in the rhizome are decidedly confused, however. The seasonal pattern of control with a wide variety of herbicides[223,227,235-43,374] agrees so well with the seasonal variations in carbohydrate flow in the frond that it is entirely reasonable to assume they are connected; but in the rhizome no such agreement is immediately obvious. With both natural[225,241] and greenhouse-grown bracken,[236,243] the only visible effects are at the growing points of the frond-bearing rhizome to which the treated frond is attached, i.e. the rhizome apex and the subsequent year's frond bud. With greenhouse-grown bracken these are also the parts of the rhizome in which most of the radioactivity is found after the application of labelled asulam or other herbicides.[243]

This is entirely in accord with Hamilton and Canny's observations on the movements of sucrose in bracken grown in the greenhouse,[245] but it cannot be reconciled with the carbohydrate metabolism of bracken growing in the field (Chapter II). While some rhizome growth undoubtably takes place more or less throughout the summer,[192] the amount of new rhizome and frond bud tissue formed is very small, and all the evidence favours the storage rhizome as the main destination of translocated sucrose.

Thus with field-grown bracken there are two alternatives:

a) the greater proportion of the herbicide follows the main sucrose stream into the storage rhizome but does no visible damage there: a much smaller quantity, proportional to the amount of sucrose required for rhizome and frond bud growth, goes to the growing points. Or:

b) the herbicide, or at least a considerable proportion of it, breaks away from the translocated sucrose stream and travels towards the growing points, propelled by some influence that is specific to itself.[375]

The field investigation carried out by MacIntyre[373] has a bearing on this question. He found that labelled 2,4-D moved towards the rhizome apex when applied to one of the pinnae on the side of the frond facing in that direction, but that when it was applied on the other side much of it appeared to go towards the storage rhizome. This is in accord with the nature of the vascular connections at the base of the frond[376-7,245] provided a reversal of direction takes place in the flow towards the apex (see Figure 7.2).

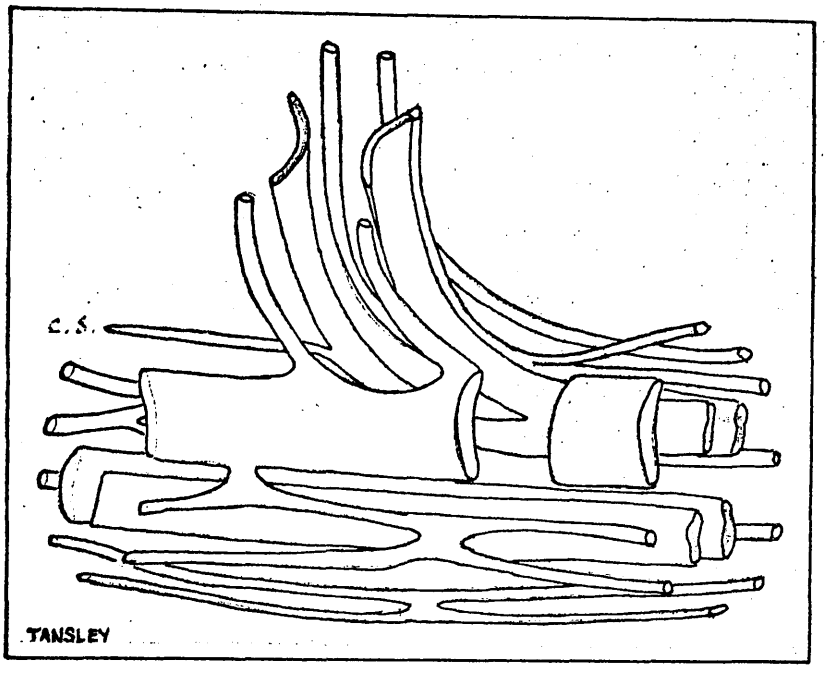


Figure 7.1 - The vascular system at the junction of frond and rhizome.

However MacIntyre did not follow the herbicide as far as the storage rhizome itself, and in any case what is true for 2,4-D is not necessarily true also for asulam (though no major differences in their translocation behaviour have become apparent so far). Also, caution is necessary when interpreting the results of experiments which depend on autoradiography: even in the fairly advanced form used by MacIntyre this technique is not strictly quantitative, highlighting localised areas of high activity rather than more diffuse regions which may contain a greater total amount of labelled material. From this evidence, therefore, it is not possible to distinguish between the alternatives (a) and (b). The question may be resolved by a field experiment with labelled asulam which is just being completed at the time of writing.[275]

If asulam and other herbicides do pass into the storage rhizome, their ability to move along it is very limited. If experimental plots

are sprayed accurately with any herbicide and reasonable control results in the following season, the fronds end very sharply at the plot boundaries and there is no obvious control outside. This means that any translocation within the storage rhizome must be limited to very short distances, certainly under 1m.

Bracken communities growing in open sites generally have a considerable reserve of dormant frond buds which are not located in the usual position between the base of the previous year's frond and the rhizome apex.[192,209] These may escape the effects of the herbicide; and in fact when the rhizome system at Sundaywell (site 5) and Carbeth (site 6) was examined in the year after spraying, most of the fronds which had emerged were attached to the rhizomes in such 'abnormal' positions and were some distance away from the nearest sprayed frond on the same rhizome.

Good long-term control must depend on cutting to a minimum the number of these isolated fronds which emerge, because they act as centres for new growth. The influence of the application rate is much more noticeable in the longer term, 2.2 kg/ha being virtually as effective as 4.5 kg/ha in the first year but poorer thereafter, so if the ideas outlined above are more or less correct it follows that translocation of the herbicide for any distance away from the rhizome base depends on the concentration gradient that can be established rather than on sucrose-linked transport. It is also one of the strongest arguments for trying to maximise the proportion of the applied herbicide that penetrates into the frond.

In theory another spraying a year after the first should allow the herbicide to reach the most inaccessible parts of the rhizome via the fronds which have emerged, resulting in greatly improved long-term control. In practice this has not been attempted, because of the drastic effect which a second helicopter spraying would undoubtably have on the exposed grass. Over a limited area it might be possible to spray each frond individually using a hand-lance, but even a 99% reduction in frond numbers leaves 1500-3000 fronds/ha and one man could not expect to spray more than about 5 ha/day.

Site of action

In bracken as in other plants, the visual symptoms of asulam treatment are those of artificially induced dormancy at the growing points.[235-43, 379] The effects take some weeks or months to appear, but whether this is due to slow penetration and translocation,[243] or slow action, or both, remains to be seen. At the affected growing points cell division, nucleic acid and protein synthesis, and respiration are all inhibited[243,274] and the deposition of lignin and accumulation of soluble phenolic compounds seem to occur more rapidly although this may be simply because they continue after the cessation of growth. Splitting and necrosis may occur at a later date.

This range of effects is rather wide, but it is not certain which of them are directly produced by the herbicide at the site where they are manifested, and which are secondary in origin. Most of the growth regulator herbicides produce rather similar physiological symptoms, but preliminary results indicate that the effect of glyphosate is quite different, involving widespread destruction of the storage rhizome rather than any attack on the growing points.[235] As yet it is not clear whether glyphosate differs from asulam in its translocation behaviour, or whether both are translocated to the storage rhizome in the same way but only glyphosate has any destructive effect there. The latter possibility seems more likely, since moderate reductions in the respiration rate of the storage rhizome have in fact been reported[243, 274] and the possibly exceptional results of spraying at Bun-an-Loch (Site IZ ; see Chapter II) included apparent loss of stored starch.

Losses of reserve carbohydrate from the storage rhizome are also a feature of the effects of glyphosate, and may perhaps be the cause of eventual rhizome death.[235] Although this newer herbicide cannot match asulam or picloram in its ability to reduce frond numbers, its more fundamental mode of attack makes it very promising for the future. A logical step would be to use mixtures of glyphosate with asulam, assuming that the two herbicides are compatible. Glyphosate does have the disadvantage of causing more serious damage to the grass.

It would not contradict the known facts if the apparent dormancy induced by asulam were caused by the herbicide interfering with the mobilisation or translocation of carbohydrates from the storage rhizome rather than to any effects at the growing point itself. It was shown by Gottlieb,[379] working with excised rhizome tips, that the rate and form of growth were largely controlled by the supply of sucrose to the growing areas. (In addition to its relevance here, this finding could be important to the autecology of the plant). There is of course no positive evidence that asulam works in this way, but the fact that it is possible to suggest a mechanism so completely different from what is normally accepted emphasises the inadequacy of our present knowledge on this subject. After rather lengthy and expensive field trials and laboratory investigations it is possible to make satisfactory recommendations for the use of asulam without being certain how or where it interferes with the growth of the plant, but future development work on other herbicides, and perhaps on glyphosate/amitrole or glyphosate/asulam mixtures, could be carried out more quickly and less empirically if the basis of translocation, at least, were understood. Questions that could usefully be answered include:

How is the sucrose produced by the frond distributed between the storage rhizome and the rhizome apex? How far does it travel along the storage rhizome? Is the transport of a particular herbicide dependent on the movements of sucrose, or on its own concentration gradients? If it goes to the storage rhizome, can it move back to the growing points during the autumn and winter? Is it accessible to other frond-bearing rhizomes? The answers can only be provided by experiments with labelled materials in the field.

Persistence

At the moment it is not clear whether the long-term effects of asulam on bracken are due to the damage which it causes to a high proportion of the growing points in the year of spraying, or to the persistence of some of the chemical in the rhizomes.[380] Radioactivity

from labelled asulam has been shown to persist in the vicinity of the growing points for at least a year,[242] but the chemical does not necessarily remain in an active form. The check in the growth of pasture grasses, particularly Agrostis spp., continues for at least two years after spraying and could likewise be due either to persistence of the chemical or to slow recovery from its effects.

As was mentioned earlier the amount of the herbicide which reaches the soil is unknown. In more acid soils it is held and eventually decomposed,[381] although at low temperatures in particular the rate of decomposition is slow. In general asulam does not seem to be as persistent in soils and vegetation as picloram, which was refused Government approval for use on bracken seemingly for this reason, but more detailed long-term information on the behaviour of asulam in vegetation would be desirable.

In soils of pH5 and above, however, asulam is not strongly adsorbed but can be leached into the drainage water.[381] Since it is not broken down in still water this must give cause for concern. All reports so far indicate that the chemical is non-toxic to animal life.[242] More confidence would be possible if the detailed results of the toxicity trials were available in published form, but it seems likely that asulam is much less toxic than bracken itself. In view of past experience with other chemicals, however, it does not appear satisfactory to rely on the apparent harmlessness of a herbicide in the laboratory when it is able to persist, and perhaps accumulate, in some part of the environment. Certainly tighter controls over spraying in catchment areas for small private water supplies[382] and perhaps also fishing waters, would seem to be required. It would be wrong, however, to encourage irresponsible or ill-informed publicity concerning the potential hazards of this undoubtably useful herbicide.

Management after spraying

On present evidence it appears that when bracken is sprayed with asulam under reasonably good conditions, regeneration is centred on fronds growing from parts of the rhizome inaccessible to the herbicide, and will be complete within three to six years unless steps are taken to destroy the fronds as they appear. During this time the frond density increases exponentially,[235] as shown in Figure 7.2.

