STUDIES ON PLANT METABOLITES

THESIS

Presented for the degree of

Doctor of Philosophy

in the

University of Glasgow

by

James J. Horton, B.Sc.

Agricultural Chemistry Section

Chemistry Department.

February 1968

STUDIES ON PLANT METABOLITES

THESIS

(Summary)

presented for the degree of

Doctor of Philosophy

in the

University of Glasgow

by

James J. Morton, B.Sc.

Agricultural Chemistry Section

Chemistry Department

February 1968

SUMMARY

The work carried out was concerned with an investigation into a possible connection between the sugars present in a mucilagenous polysaccharide from bracken rhizomes and the sugars present in the glycolipid fraction from the same plant source.

This work was initiated after a survey of the literature concerning the role of nucleoside diphosphate sugars in glycosidic bond formation and in particular polysaccharide synthesis, revealed a number of unexplained observations which suggested a possible involvement of a third group of sugar containing compounds. The nature of these observations inferred that the compounds involved could well be lipoidal in character.

BRACKEN POLYSACCHARIDE.

Part 1 of the thesis is devoted to a description of the extraction, purification and analysis of an acidic mucilagenous polysaccharide present in the rhizomes of the bracken plant (Pteridium aquilinum).

The polysaccharide was extracted using a cold 5% solution of trichloroacetic acid and subsequently purified by repeated precipitation in alcohol followed by titration with a 10% solution of cetyl trimethyl ammonium bromide. The sugar content of the purified polysaccharide was analysed qualitatively by both paper and gas-liquid chromatography. The following sugars were detected: D-galactose, D-mannose, L-arabinose, D-xylose, L-fucose and a uronic acid.

An investigation into the acidic function present revealed that it was probably entirely due to the uronic acid moiety. Sulphate content was shown to be very low and to decrease with purification, suggesting that its presence was due to contamination rather than as an integral part of the structure.

Quantitative analysis of hexose, pentose and methyl hexose was performed using the colorimetric methods of Dische and Borenfreund. This showed that L-fucose was present in as much as 20% weight of the polysaccharide.

GLYCOLIPID FRACTION.

Part 2 of the thesis gives an account of the investigation carried out into the glycolipid fraction present in bracken.

Section A is devoted to a preliminary investigation into different methods of extraction and purification. These included the use of methanol and chloroform-methanol, 2:1 v/v, as extracting reagents. Various bracken preparations were also used: fresh bracken, bracken preheated at 110° and freeze-dried bracken. These different trials were studied as to their effectiveness in reducing enzymic action during the extraction process. The purification processes studied included cellulose column chromatography for the removal of non-lipid contaminants, dialysis, and the diffusion of a chloroform-methanol mixture of the extract against a large excess of water, as used by Folch.

A preliminary examination of the lipid-bound sugars present in the lipid mixture revealed D-glucose, D-galactose, D-mannose, L-arabinose, D-xylose and two unknowns, one of which was suspected of being a sulphonated glucose. These results were confirmed by both paper and gas-liquid chromatography.

One of the major problems during this initial series of investigations was the difficulty in obtaining reproducible results, with regard to the kind and amount of sugars found.

Confirmation of the sugars found in the glycolipid fraction was obtained from the work described in Section B of Part 2. This section is concerned with attempts to fractionate the lipid mixture, firstly by removing neutral lipids and other nonglycolipid material such as phospholipids, followed by

the fractionation of the individual glycolipids themselves. This was performed after preliminary trials, using a combination of silicic acid, magnesium silicate and DEAE cellulose column chromatography. The fractions obtained were analysed for phosphorus and sugar content and they were also monitored using two dimensional thin-layer chromatography. After column fractionation the groups of purified glycolipids were finally separated into individual compounds by preparative thin-layer chromatography on silicic acid. The individual glycolipids were then analysed for sugar and fatty acid content by gas-liquid chromatography.

PREFACE

The experimental work described in this thesis was carried out in the Agricultural Chemistry Section of the Chemistry Department, University of Glasgow, under the supervision of Dr. W.R. Rees, from October 1963 to February 1967.

I would like to record my thanks to Dr. Rees who showed continual interest in the work from its inception to its conclusion.

I would also like to thank Professor J.M. Robertson, F.R.S. for his generosity in allowing the use of facilities available in the rest of the Chemistry Department.

James J. Morton

CONTENTS

PART 2 Studies on the Glycolipid Fraction from Bracken Rhizomes.

SECTION B Quantitative Fractionation of the Glycolipids from Bracken Rhizomes

ABBREVIATIONS USED

DEAE cellulose	-	diethylaminoethyl cellulose
ADP	-	adenosine - 5 - diphosphate
UDP	-	uridine - 5 - diphosphate
GDP	-	guanosine - 5 - diphosphate
CDP	-	cytidine - 5 - diphosphate
Kg.	-	kilogram
BDH	-	British Drug Houses Ltd.
ug.	-	microgram
mg.	-