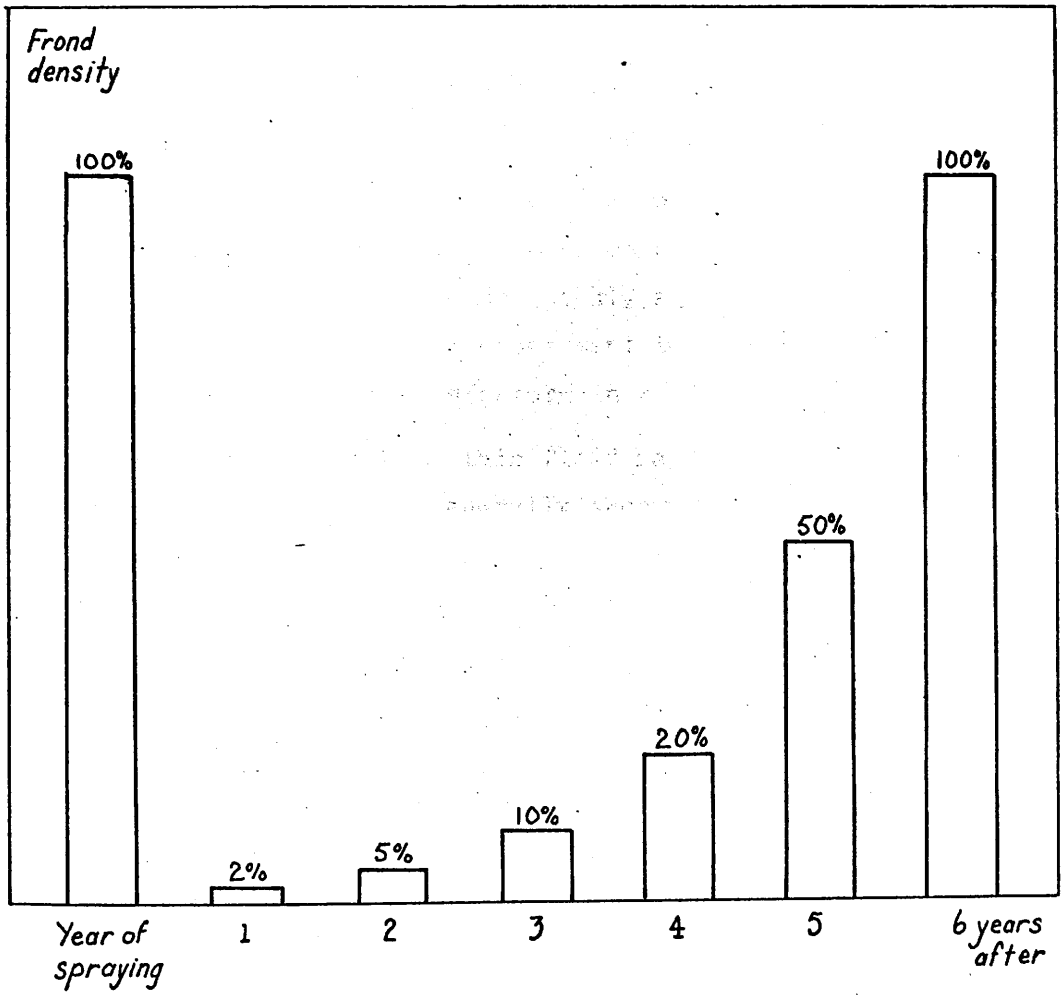


Figure 7.2

Exponential increase in frond density after asulam treatment. Optimum spraying conditions but no attention thereafter.[235] (Conjectural after 4 years).

Thus if only 95% control is achieved, the frond numbers will return to normal after four years instead of six, on this basis.

If true long-term control is to be achieved the relatively short period when fronds are almost absent must be used as an opportunity to increase the carrying capacity of the land. Stock, particularly cattle, trample the fronds as they emerge and are capable of holding frond numbers at a low level as long as the stocking rate is high. If this can be continued for a number of years the carbohydrate reserves of the rhizomes will be depleted by normal respiration,[261] and if they fall below a certain critical level (about 8-9% 'reserve' carbohydrate, but the equivalent starch content is not known), rhizome decay and death will occur, the frond-bearing rhizomes being affected first.[262] It then becomes possible to talk of at least partial eradication, rather than merely control of frond emergence.

The key to effective long-term control of bracken is therefore the establishment of a productive grass sward capable of carrying reasonable numbers of stock, as quickly as possible after spraying. It must be admitted that the management techniques currently available for this purpose are unsatisfactory in a number of ways.

Development work in this field has been rather seriously hampered by the lack of a generally accepted basis for classifying or even describing bracken stands. The two authoritative classifications of Scottish plant communities in phytosociological terms,[383] which appeared during the 1960's, made no provision for communities dominated by bracken.[384-5] Instead the community types in which bracken is an important component were classified on the basis of the underlying grass layer. It would not be appropriate here to criticise this decision on ecological grounds, but a separate classification of bracken communities would perhaps have been more valuable from an agricultural point of view than any other single feature of these classification projects. This must be regarded as a lost opportunity, since in plant ecology the emphasis has now moved away from the classification of community types.

It is likely that bracken communities show continuous, multidirectional variation,[131] but an arbitrary classification into three loosely-defined groups (excluding woodland bracken) has been suggested independently on a number of occasions[186, 235, 183] and seems convenient in practice. In all cases the basis for classification is frond density and vigour (cf Mitchell's 'interference index', number of fronds x mean height ÷ 100),[181] and fortunately the divisions proposed by different authors are approximately the same. No attempt will be made here to define these classes, but a brief description of each is given below. Poel[186] provides full species lists for his site at Ballochraggan in W. Perthshire.

Class I. *Agrostis-Festuca* grassland of whatever type is normal in the area in question, infested with bracken to an extent sufficient to affect its management but not sufficient to modify its composition seriously. *Festuca-Agrostis* communities of King and Nicholson's type 5 are most commonly involved, but their type 9 and, except on the more ill-drained soils, the *Festuca-Agrostis-(Nardus)* type 6 communities of the Southern Uplands may also carry bracken of this density.

Class II. Moderately dense bracken under which there is still more or less continuous grass cover, but the species composition of the grass layer is substantially modified by the influence of the bracken. The shade-tolerant species which appear are more familiar in woodland, and in general are of very limited feeding value to stock. *Nardus stricta*[181] is apparently a common member of such communities in the Eastern half of the country and *Holcus mollis*[186] in the West, although there has been some disagreement about this. In sheltered areas on the West Coast *Rubus* spp. and *Endymion non-scriptus* are associated with *Holcus mollis* under bracken to give a dense scrub similar to oakwood undergrowth and almost impenetrable (cf Louseley[387]). All these species can be troublesome when the bracken is removed.

Class III. Dense bracken stands in sheltered areas on deep, fertile soil, including woodland clearings, where the underlying vegetation is suppressed altogether or reduced to a limited growth of very shade-tolerant species such as *Deschampsia flexuosa*[181] and *Galium saxatile*.

The best methods of treating one kind of bracken stand are not necessarily suitable for another. A factor which was seriously underestimated in early trials is the effect of asulam on the pasture grasees which grow under bracken.[388] They are to some extent protected by the frond canopy, but even in a dense bracken stand some of the herbicide will be washed down by rain unless penetration is very rapid. Agrostis tenuis and A. canina are particularly sensitive to asulam, Festuca ovina rather less so. Clover, if present beforehand, will be eliminated. Holcus mollis is sensitive but will reappear in the following season, and is quite capable of dominating the sward in the absence of bracken at the less exposed sites where it is normally found. The results of field trials on numerous other species have been reported by Robertson,[389] and by Cadbury.[390]

For this reason there is general agreement that spraying Festuca-Agrostis pasture which carries only fairly sparse bracken fronds, less dense than Class I, will do more damage than good. The grass is completely exposed and suffers very badly, and bracken at this density does little harm in any case.

Almost all the bracken-infested land which is being treated at the moment is of Class I or at the lower-density end of Class II. It is difficult to be wholly enthusiastic about the results up to the present, although there is no doubt that more can be achieved, with less effort, than by cutting. In the year after spraying the loss of grass production due to the effects of asulam is typically about 50%,[235] and Agrostis tenius and A. canina are almost eliminated from the sward. It has been held that after one year the grass recovers,[242] but two years after spraying at Sundaywell and Cosandrochaid (sites 5 and 7) the appearance of the ground was still rather depressing, with reasonably good grass, heavily grazed, amongst clumps of old, decaying fronds which had provided cover in the year of spraying, but with stunted and almost ungrazed grass between.

4 cwt/acre (0.4t/ha) of ground mineral phosphate is the minimum requirement before the 50% grant will be given for spraying, and ought to help in overcoming the check in the growth of the grass caused by the herbicide. Recent field trials to investigate this have had rather peculiar results, but these may have been due to the unusual weather conditions in the summer of 1974.[235] Ground mineral phosphate is now very expensive, but spraying (without the grant) when no after-treatment is intended does not seem at all advisable.

Liming may also be an advantage if the terrain permits and if the introduction of clover is contemplated, but the lack of satisfactory, proven methods for re-seeding rough ground of this type after bringing the bracken under control is a serious obstacle to progress, and any developments would be welcome. The situation in parts of Aberdeenshire and Galloway where the terrain is less broken is more encouraging.

Although it may seem tempting to make an assault on Class I bracken, therefore, the economic advantages and disadvantages must be considered coldly beforehand. Rough pasture of moderate quality reclaimed from bracken may not be a great asset if it only adds to the summer grazing when winter feed effectively determines stock numbers, and if the ground is too broken for this excess of summer production to be conserved. Draining and fencing pasture of this quality is unlikely to be economic, although one cannot generalise and each case has to be considered on its own merits.

Some of the Class II bracken stands present a still greater problem due to the poor feeding value of the underlying grass. Holcus mollis, the main component at many West Scottish sites, is of much lower palatability and digestibility than the bents and fescues. Some of these sites, however, and many of Class III are on comparatively level, fertile, and well-sheltered ground at low altitudes - often old in-bye land. In many cases they have the potential to produce grazing, in a limited area, of an altogether higher quality than is possible amongst the high hills, and warrant considerably more expenditure on improvement.

Provided a tractor can be taken onto the land after the bracken has been treated, this should now be possible. The following sequence of operations is suggested as an example, but the strategy would obviously vary from one site to another.

The bracken is sprayed with asulam in the normal way. (Glyphosate or a mixture could be used if desired, as its effect on any underlying grass will not matter). As soon as the fronds have withered down enough to give access for a tractor 4-6 cwt of ground mineral phosphate and an appropriate amount of lime are applied and left to leach into the soil, protected from the wind by the remains of the fronds. Even if it is possible discing is better avoided unless the rhizomes are very deep, since they will be cut up and this may conceivably allow new frond growth. As soon as the old fronds are dry enough to catch fire in the spring, they are set alight and the litter burned thoroughly and deeply. A ryegrass/clover mixture of the type used for hill land is broadcast directly into the warm ash and rolled in with the tractor wheels or trampled by running sheep over the land.

If a productive sward can indeed be obtained by these or similar methods at a relatively sheltered site, the much higher stocking rate thus made possible will be of great assistance in securing long-term control over the bracken. In certain cases a second, hand spraying of the fronds which emerge may be justified, with due care to avoid damage to the clover.

For this type of utilisation fencing would certainly be necessary, but draining is unlikely to be justified. It is well-known that bracken does not grow on poorly-drained soils,[186,392] but the appearance of some of the sites investigated here, particularly site , does suggest that the bracken plant may itself improve the aeration in its own rooting zone and that of the grass, or render the grass less susceptible to waterlogging by some other means. Possibly old frond stumps and the interconnecting air spaces in the rhizomes[393] allow gas interchange with the root area.[394] It is not known if this would continue after the suppression of the bracken:

there is an unverified report[395] that rushes (which are never found within a dense bracken stand on smooth ground) may have appeared at one site a year or two after spraying. This danger is unlikely to be serious, but should be kept under observation.

The unit cost of improving bracken-infested ground in this way is bound to be considerable, probably £50-£70/acre to the farmer at current price and grant levels. The areas which could be treated are in most cases small, however, and the object may be seen as similar to that of the 'mosaic' improvement schemes for rough pasture advocated by the HFRO [11] - the introduction of small, high-quality areas of fenced grass to extend the productive capacity of hill land on either side of its narrow summer peak. In certain areas, of course, similar techniques could be used to add to the useful pasture acreage of lowland-type farms on the fringe of upland areas, either for beef in the North-east or for milk production in the South-west. In such a situation the economics are perhaps more favourable, and long-term control of the bracken by cattle is better than by sheep.

From the mid-1950's until about 1972 the acreage of bracken being cut by Scottish hill farmers, under grant aid, fell steadily as the cost of labour increased and the economics of hill land reclamation became less favourable. In the past two seasons, however, a rapidly-increasing area has been sprayed with asulam, largely by helicopter, in schemes involving some follow-up treatment and qualifying for a 50% government grant.

For the moment this trend seems likely to continue, but in the longer term the willingness of farmers to undertake bracken eradication schemes will depend on the success of those in progress now; the results obtained so far have been rather mixed, mainly because the techniques available for establishing a satisfactory grass sward after removal of the bracken are as yet inadequate. Out of the 200,000 hectares of bracken-infested land in Scotland, it has been estimated that about 50,000 hectares are suitable for reclamation : with improved techniques for after-treatment this figure could possibly be increased, but inevitably the amount of bracken which is brought under control during, say, the next decade will depend on economic as much as on technical factors.

It is often suggested that greatly increased use of improved hill land for livestock production would be in the national interest, but this can never become a reality unless, regardless of changes of Government or governmental policy for a number of years into the future, the farmer is assured on adequate financial return on the additional stock which the improvement of this ground would allow him to keep.

Chapter VII

GENERAL METHODS

Colourimetric methods for carbohydrates.

G.M.1 Phenol-Sulphuric method [396]

In the most common version of this method, 0-100 μg aliquots of carbohydrate in 1 ml of water are added to 1 ml of 5% aqueous phenol in matched, wide-bore test tubes. 5 ml of analytical grade sulphuric acid (S.G.1.84) is run in rapidly and evenly from a 10 ml graduated pipette, at which the temperature rises immediately to well over 100°C. The tubes are cooled and the absorbance measured at 390 nm. The conditions are severe enough to hydrolyse soluble oligo- and polysaccharides (with the exception of polyuronides) more or less quantitatively,[397] but separate calibration curves must be constructed for each carbohydrate, as a rule.

Lipid materials, ferric ions, urea, commercial grade glycerol, and (to a small extent) methanol all interfere. Certain natural phenolic compounds artificially increase the sensitivity to an unpredictable degree.

G.M.2 Carbazole method for free or combined uronic acids [398]

Reagents; A. Sodium tetraborate decahydrate (analytical grade) 0.025 M in analytical grade conc. H_2SO_4.

B. Carbazole, 0.125% in absolute ethanol. Stable for 4 weeks in the dark.

1 ml of sample solution containing 0-50 μg of uronic acid or equivalent is carefully layered onto 5 ml of reagent A, pre-cooled to 4°C. The tubes are stoppered and shaken under the cold tap, first gently and then vigorously for 1 min, heated on a boiling water bath for 10 min, and cooled to room temperature. Reagent B (0.2 ml) is then added, and the tubes shaken and heated at 100° for a further 15 min. The absorbance is read at 530 nm after cooling.

Dust, some cations, and chlorinated tap water interfere. This method is less sensitive than most to the presence of neutral sugars, but hexoses do give a considerable absorbance and must be allowed for by suitable blanks or by calculated adjustments if the neutral sugar composition of the sample is known.

Most polyuronides are depolymerised under the reaction conditions, but separate calibration curves should be constructed where possible for different materials.

G.M.3 Roe method for ketohexoses[399]

Reagents; A: 0.15% w/v resorcinol in absolute ethanol
B: 7.5 mg/l ferric chloride in conc. HCl.

0-80 μg of fructose in 3 ml of water or alcohol is mixed with 4 ml of reagent A and 5 ml of reagent B. The tube is stoppered with a marble and heated at 77-79°C for 30 min. The absorbance is read at 460 nm after cooling. No serious interferences were encountered.

G.M.4 Somogyi-Nelson method for reducing sugars[400]

Reagents; A: 12g of potassium sodium tartrate and 24g of anhydrous sodium carbonate are dissolved in 250 ml of water. 4g of copper sulphate in a little water and 16g of sodium hydrogen carbonate are added with stirring. A solution of 180g of anhydrous sodium sulphate in 500 ml of water is boiled to expel air. The two solutions are combined, diluted to 1l, allowed to stand for a week, and filtered. The reagent is stable if kept warm.

B: 25g of ammonium molybdate is dissolved in 450 ml of water and 21 ml of conc. sulphuric acid added. To this is added a solution of 3g of disodium hydrogen arsenate in 25 ml of water. The reagent is kept at 37°C for 2 days and thereafter is stable for about 2 months in the dark.

0-30 µg of reducing monosaccharide in 1 ml of water is mixed with 1 ml of reagent A and heated in a boiling water bath for 20 min. The tubes are cooled and shaken very vigorously before and during the addition of 1 ml of reagent B. The solution is made up to 10 ml or 25 ml and the absorbance measured at 690 nm.

This method is not very reliable and a number of substances, particularly fats and phenols, interfere. Samples should be analysed in duplicate or triplicate with a comprehensive series of standards and blanks.

G.M.5 Leloir-Roe method for simultaneous determination of glucose, fructose and sucrose

Total fructose is estimated by the Roe method, which uses acid conditions and therefore hydrolyses sucrose. Reducing sugars are then destroyed by adding 0.6 ml of 1M NaOH, making up to 3 ml, and heating for 10 min at 100°C [401]: the remaining sucrose-derived fructose is determined by the Roe method. Interfering colours arise from the alkali degradation of the reducing sugars, and are allowed for by a blank in which the resorcinol reagent is replaced by ethanol. Reducing sugars are determined by the Somogyi-Nelson method, and the glucose, fructose, and sucrose levels can then be calculated as long as raffinose, fructosans etc. are absent. As a check, total sugars can be determined by the phenol-sulphuric acid method, calibrated with a 1:1 or other suitable mixture of glucose and fructose.

Separation of Carbohydrates

G.M.6 Paper chromatography of sugars

Partition chromatography on paper (PC) is a long-established technique that can still be very useful in certain circumstances. Its main virtue for the present work was that the separation characteristics of a particular sugar are the same whether the method is being used for qualitative, quantitative, or preparative work; hence it was easier to avoid ambiguity in identification during these non-routine studies. Using a series of slow-running solvents that were developed specifically for plant sugars the resolution and sensitivity are comparable with good GLC techniques, and superior to high-pressure liquid chromatography at its present state of development.[326] The running time is much longer than GLC, but the time required for preparation is not, for qualitative work. Quantitative analysis by PC gives satisfactory results but is a good deal more laborious than GLC.

Descending PC on Whatman no.1 paper was used exclusively here. Provided salts, lipids and polymeric material are removed from the sugar solution to be analysed and its concentration is such that the required amount of sugar can be applied in a single spot 4 mm in diameter (0.5 μl), resolution is limited by the following factors:

a) The amount of each sugar applied, which should be as small as possible to minimise spreading by diffusion - this necessitates a sensitive detection method.

b) The mobility of sugars in the solvent; resolution is increased by using a less polar solvent and running for longer periods. This not only gives a wider spread of R_f values (figure G.M.6.1), but also reduces the size of the spots since spreading by diffusion depends on mobility in the solvent chosen but only on the square root of the running time.

c) The ultimate resolution is limited by the unevenness of flow through the paper.

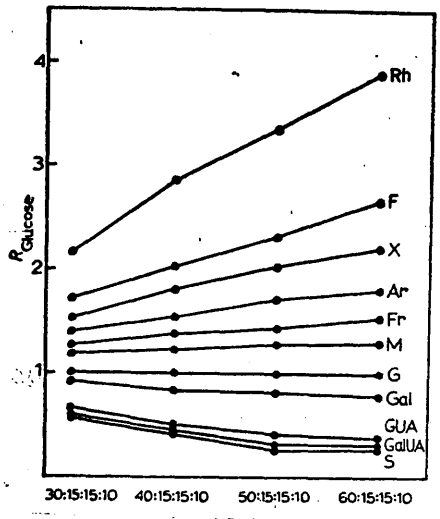


Figure G.M.6.1: Variation in $R_{glucose}$ with increasing proportions of ethyl acetate in the solvent system ethyl acetate-acetic acid-pyridine-water.

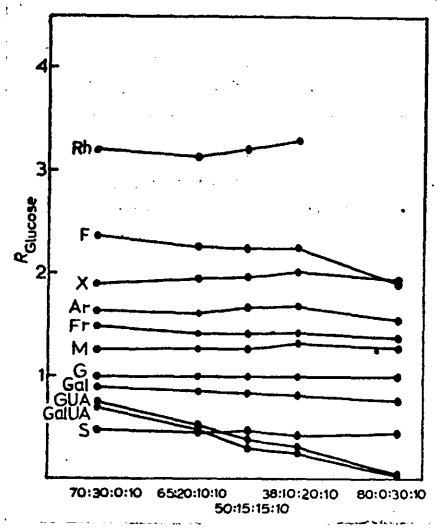


Figure G.M.6.2: Variation in $R_{glucose}$ with different proportions of acetic acid to pyridine. The proportion of ethyl acetate was adjusted to keep sucrose near $R_{glucose}$ = 0.45.

Abbreviations: Rh = Rhamnose; F = fucose; X = xylose; Ar = arabinose; Fr = fructose; M = mannose; G = glucose; Gal = galactose; GUA = glucuronic acid; GalUA = galacturonic acid; S = sucrose.

The mobilities of most sugars are affected, to a lesser extent, by the acidity or otherwise of the solvent (Figure G.M.6.2). Thus glucose and galactose are poorly separated in most acidic solvents; arabinose and fructose, and sometimes also xylose and fucose, in basic solvents. The solvent ethyl acetate-acetic acid-pyridine-water (50:12:18:1 (EtAcAcPy) is a suitable compromise, and can give a satisfactory separation of all the neutral sugars listed in 30 h, though if rhamnose is absent the resolution can be improved by running for 40 h.

If time is at a premium the proportion of ethyl acetate may be reduced with some loss of resolution. Longer running times, around three to four days, are required to separate uronic acids.

Before the development of EtAcAcPy the following two solvents were used in conjunction with one another for general work, samples being duplicated and run in each solvent in turn:

EtAcPy, ethyl acetate-pyridine-water (12:5:4)[402]

EtAcAcF, ethyl acetate-acetic acid-formic acid-water (18:3:1:4)[155] (all proportions are by volume).

These two solvents were also used for oligosaccharide separations, EtAcPy for neutral oligosaccharides as it does not cause hydrolysis and EtAcAcF for acidic oligosaccharides as it suppresses ionisation and thus gives reasonable mobilities.

Methods:

a) Qualitative. A sensitive modification of the alkaline silver detection reagents of Trevelyan et al.[403] is necessary because such small amounts ($< 1\mu g$) of each monosaccharide must be applied to the chromatogram for maximum resolution. The detection limit with these reagents is about 10-20 ng for glucose if the spot covers no more than 0.5 cm^2.

Reagents; A 0.3 ml of saturated silver nitrate solution is added to 50 ml acetone, and sufficient water added to redissolve the resulting precipitate.

B. Potassium hydroxide, dissolved in the minimum amount of water and made up to 1M with ethanol.

The chromatogram is dried thoroughly, and dipped through reagents A and B in turn, drying between. Dark brown spots on a light brown ground appear after 1-30 min. This reagent in theory depends on reducing power, but gives positive results with glycerol, inositol, sucrose, and other non-reducing substances.

Sensitivity: 10-20 ng for glucose if the spot covers no more than 0.5 cm^2.

b) Quantitative. The method of Wilson[104] was adapted for use with the aniline oxalate detection agent, which is easier to handle than aniline phthalate. 10-100 μg of each sugar are required, so that some loss of resolution must be accepted.

Reagent: 4.5 ml of redistilled aniline and 6.3g of oxalic acid (reagent grade) are dissolved in 100 ml of water, and 5g of trichloro-acetic acid added. The solution is made up to 1l with acetone.

The paper is dipped evenly through the reagent, dried, and heated for 4 min at 100°C. The spots are cut out, shredded, and leached for 2 hr with 0.7M HCl in 80% ethanol, with occasional shaking. The solutions are made up to 5 ml or 10 ml with leaching solution and the absorbance read at 360 nm (pentoses and uronic acids) or 390 nm (hexoses and deoxyhexoses). It is essential that standards are run on the same paper, and blanks should be measured in the same R_f region as each sugar. With attention to these details the reproducibility is $\pm$ 2-4% in the range 50-100 μg.

c) Preparative. Milligram quantities of sugar mixtures were applied as bands along the baseline, and the separated components were located by cutting 5 mm-wide strips from each side of the chromatogram and from the centre, and dipping in aniline oxalate, and were then leached from the paper with 30% aqueous ethanol. Hydrolysis of neutral oligosaccharides occurs with the solvent EtAcAcF or if the pH is allowed to fall below about 5 at any stage in the process. The technique is unsatisfactory with very small amounts of sugars (< 5μg) as detectable quantities of galactose, xylose, and sometimes glucose and mannose are released from control papers under these conditions. Whatman no.3 paper (or at least the rather old batch which was available) caused more serious artifacts than no.1 and was therefore not used.

G.M.7. Desalting of polysaccharide hydrolysates prior to chromatography

The presence of acids or salts interferes seriously with most of the chromatographic methods used for sugars, and a variety of methods were used for desalting.

a) Hydrolysis with H_2SO_4 and neutralisation with solid $Ba(OH)_2$ powdered just before use. The insoluble $BaSO_4$ is difficult to remove by centrifugation unless neutralisation is halted at pH 4-5.

b) Neutralisation to about pH 4 followed by adsorption of the sugars on activated charcoal ('Ultrasorb' grade) which is washed with water to remove inorganic salts.[405] Sugars are then eluted with 40% ethanol. This method has the advantage that phenolic and lipophilic contaminants are retained on the charcoal, but the presence of any ethanol or methanol in the hydrolysates (e.g. for wetting the polysaccharide prior to dissolution) must be carefully avoided. A small Büchner funnel containing a 10 mm x 5 mm thick pad of charcoal is quite effective. A modification of this method using the less active BDH activated charcoal was used for oligosaccharides (see Chapter V).

c) Neutralisation with an anion-exchange resin in the carbonate form. This is only satisfactory for relatively dilute acid hydrolysates, i.e. below about 0.2M. The resin must be washed well with water as it adsorbs sugars to some degree.[326]

d) Very small quantities of dilute acid hydrolysates were neutralised with pyridine and chromatographed directly on paper in a pyridine-containing solvent.

G.M.8 Electrophoresis of polysaccharides[155]

This was carried out on 35 x 10 cm strips of glass-fibre paper (Whatman GF-A) suspended between troughs of buffer solution into which carbon electrodes, incorporated in a perspex safety cover, were inserted when the apparatus was set up. The paper strips were cooled either by allowing them to dip into a trough of CCl_4 or by sandwiching them between a sheet of plate glass and a water-cooled tank with glass faces. The latter cooling system was used for high-voltage work (> 500V DC).

The buffers used were (a) pyridine acetate pH 6.5 containing 5% pyridine and 0.15% acetic acid by volume; (b) 0.05M sodium tetraborate, pH 9.2. In either case EDTA (0.005M) was added to prevent adsorption at the baseline. The papers were soaked in buffer from each end immediately after the polysaccharides had been applied and before the spots had dried out.

Polysaccharide components were visualised after separation with sulphonated α-naphthol (5g α-naphthol in 25 ml conc. H_2SO_4, made up to 500 ml with acetone). The glass-fibre paper was heated for 1 hr at 400° before use to remove small amounts of an organic contaminant which reacts with this detection agent.

G.M. 9. Molecular-sieve chromatography[322-3]

As stated in Chapter V only polysaccharide-based gels of the 'Sephadex' and 'Sepharose' series (Pharmacia Ltd., Uppsala) were used in this work. Good results with these materials are very much dependent on the standard of column packing. Since the retention of any component depends on the ratio of the accessible volume within the beads to the total volume of the column, the tightness with which the beads are packed, and hence the amount of liquid around them, is highly critical. Complete homogeneity of column packing is the aim, but especially with narrow columns it is difficult even to approach. Edge effects are the main problem: as would be expected on hydrodynamic grounds the beads sediment faster down the centre of the column and tumble out towards the edges when they reach the packed surface. Thus the packing at the edges is looser than at the centre, and a polymer large enough to be excluded from the gel moves faster at the edges.

These effects can be minimised by careful choice of column diameter, by silanating the walls of the column so that they are not wetted[328], and by avoiding any disturbance or turbulence when the slurry passes into the top of the column. Silanation was carried out by rinsing with a few ml of 10% HMDS (hexamethyldisilazane) in toluene, for 10 min. The preferred method of column packing was to use a longer column than necessary and pour in the gel beads as a slurry until they reached the top, rather than using a funnel which inevitably causes turbulence at its junction with the top of the column. The beads were allowed to settle under gravity, and only after this was the column tap opened very slightly and cautiously, permitting a slow flow while the beads settled a little further. With Sepharose gels in particular

the flow rate must not be allowed to exceed about 0.1 ml/min for a 1.5 cm-diameter column, as the beads distort very easily under pressure. All columns were checked before use with dyed dextran 2000 ($\bar{M}_w$ 2 x 10^6). This polysaccharide is not wholly excluded from Sepharose 4B but its larger components give a clear exclusion peak.

The dyeing of the polysaccharides prior to chromatography was carried out as described by Dudman and Bishop.[327] 50 mg of Procion Blue MX2B was added to 10 ml of polysaccharide solution (0.05 - 1%). After 5 min 2g of NaCl was added, followed by 100 mg of Na_2CO_3 after 30 min. The reaction mixture was left overnight and applied to a 3 cm x 30 cm column of Sephadex G-15. The dyed polysaccharide was eluted in the void volume with water and concentrated under reduced pressure if necessary.

Samples (up to 0.3 ml) were applied to the analytical columns as shallow layers weighted with NaCl. The eluting liquid was generally water, but 1M NaCl could be used with apparently identical results. The column effluent was monitored at 260 nm with a recording system designed for nucleotides (LKB 'Uvicord' model 4701 with additional purpose-built x 10 amplifier unit).

Analytical methods for lipids

The mixtures of polar lipids found in plants are in general a little too complex to be resolved by one-dimensional TLC, and some kind of preliminary separation is in general necessary. Magnesium silicate ('Florisil') has been used to separate phospholipids from glycolipids,[406] but the separation is by no means clear-cut and is not a satisfactory preliminary to TLC.[287] Nor is the separation that can be achieved on silicic acid columns suitable, since it is on the same basis as the TLC itself and what cannot be resolved in the one is unlikely to be resolved in the other.

A preliminary separation into acidic lipids on the one hand and neutral and zwitterionic on the other, using small columns of DEAE cellulose, fulfils the necessary criteria.[287] It also has the advantage

that some contaminating substances are held by adsorption under the conditions used, and as a result the subsequent TLC separations are cleaner. The methods to be described were developed from those used by Roughan and Batt for photosynthetic tissue[285,287], but their satisfactory overall resolution and freedom from degradative tendencies make them suitable for any other plant tissues containing polar lipids in appreciable quantities.

It should be noted that where the term 'phosphatidyl choline', for example, is used as though to describe a single chemical entity, it is recognised that a wide range of molecular species differing only in the combinations of fatty acids which they contain are actually involved. Under the experimental conditions chosen these were not resolved, and during TLC all the 'phosphatidyl choline' species moved as a single band.

G.M.10 Lipid extraction

Approximately 10g of leaf tissue or 10-20g of storage tissue was homogenised with 100 ml of chloroform-methanol (2:1 v/v) for 2 min. The slurry was filtered and washed on the filter paper with a further 100 ml of the same solvent in small portions, but without allowing the solid to dry out. Extraction of lipids was judged to be complete when the chlorophyll had been removed. The combined filtrates were mixed with 40 ml of 0.1M KCl and left overnight at $4^{\circ}C$ for the phases to separate. The organic layer was dried with anhydrous sodium sulphate, decanted, the hydrated sodium sulphate washed with chloroform, and the combined lipid solutions filtered and evaporated carefully to dryness at reduced pressure. The lipids were immediately redissolved in a known amount (5-15 ml) of chloroform-methanol (2:1 v/v).

G.M.11 Separation of lipids on DEAE cellulose

This was carried out in non-ionising solvents based on chloroform and methanol. As a result the capacity is at least an order of magnitude less than the nominal capacity in aqueous solvents

(0.7 milliequivalents/g) and zwitterionic lipids such as PC and PE behave as though they were neutral.

Preparation of the DEAE cellulose was carried out as described by Rouser et al.[407] Whatman DE-11 was washed successively with HCl, NaOH, methanol, chloroform, and glacial acetic acid, and finally washed with methanol until the pH of the washings on dilution with water reached 6. It was then stored as a slurry in methanol. 5 x 2 cm columns were packed and washed with 20 ml of methanol and 20 ml of chloroform, then allowed to settle again before application of 5 ml of lipid solution. Neutral and zwitterionic lipids were eluted with 50 ml of chloroform-methanol (2:1 v/v) and acidic lipids with 50 ml of the same solvent containing 7.5% NH_3 (S.G.0.88).

The lipid fractions were evaporated to dryness at reduced pressure and dissolved in a known volume of chloroform (0.5-2.5 ml). The acidic fraction contained ammonium acetate, which could be removed by partitioning with 2 ml each of chloroform, ethanol and water. The aqueous phase was washed with 1 ml of chloroform and the combined organic phases dried with anhydrous Na_2SO_4, evaporated, and redissolved in 0.5 ml of chloroform.

G.M.12 TLC separation of lipids on silicic acid

This method was used both for general purposes of qualitative monitoring and for quantitative determination of phospholipids and glycolipids by the methods of Rouser[408] and Roughan and Batt[287] respectively. All lipid components were checked for homogeneity by 2-dimensional TLC in the solvents CMAc and $CMNH_3$ (see below) and were identified by their chromatographic behaviour on DEAE-cellulose and silicic acid, by their reaction with 50% H_2SO_4, α-naphthol/H_2SO_4, the Liebermann-Burchard reagent and ninhydrin,[409] and by their carbohydrate and phosphate contents.

Glycolipid bands were scraped from the plate and hydrolysed with 0.5M H_2SO_4 for 1 hr at 100°C, without prior removal from the adsorbent.[287] The supernatant was neutralised with ion-exchange resin (AG1-X4 200-400 mesh, carbonate form, and a little AG50-X1, 200-400 mesh, hydrogen form) and evaporated to dryness at reduced pressure.

The sugars released were dissolved in saturated benzoic acid and identified by PC in EtAcPy and EtAcAcF. The sugar composition of a number of unidentified lipid fractions from bracken rhizomes was determined by Morton, and found, interestingly, to be comparatively complex.[260] However commercially available silicic acid was found to contain very substantial amounts of carbohydrate as measured by the phenol-sulphuric method. Typical figures were:

Kieselgel G(Merck A.G., Darmstadt), 30 $\mu g/g$;

Kieselgel H, 55 $\mu g/g$, Kieselgel HR, 9 $\mu g/g$.

When control samples were hydrolysed and the contaminating sugars examined by PC a considerable range of monosaccharides was found. A batch of Kieselgel G thought to have been used by Morton contained detectable quantities of galactose, glucose, mannose, arabinose and xylose, all of which appeared in Morton's polar lipid fractions.[260]

It therefore appears that artifacts can be produced if untreated commercial silicic acid is used for glycolipid separations.

It was therefore purified as follows:

Kieselgel HR was washed on a Büchner funnel with methanol, chloroform, and more methanol. It was then sieved (100 mesh) in methanol or acetone and dried at 110°C. Calcium sulphate (analytical grade) is sieved in acetone and dried at 110° for 1-2 days to reduce it to a fine powder. 34g of silicic acid and 6g of calcium sulphate were mixed with 80 ml of water and stirred vigorously for a few seconds until the mixture reached a suitable consistency. The plates (generally 20 cm x 5 cm, layer thickness 0.25 or 0.5 mm) were spread in the usual way. This procedure gave thin layers which contained negligible amounts of carbohydrate and phosphate, had a high capacity, and were robust enough to withstand normal handling and spraying.

Solvents

The solvents used (all proportions by volume) were:
HEAc; hexane-ether-acetic acid (90.10:1) Polar lipids remain at the origin in this solvent, which is used for separating triglycerides, free fatty acids, free sterols etc.

CMNH$_3$; chloroform-methanol-7M aqueous NH$_3$ (65:30:4). Used in qualitative 2-dimensional TLC as the second dimension after CMAc.
CMAc; chloroform-methanol-acetic acid-water (85:15:10:3) This was the solvent generally used for quantitative work, ca. 1 μmole of total polar lipid material being applied as a 4 cm-long band with a comb-type applicator. The plates were normally activated for 30 min at 120° and allowed to cool for 1 hr before use: when this was done a biphasic separation occurred due to uptake of water from the silicic acid by the solvent. This was an advantage since monogalactosyl diglyceride travelled close to the second solvent front and interfering neutral lipids and pigments with the first.

All solvents used were of analytical grade.

Determination of phospholipid and glycolipid components.

Lipid bands were located with iodine vapour, and scraped from the plate after removal of iodine. Phospholipids were determined in the digests by the sensitive phosphomolybdate method of Rouser et al.[408] The silicic acid with phospholipid components adsorbed was refluxed for about 1 hr with 1 ml of 72% perchloric acid (analytical grade) in 2.5 cm-diameter tubes on a micro-kjeldahl rack with rheostat control. If there was any doubt about the completeness of digestion a further 1 ml of perchloric was added, as residual organic matter can produce artificially high results. The tubes were then cooled and the contents transferred to 10 ml centrifuge tubes, rinsing with 4 ml of water. 1 ml of 2.5% aqueous ammonium molybdate and 1 ml of 10% aqueous ascorbic acid were added and the tubes heated for 5 min on a boiling-water bath with occasional shaking. The volume was adjusted to 7.5 ml with water (normally 1 ml), the tubes centrifuged briefly to bring down the silicic acid, and the absorbance of the supernatant measured at 700 nm. Phosphate determinations which use ascorbic acid as the reducing agent are normally sensitive to interference from dissolved silica,[410] but since the silicic acid is thoroughly dehydrated during the digestion of the organic matter this is not a problem here.

The adapted phenol-sulphuric method of Roughan and Batt[287] (cf GM1) was used for the glycolipids. The lipid bands were scraped from the plate into a 10 ml centrifuge tube and 1 ml of 5% aqueous phenol added. 4 ml of concentrated sulphuric acid (analytical grade) was run in quickly with constant shaking the tubes centrifuged, and the absorbance of the supernatant measured at 490 nm. In this case the other degradation products of the glycolipids, which would normally interfere, remain attached to the silicic acid. The separate determination of MGG described by Roughan and Batt was found to be unnecessary when a biphasic separation was achieved.

These analytical methods were calibrated with appropriate quantities of phosphate, glucose and galactose in the presence of silicic acid. Sulphoquinovose conveniently gives the same asorption, weight for weight, as galactose under the conditions used.[287] Slight variations in the phenol-sulphuric blank were sometimes found in different parts of the plate, and two or three blank determinations were normally carried through for each plate.

A note on experimental work with bracken rhizomes

In the Introduction (p.29) it was mentioned that the fundamental differences between bracken plants grown in the greenhouse and mature communities on the open hill-differences in carbohydrate transport, leaf structure, and reaction to herbicide treatment - make it very difficult to apply the results of experiments with greenhouse-grown bracken to the field situation. Nevertheless much experimental work has been done on the 'pot' scale, often because of the extreme difficulty of obtaining suitably uniform material in the field.[243]

It might be expected that bracken growing in a restricted area, and probably drawn entirely from a single clone, would have the necessary uniformity. In fact this is by no means the case. The reason became apparent when bracken at Carbeth (site 6) was being examined in connection with spraying trials. Four 0.5 m x 0.5 m soil pits were dug within a very limited area. Data relating to the frond buds developed during March are shown in Table 8.3. (The effect of herbicide treatment is irrelevant as the buds were initiated before spraying).

Pit:	a	b	c	d
% of rhizome segments bearing frond buds.	63	57	68	50
No. of frond buds/kg.	9.5	7.5	8	11.5
Average number of nodes in frond-bearing rhizome.	1.8	3.9	4.0	1.3

Table 8.3

Data on frond buds from 4 pits in a single bracken stand (Carbeth, March 1974).

The rhizomes from pits (a) and (d) have more frond buds on younger frond-bearing rhizomes than in pits (b) and (c), and appear to belong predominantly to an earlier phase in the cycle of change described by Watt.[193,210] This is in keeping with Anderson's conclusion that in established bracken communities (corresponding to the 'hinterland' areas of Watt[193]) the pattern of the phases is jumbled and on a small scale.[411] Criss-crossing arcs of a few metres radius can be discerned in the pattern of the fronds, and it is not unlikely that the phases are mingled even within the tangle of rhizomes from a single pit. It is only to be expected that the composition of the rhizomes, (particularly their starch content) will be affected by the phase to which they belong within the community: hence the lack of reproducibility in analysis.

The large changes in rhizome starch content which take place during the growing season are another problem. The resulting variations in dry matter content make it difficult to find a realistic basis on which to express the analytical results. Percentage dry matter has been used uniformly in this thesis to facilitate comparison, but some German workers expressed all other quantities in terms of the percentage of crude fibre, which should be relatively constant through the year (i.e. not affected by metabolic activity etc.),[248] and Williams and Foley[261-2] expressed their carbohydrate results on a square metre basis, which has obvious advantages when quantitative measurements are extended

to the fronds in addition to the rhizomes. Both of these approaches make interpretation of the results simpler, but by introducing an additional factor (percent crude fibre or rhizome weight/m^2), they cause a further increase in experimental error.

APPENDIX I

LIST OF BRACKEN SITES

The sites listed below are those at which material was collected for experimental purposes, or at which the bracken-rhizomes as well as fronds - was examined in some detail for one purpose or another. Naturally the behaviour of the plant has been observed at numerous other sites in the West and South of Scotland, together with some elsewhere, but those described here are a fairly representative selection.

Site 1: Drumclog Muir, Stirlingshire. Grid ref. NS 552757, altitude 75 m O.D. Open: aspect W.

This area of about 3 ha of class II and class I bracken has been used for experiments for over sixty years,[412-3,223] but still seems quite healthy except in the vicinity of paths, where trampling by the large numbers of local people who use the Muir for recreational purposes keeps the bracken very effectively suppressed. Various other stands in the vicinity have been used by different workers.

Site 2: Drumclog Wood, Stirlingshire. Grid ref. NS 546766, altitude 110m O.D. Woodland, but with some cleared areas up to 5 ha in extent which carry excellent examples of class III bracken stands. The comparison between this site and Site 1, which lies less than 1 km to the S., is most instructive. In the wood the fronds emerge approximately a month earlier than on the moor, and at the time of writing (late October) they are only just turning brown, whereas at the previous site they reached the same stage six weeks ago. Perhaps for this reason, the amount of storage rhizome appears rather small considering the height and density of the fronds (over 2m and 20/m^2

in places, though much sparser in moderate shade). Differences in frond posture and structure such as have been described by Tansley[182] and Boodle[246] are also very apparent.

Site 3: Auchentorlie, Dunbartonshire. Grid ref. NS 447739, altitude 65m O.D. Woodland: situated on a bank of loose gravel overlooking the N carriageway of the A82. With its southerly aspect this site regularly has the earliest frond emergence date of any in the Glasgow area, which influenced its selection for the experiments in the first part of Chapter III. The rhizomes are unusually easy to extract without damage from the gravelly soil.

Site 4: Conic Hill, Stirlingshire. Grid ref. NS 423928, altitude 155m O.D. Open, aspect W. The tongues of high ground that project westwards from the col N of Conic Hill, divided by small, steep-sided valleys, present an interesting picture of the competition between bracken and heather. Heather, on peaty gleys, covers the flat tops: the S-facing sides are covered by class I bracken and the slopes facing N have both bracken and heather in a sharply-defined mosaic, with the heather areas predominating. A bracken-heather boundary on a slight W-facing slope at the tip of one of the spurs was selected for the investigations to be described in Appendix II.

Site 5: Sundaywell, Dumfriesshire. Grid ref. NX 786846, altitude 220m O.D. This area of mixed Class I and Class II bracken was sprayed by helicopter with 4.5 kg/ha of asulam in 197?, under the auspices of the West of Scotland Agricultural College. It has been kept under observation since then without receiving any overall treatment, but limited areas have been fertilised and reseeded by various means.

Site 6: Carbeth, Stirlingshire. Grid ref. NS 527798, altitude 160m O.D. Open, aspect W. Class II bracken amongst which a small number of Scots Pine seedings have been planted: these are making little progress as they are not yet as tall as the bracken. A small field experiment was conducted here in 1973.

Six 15 ft x 15 ft (4.5m x 4.5m) plots were sprayed with 4.5 kg/ha of asulam at a volume rate of 1151/ha, with the addition of two kinds of surfactant. The object was to investigate the control obtainable by late-season spraying and to determine whether or not this could be improved by the addition of wetting agents to hasten penetration. It was thought at the time that low-volume spraying with no surfactant might cause some scorching of the fronds, but in fact other workers have found that this is not a problem after the beginning of July.[395]

	March 1974 % of frond buds stunted, 3cm	August 1974 % reduction in frond numbers
Plot 1 (no surfactant)	65%	81%
Plot 2 (0.1% SDS)	67%	79%
Plot 3 (0.1% Tween-20)	58%	80%
Plot 4 (no surfactant)	not determined	80%
Plot 5 (control)	0%	0%
Plot 6 (0.1% Tween-20)	not determined	87%

Table A1.1

Results of field experiment: 4.5 kg/ha Asulam applied September 1973, assessment March and August 1974.

The short-term (1 year) control achieved was thus not as good as is possible with earlier treatment, and is not consistently improved by the addition of a wetting agent. When these results are compared with those of late-season applications elsewhere it is the variability of control that is most evident.

Site 8: Glen Fruin, Dunbartonshire. Grid ref. NS 280903; altitude 150-330m O.D. Open; aspect SW. The growth of bracken (Class I) on the N side of this U-shaped glen, on a partial hydrologic series from freely or imperfectly drained brown forest soils at the top to peaty gleys at the bottom, demonstrates with unusual clarity the effect of altitude and natural drainage on the plant. The site is too extensive and complex to describe in full here; it will suffice to say that in the lowest part of the area, where drainage is obviously the main factor controlling all the vegetation, the boundaries between bracken stands and the surrounding mire communities dominated by Molinia and Juncus acutiflorus/J. articulatus (cf Ratcliffe pp 435 & 437)[414] are very clear-cut yet not obviously related to topography or to the degree of gleying in the underlying mineral soil. The range of grass species found under these bracken stands quite similar to those of the drier areas higher up on the slope, and in striking contrast to the mire vegetation 2-3m away outside the bracken.

Site 9: Loch Arail, Argyll. Grid ref. NR 805796, altitude 225m O.D. Open, aspect S.E.. This predominantly heather-covered hillside has a number of small, flushed areas carrying Class I bracken. The contrast between the soils under the bracken and the heather is similar to that at Conic Hill, and if anything more marked. However here the base status of the soil is clearly influencing the vegetation. It might in general be preferable to think of the soil and the vegetation upon it as a single developing entity, with the balance of soil-plant and plant-soil interactions varying from one site to another.

Site 10: Monksfoot, Lanarkshire. Grid ref. NS 780292, altitude 270m O.D. Open aspect S. Class I bracken. Another West of Scotland Agricultural College site. Trials with glyphosate and picloram are in progress here as well as with other herbicides.

Site 11: Erraid, Argyll. Grid ref. NM 289196, altitude 20m O.D. The granite rocks of this stormswept island carry no true

soil; they are either bare or covered directly with peat. A few sheltered gullies are filled with dense bracken with its rhizomes in a light brown, relatively undecomposed and loose upper layer of the peat. The ground layer of vegetation under the fronds is surprisingly rich, including not only luxuriant Agrostis spp., Festuca ovina, Anthoxanthum odoratum and a variety of herbs but also basiphilous species including clover. The contrast with the austere vegetation on the surrounding peat is remarkable.

Site 12: Bun-an-Loch, Argyll. Grid Ref. NR 707773
altitude 20m O.D. Class I-Class II bracken. The proprietor treated two areas at this site with asulam in 1972, the first in June and the second in August. In both cases the application rate was very heavy, at least 12kg/ha in about 400 l/ha volume. Reasonable control was achieved with the June as well as the August application. An adjacent area has been cut repeatedly, and it will be interesting to follow the long-term control with these three treatments.

APPENDIX II

SOIL-FORMING PROCESSES UNDER BRACKEN AND HEATHER

Introduction

It has long been recognised that bracken (Pteridium aquilinum) is most vigorous and widespread on the leached brown earths, with mull or moder humus, which underlie the better upland grazings of the British Isles, and is less common on podzolic or gleyed soils, with mor or peaty surface layers, characteristically found under heather (Calluna vulgaris). It has recently been suggested, however, that the bracken plant itself is able to influence the soil in which it is growing.[181] Because of their contrasting growth forms and community structures, the boundary between stands of these two species if frequently well-defined, with a relatively narrow transition zone.[415] The change in vegetation across such a zone is often accompanied by a change in soil morphology, from a brown earth or soil of similar appearance under the bracken to an obviously podzolised soil under the heather.[181, 416] In many cases the boundary is stable, its position determined by drainage, aspect, or clear differences in the soil parent material; but on sites which are entirely uniform in these respects the dynamic structures of the competing bracken and heather communities seem to be the determining factor.[415]

It has not been established, however, whether the podzolisation process under the bracken (when defined as downward redistribution of iron, aluminium, and manganese) is retarded or reversed, or whether it is merely hidden by a more uniform distribution of organic matter down the soil profile. To answer this question a suitable site was chosen for detailed study of the distribution of sesquioxides in the soils across a bracken-heather transition zone.

METHODS

Site

The area chosen (Site 4) lies on the top of a spur running westward from one shoulder of Conic Hill, Stirlingshire. The site has a westerly slope of 5^{o}, and in a N-S direction is predominantly level with slight undulations. The parent material is a homogeneous red till of sandy loam texture, not stony, derived from local Upper Old Red Sandstone rocks. Drainage is generally free, and despite weak induration of the lower B and C horizons there are no morphological signs of gleying.

A N-S transect was drawn across the boundary between stands of heather and bracken occupying the N and S halves, respectively, of the top of the spur. Four soil pits (0.5 m^2) were dug at 4 m intervals along this transect (profiles 2-5). For comparison two further pits, profiles 1 and 6, were dug 20 m from the ends of the transect in the midst of the heather and bracken communities respectively.

Soil Profiles

Since it was apparent that the variation in both the soils and the vegetation, across the transition zone, was more or less continuous, it was considered preferable to follow changes in individual soil properties along the length of the transect rather than to adopt a classificatory approach to the description of the soils.

For comparison with other work, however, all the profiles would probably be considered as iron podzols in the wide sense used by the Scottish Soil Survey,[417] but profiles 5 and 6 resemble brown podzolic soils in other classifications,[418-9] while profiles 1-3 are almost typical iron podzols but have some of the features of iron-pan soils (peaty podzols with thin iron pan;[417] stagnopodzols[418]) developed to a minor degree.

Nomenclature of soil horizons follows the system of the Scottish Soil Survey.[417]

Progressing from the heather end of the transect (profile ?) towards the bracken end (profile 5), the most important visible change is that the A_2 and B_2 horizons of profiles 1-3 merge to become the A/B horizon of profile 4 and the B_2 of profile 5. In the field these last two horizons appeared fairly homogeneous, but in case their composition did not reflect their appearance they were divided into two for analysis, above and below an arbitrary line 7 cm from the top of each horizon.

The A_2 horizons of profiles 1-3 contained dark, vertical streaks which merged in places at both top and bottom of the horizon to form fragmentary, soft layers up to 2 cm in thickness. A layer of this nature above the A_2 is unusual, but has been described elsewhere.[420] A sample large enough for analysis was taken from profile 3 and proved (vide infra) to be similar in composition to a normal thin iron pan.[421] Such fragmentary areas of iron deposition were more frequent at the bottom of the A_2 horizon, though certainly never continuous and impermeable as in typical iron-pan soils such as those to be found on steep, N-facing slopes adjoining the site, similarly heather-covered but with considerably thicker F layers.

Vegetation

For vegetation sampling two 2 m x 2 m squares were laid out adjoining each soil pit to E and W. Thus straddling each point on the transect there was one pair of sampling squares and one soil pit. Percentage cover of Calluna vulgaris was determined using a grid of 100 points, spaced at 20 cm, within each 2 m square. The squares were each sub-divided into four 1 m^2 areas for analysis of parameters relating to Pteridium aquilinum.

A general description of the vegetation at this site has been prepared in the course of another investigation.[422]

Soil Analysis

Soil samples were sieved within 2 days of collection and the <2 mm fraction air-dried at 30°C. Moisture content was determined

by oven-drying at 110°C. All results are expressed on an oven-dry weight basis. Loss on ignition was determined after 3 hr at 450°C. Both air-dry and ignited soils were lightly crushed and rubbed onto strips of double-sided adhesive tape attached to stiff card, and scale facsimiles of each profile reconstructed in this way.[423]

Sesquioxides were extracted with the following three reagents:

(a) 0.1 M pyrophosphate,[424] pH 10. 1 g of air-dry soil was shaken for 18 hr with 80 ml of pyrophosphate solution.

(b) Acid oxalate,[425] pH 3.0. 1 g of soil was shaken for 3 hr in the dark with 50 ml of acid oxalate solution.

(c) Dithionite-citrate-bicarbonate;[426] one 30 min extraction.

Aluminium, iron, and manganese in the centrifuged and filtered extracts were determined by atomic absorption, using a nitrous oxide-acetylene flame for the aluminium. For both the aluminium and the lower manganese values, the precision was improved by integrating the absorbance readings over a 10 sec interval. A number of samples were extracted in quadruplicate, and from these it was calculated that relative errors amounted to ± 4% for the pyrophosphate and oxalate extracts and ± 8% for the dithionite. The largest part of the error comes from the extraction process and the inhomogeneity of the soil, rather than the analysis of the extracts. Very small values are subject to larger relative errors: the minimum standard error is ± 3 ppm for manganese, ± 0.01% for both iron and aluminium in the pyrophosphate and oxalate extracts; and ± 8 ppm and ± 0.02% respectively in the dithionite.

It was originally considered that pyrophosphate extracted those hydrous, amorphous oxides of iron in intimate association with organic matter (Fe_p), while oxalate extracted all non-crystalline iron oxides (Fe_{Ox}), and dithionite, all amorphous or crystalline iron oxides not incorporated in silicate lattices ($Fe)_D$.[424,427] It now seems unlikely that the distinctions are as clear-cut as this; for example both oxalate and dithionite attack silicate minerals to some degree.[421] Nevertheless there is little doubt that pyrophosphate, oxalate, and dithionite extract iron oxides of increasing degrees of dehydration and crystallinity, the crystallisation processes being retarded by the presence of organic matter.[428]

The differential extraction of aluminium and manganese with these reagents is not so well established. The relative amounts of aluminium extracted in the present experiment (Al_p, Al_{Ox}, and Al_D), are quite close to one another and highly correlated, and so are the oxalate and dithionite-soluble manganese (Mn_{Ox} and Mn_D), as has been found elsewhere.[429-30] Manganese is not usually determined in pyrophosphate extracts, no doubt because the amounts present are small: the Mn_p values reported here are therefore of wider interest.

The low proportion of extractable aluminium to iron is an unusual feature of all these soils. Aluminium may have been leached out of the profile, but the highly ferruginous parent material is more probably responsible.

RESULTS AND DISCUSSION

Soils

The vertical distribution of the organic matter, which to some degree affects the way in which these soils would be classified in the field, can be assessed from Figure A1 (loss on ignition). The loss on ignition is a satisfactory approximation to the organic matter content in acid, highly organic soils of this type, though over-estimating it somewhat in the lower horizons. From profile 1 to profile 6 the loss on ignition in the H horizon falls from 67.1 to 26.7 per cent, while in the A_1 and lower horizons it rises substantially. These changes are in accord with the appearance of the soils in the field.

The reconstruction of 'profiles' from the ignited horizon samples[423] showed that this more uniform vertical distribution masked a considerable depletion of iron in the H and A, and accumulation in the B horizons, of profiles 4-6. However, there was clearly more iron in the upper horizons of these soils than in those of profiles 1-3. The A_2 horizons of profiles 1-3, and the A horizons of profiles 5 and 6, had a speckled appearance after ignition, the iron being unevenly distributed on the soil particles. We have observed this feature elsewhere in the $A_{2(g)}$ horizons of some iron-pan soils.

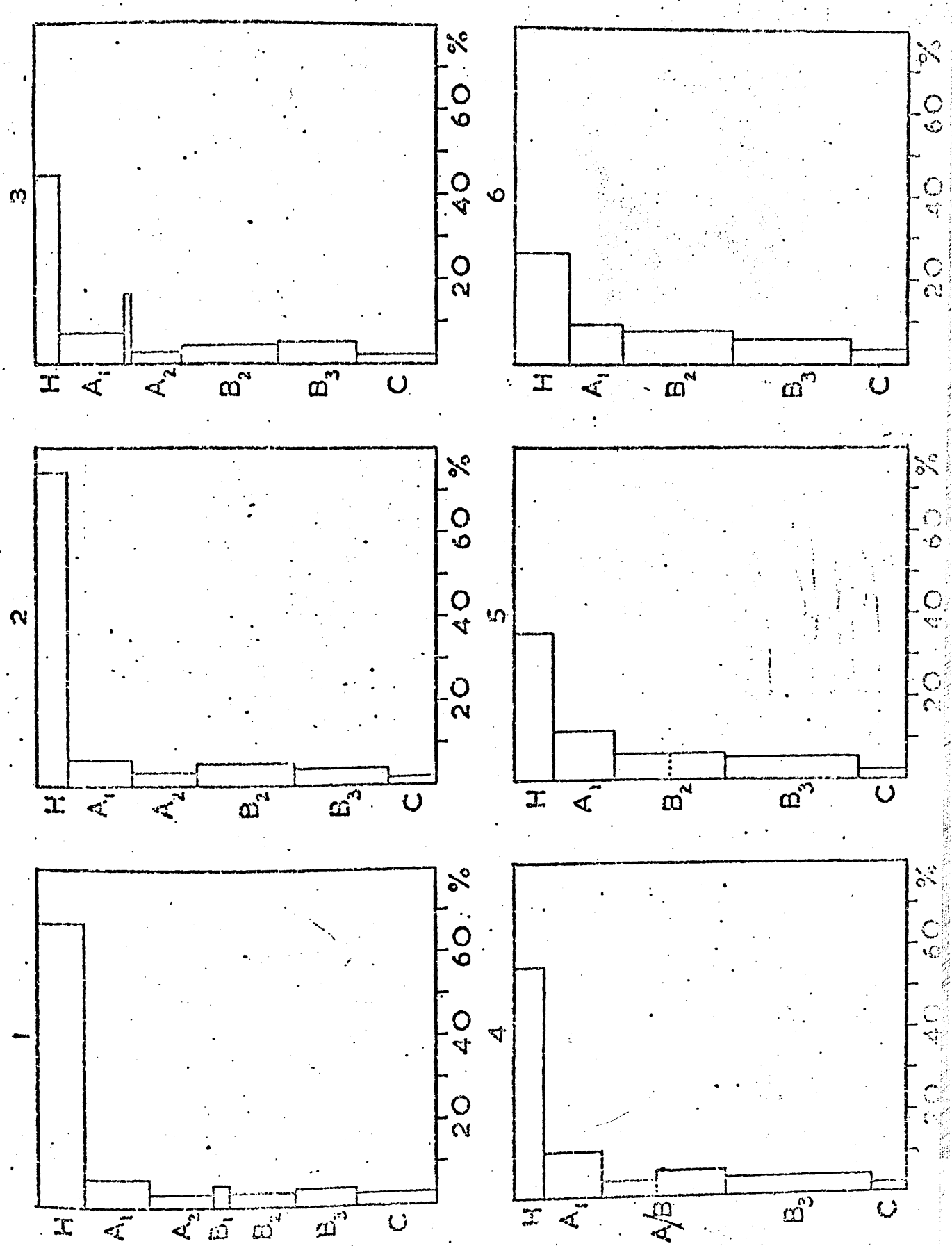


Figure A1 - Loss on Ignition.

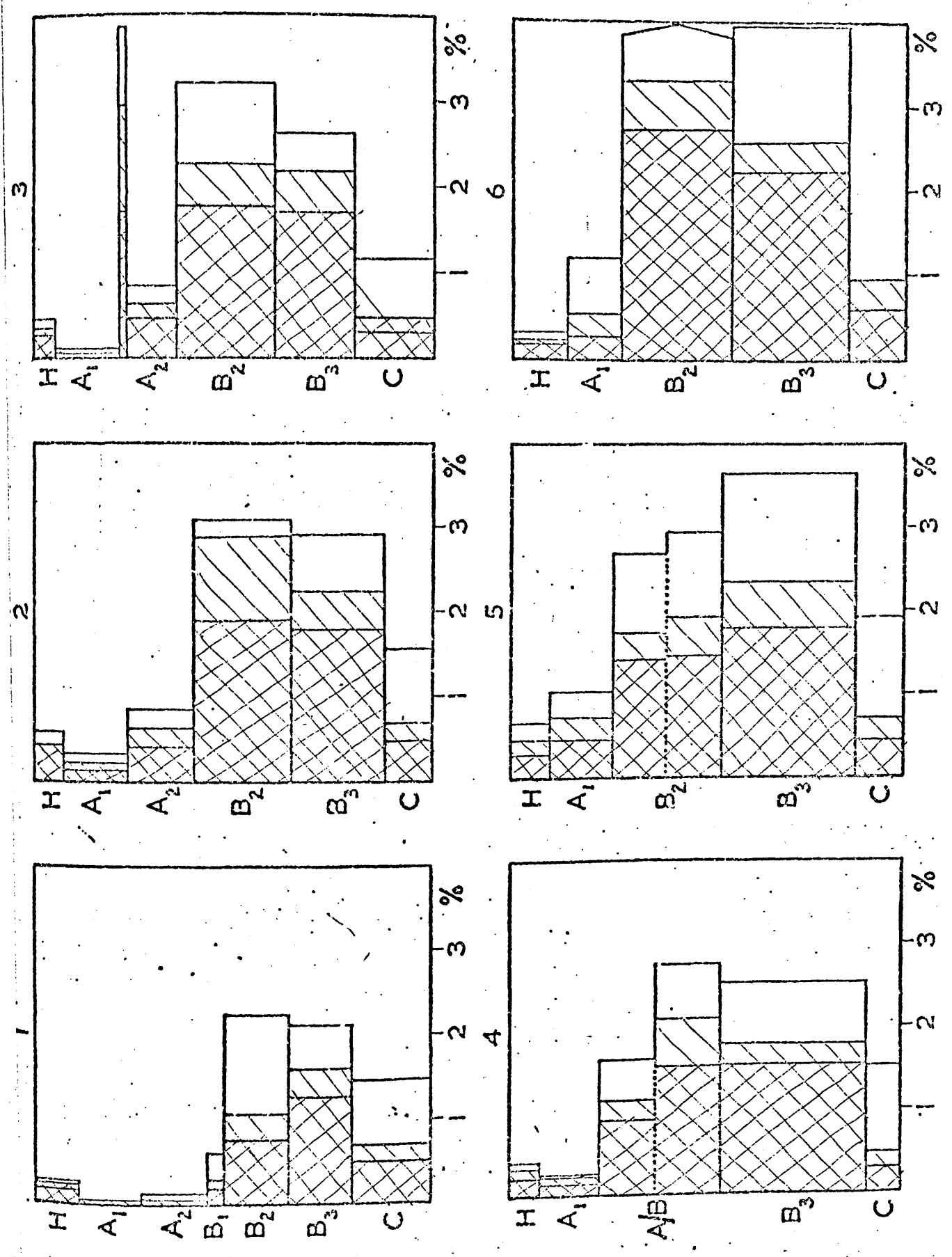


Figure A2 - Iron

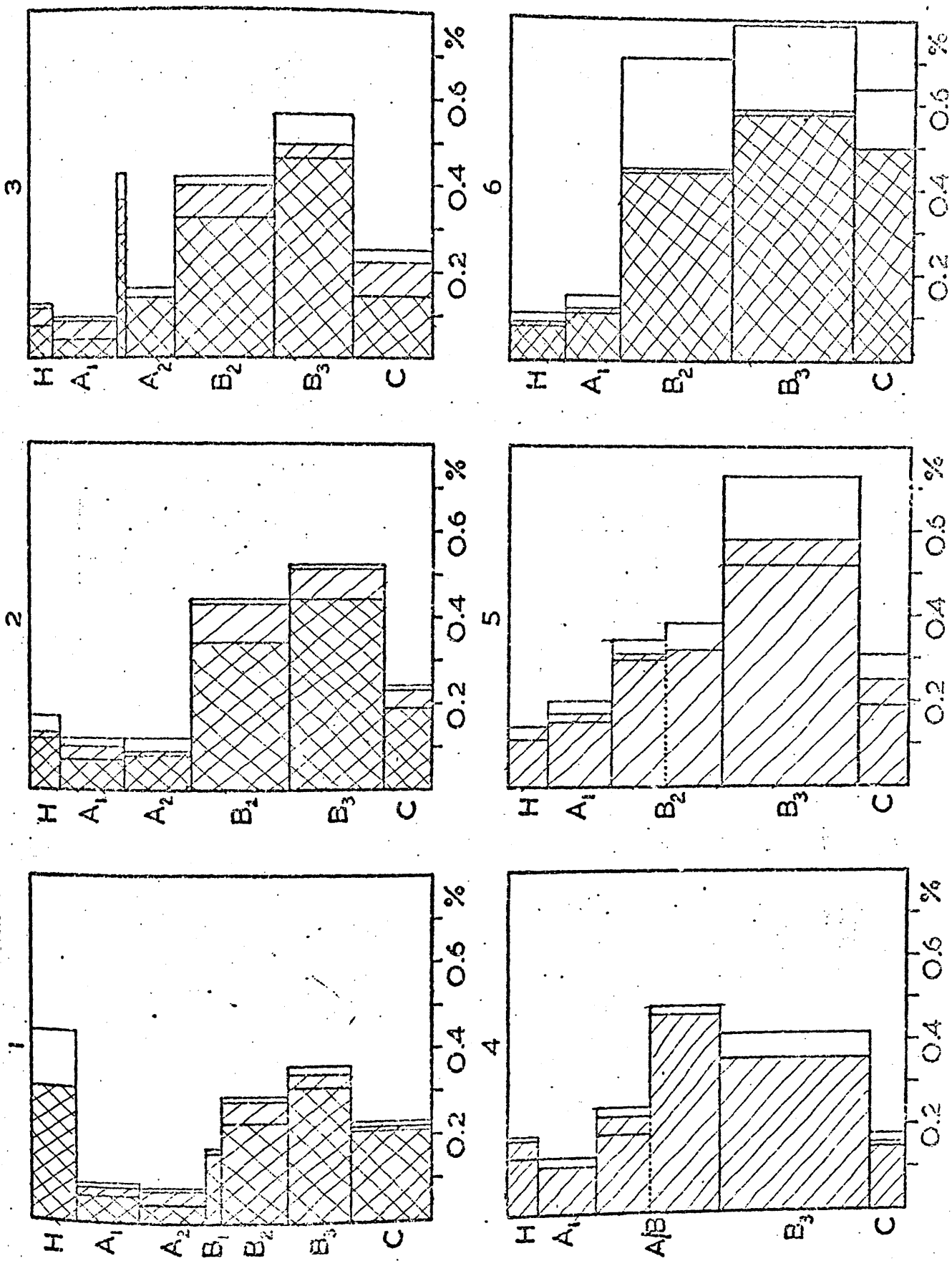


Figure A3 - Aluminium.

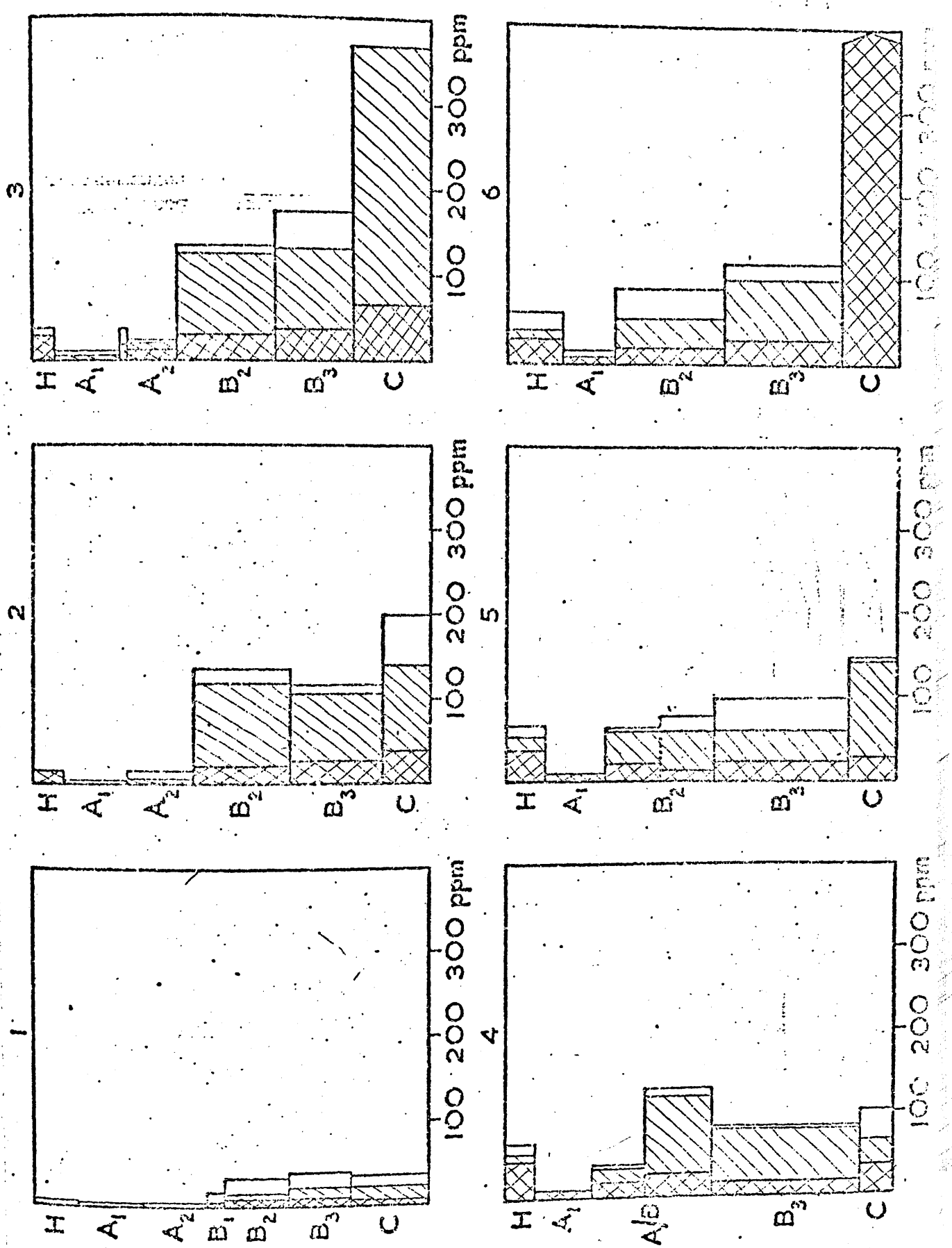


Figure A4 - Manganese.

The extractable iron, aluminium, and manganese results are shown in Figures A 2-4. It is considered that the most suitable criteria for the degree of mobilisation and vertical movement of sesquioxides in the soils included in the transect are the amounts of iron and aluminium extractable from the A horizons, and the 'activity ratio' Fe_{Ox}/Fe_D which is diagnostically high in the B horizons of podzolised soils[429] (also in strongly gleyed horizons).[430] Profiles 1 and 6 will be included in the following discussion only for qualitative comparison, since the essential constancy of parent material and drainage status can be relied upon much more safely within the limited area of the transect itself.

The amount of extractable iron in the A_1 horizons increases progressively from profile 2 to profile 5. The same trend is evident in the horizons immediately below, viz. the A_2 horizons of profiles 2 and 3, the upper A/B of profile 4, and the upper B_2 of profile 5 (the pan shown in profile 3 may be ignored here as it is very fragmentary). The differences in Fe_p, Fe_{Ox} and Fe_D between the corresponding horizons in profile 2 and profile 5 are highly significant ($P < 0.01$), profiles 3 and 4 being intermediate. Aluminium shows the same pattern, the differences being smaller but still significant at $P < 0.05$ for all three reagents. Profile 6, like profile 5, has high iron and aluminium levels in the A_1 and B_2 horizons.

The Fe_{Ox}/Fe_D ratio in the B horizons tends to increase towards the heather end of the transect; in the lower B_2 and B_3 of profile 5 it is 0.65 and 0.64 respectively, significantly lower ($P < 0.05$) than in the corresponding horizons of profiles 2 and 3 (0.76 - 0.94). The ratio is low also in the B horizons of profile 6, while profile 4 is intermediate.

It is thus confirmed that leaching of sesquioxides, as expressed in the vertical distribution of iron and aluminium in the profile and the 'activity ratio' for iron,[429] is distinctly more advanced under the heather than under the bracken.

To some extent the distribution of extractable manganese shows the same trends as iron and aluminium, with increasingly high levels in the upper A/B of profile 4 and B_2 of profile 5. There are certain anomalous features, however, particularly in profiles 1 and 6. The extractable manganese in the C horizons fluctuates over a wide range, apparently due both to translocation and to small variations in the general freedom of drainage:[429,431] the micro-relief around profile 1 was slightly convex, and that around profile 6, slightly concave (this was not characteristic of the heather and bracken stands as a whole). High extractable manganese levels are apparently a very sensitive indicator of temporary waterlogging in soils of this type, appearing before any visible signs of gleying. (In strongly gleyed soils the manganese becomes not merely easily extracted but actually mobile, and there is a net loss of Mn_D from the waterlogged horizons).[430] Variations in micro-relief rather than parent material may be responsible for the larger total amount of sesquioxides throughout profile 6 than in profile 1, if translocation takes place laterally as well as vertically.

The Mn_P values are generally low, the ratio Mn_P/Mn_D averaging 0.2 in the lower B and C horizons. In the H horizons this ratio is significantly greater ($P < 0.01$) increasing from 0.5 in profile 6 to 1.0 in profile 2, in parallel with the loss on ignition. A connection with organic matter seems possible, as with Fe_P.[424] A considerable proportion of the manganese in podzolic soils is known to be involved in the organic cycle.[429,432] The amounts of manganese in the above ground sections of heather[433] and bracken[272] ecosystems are of the same order of magnitude, as far as is known, but of course there is an annual turnover of bracken fronds and a slower turnover of heather shoots.

It will be noted that although the upper and lower parts of the B_2 horizon of profile 5 are not significantly different in their content of extractable iron, manganese and aluminium, there are substantial differences between the upper andlower parts of the A/B horizon in profile 4. The common, tacit assumption that chemical soil properties are constant within a horizon and change only at its visible boundaries, does not seem to hold in this case.

Vegetation

Until this point the variations in the soil along the transect have been considered only in their spatial context. If the heather/bracken boundary is moving, however, the associated changes in the underlying soil must also be changes occurring in time at a given place.

The cycle of change that occurs when bracken is in competition with heather, in the Breckland of East Anglia, has been very fully described.[415] Similar phenomena can often be seen in the altogether different conditions of the Scottish uplands, with the exception that in exposed areas such as the one being examined here, much of the litter is removed by wind during the winter and it is frequently not possible to discern anything corresponding to the 'mature' phase in the Breckland cycle, where the decaying fronds form a continuous, insulating carpet and the grass layer is completely suppressed.[210] In places where the bracken is advancing, therefore, the 'building' phase often gives way directly to a hinterland region in which the phases are mingled, with a relatively small scale of 'pattern'.[411]

If profile 4 is regarded as being within the 'pioneer' and profile 5 within the 'building' phase, then the data presented in Table 1 are almost entirely in accord with the published description of an advancing bracken front[210,415] and are supported by the fact that the main rhizomes are predominantly oriented along the line of the transect, in the vicinity of profile 4 especially. The differences between the two phases in lamina/petiole ratio, percentage of dead rhizome, and number of nodes on the frond-bearing rhizome before the current year's frond, are particularly diagnostic (the number of nodes being an indication of the age of the rhizome branch, though not necessarily equal to its age in years).[192] There is no significant difference in the depth of origin of the fronds, since the rhizome system at this site is shallow (10-15 cm) and the frond-bearing rhizomes have no clear tendency to grow upwards.

It may be concluded that the bracken is encroaching on the heather, and that its presence brings about not only a progressive change in humus type and distribution, but also some redistribution of sesquioxides in the soil. The rate of advance is not known, but is likely to be considerably less than the figure of 1 m/year accepted as

normal where conditions are ideal for bracken:[185,188-90] the 4 m distance between points on the transect more probably represents some decades of irregular advance.[191]

The reversal of podzolisation by some types of forest vegetation has been suggested in the past,[434-6] although previous studies were concerned mainly with the interconversion of mor, moder, and mull humus. It is not clear how changes in the pattern of sesquioxide distribution might come about, particularly in soils too acid for earthworm activity. A chemical mechanism for reversal would not be easily reconciled with current theories on podzolisation.[436-9] When considering the present results in this context, it should be borne in mind that the soil profiles at this site have certain features in common with iron-pan soils, the genesis of which is incompletely understood but certainly different from that of conventional podzols.

Adjacent to profile:	2	3	4	5	
Calluna vulgaris, percentage cover	76	78	40	0	
Pteridium aquilinum, fronds/m^2	0	0	12.6	23.7	differences significant ($P < 0.01$)
average frond height, cm	-	-	64.9	78.6	
average petiole length, cm	-	-	22.8	34.9	
average lamina/petiole ratio	-	-	1.85	1.22	
average number of nodes between frond base and main rhizome	-	-	2.5	4.2	$P < 0.05$
percentage dead rhizome, by length	-	-	10	35	
depth of origin of fronds, cm	-	-	8.0	8.7	not significant

Table 1

Vegetation Data

(Submitted to 'Plant and Soil')

APPENDIX III

THE COLONISATION OF ISLANDS BY BRACKEN.

Introduction

Bracken spreads in two ways: by the germination of wind-borne spores and subsequent establishment of new colonies via the gametophyte stage of the plant's life-cycle; and by vegetative extension of the complex underground rhizome system. Despite earlier suggestions to the contrary,[188-9] it is now considered that vegetative spreading is predominant in Britain. Until numerous bracken colonies were seen springing up in the ruins of bombed cities during the 1939-45 war,[187,441] Braid and Conway found newly-established colonies on only six occasions in the course of their long and intensive investigations on bracken in Scotland.

Establishment from spores is therefore at least moderately rare; it is limited by the gametophyte's sensitivity to desiccation, soil acidity, frost and attack by insects and fungi. The young sporophyte, too, is more sensitive to frost than the adult plant.[187] In Southern England the spores are shed in July, but in Scotland not until mid- or late August, allowing little time for development before the onset of winter frosts. The spores are dispersed widely by the wind, however; it is thought that those responsible for forming new colonies in the Finnish forests originate from southern Sweden, where they are shed considerably earlier than in Finland.[191]

The presence of bracken on small islands can give some information on how rate establishment from spores actually is. The likelihood that an island will have been colonised depends on its size - the area upon which spores can fall - as well as on the above environmental factors. All the major Hebridean islands carry bracken, usually in abundance, but the position regarding small islets was

not clear. Accordingly, in the course of fieldwork for the main part of this thesis, 24 small islands off the coasts of Knapdale and Jura (mid-Argyll) were visited and the presence or absence of bracken established. This district has more favourable conditions for colonisation by spores than much of the Scottish mainland, with no frost before the beginning of November on average and 120-150 cm rainfall (Climatalogical Atlas, 1952).[442]

The parent rocks are a complex group of epidiorite and hornblende schists and quartzites, for the most part not severely deficient in bases, and the soils are mainly braunerden of varying base status, depth and drainage, with some rankers and thin ironpan soils, mainly on the quartzites. Eilean Mor has an area of shallow rendzinas on limestone.

Methods

On all of those islands which had been colonised the bracken had spread extensively. On the others, damp crevices and other sheltered areas suitable for colonisation were examined with particular care. Such sites, in the absence of bracken, were often occupied by a luxuriant growth of Dryopteris. Areas were measured by cutting out and weighing outlines traced from the 6 in O.S. map. Soil nomenclature follows Fitzpatrick (1964).[419]

A possible complicating factor, briefly suggested by Braid,[195] is that bracken may have been deliberately introduced in some places as a convenient source of bedding and thatching material. However, there is certainly no tradition of such a practice in the area of the investigation, nor is bracken noticeably either more or less often present on those islands which appear, from the remains of buildings of a particular local type,[cf 443] to have been inhabited in or around the 18th century (Nos. 9, 12, 14 and 15), or at any earlier period.

Results and Discussion

The results are shown in Table 1. The presence of bracken seems unrelated to the predominant soil parent material on islands within this area, although on more acid soils elsewhere colonisation is no doubt less likely.

If we exclude the Upper Loch Sween group, it is clear that large islands tend to carry bracken while small ones do not, with a considerable overlap. The average area of the islands in the overlap range (Nos. 9, 10, 11, 19, 21 and 22) is 8.4 ha.

In the Upper Loch Sween group, however, bracken is present everywhere except on the tiny Sgeir Bhuidhe (0.2 ha). On the whole, the islands in this group are less exposed than the others, but the most striking difference is that, probably for the above reason, they all carry woodland or scrub (mainly Betula and Querous with some Pinus). They appear to have done so for at least 160 years,[444] and probably much longer.[202] Elsewhere there are only small patches of scrub, on Taynish Island (No.9) for example. It seems, therefore, that colonisation is more likely where shelter, particularly that provided by woodland, is available. In Finland, it is known that forest fires have provided suitable sites for colonisation.[191]

The above results indicate that in mid-Argyll the minimal area, within which there is an even chance of a colony having established itself during the time when conditions have been suitable for the growth of bracken, is probably a few hectares in the open and less than one hectare in woodland. At higher altitudes and in less favourable terrain these areas would be increased.

For comparison, of all the islands in the Firth of Forth, bracken is present only on the wooded Cramond Island (11 ha), although there are five others in the range 5-50 ha.[445-6] It would appear that establishment from spores is rarer on the East coast than in the West, possibly due to drought.[181]

Table 1
Presence of bracken on Argyllshire Islands.

		O.S. Grid Ref NR	Area (ha)	*Geology	Bracken
I.	Upper Loch Sween				
	1. Unnamed	766885	0.4	X	present
	2. Unnamed	767884	0.6	D	present
	3. Unnamed	766882	0.6	X	present
	4. Eilean a'Bhrein	765881	2.2	X/D	present
	5. Unnamed	763878	1.2	D	present
	6. Sgeir Bhuidhe	762875	0.2	D	absent
	7. Eilean Loain	754854	16.6	X/RB	present
	8. Cala	753856	0.9	D	present
II.	Taynish Group				
	9. Taynish Island	727827	9.9	X	present
	10. Ulva Island E.	721824	10.3	X/D/RB	absent
	11. Ulva Island W.	717823	7.6	RB	absent
III.	MacCormaig Islands				
	12. Eilean an Leac	689755	4.6	D	absent
	13. Eilean Gamhna	680759	3.2	D	absent
	14. Corr Eilean	676757	10.8	X/D	present
	15. Eilean Mor	665753	16.7	D/L	present
	16. En.Mor N.E. peninsula	667754	2.5	D	absent
	17. Carraig an Daimh	662789	1.4	D	absent
IV.	Carsaig Group				
	18. Eilean Dubh	719873	16.6	X/D	present
	19. Eilean Traighe	723875	9.3	D	present
	20. Carsaig Island	732892	11.5	X/D	present
V.	Small Isles Group				
	21. Eilean Bhride	555698	7.4	D & others	present
	22. Eilean nan Coinean	544685	6.2	D & others	present
	23. Pladda	542688	3.3	RB	absent
	24. Eilean nan Gabhar	537675	14.6	D	present

* X : Quartzites
D : Epidiorite and hornblende schists, with igneous intrusions
RB : Raised beach deposits; L : Limestone.

The length of time involved is not known, but may amount to some millennia unless established colonies are ever wholly wiped out, for example by severe frost; thus successful establishment of bracken from spores is indeed a very rare event and certainly need not worry hill farmers. However under good conditions the plant spreads vegetatively at around 1 m/annum,[194-7] so that a colony may occupy 10 ha within two centuries. On the mainland, at least in the West at lower altitudes, bracken has probably spread sufficiently from the original colonies for its present distribution to reflect only the ability of the established sporophyte to withstand environmental conditions and to compete with other vegetation.

(Trans. Bot. Soc. Edinburgh, in press).

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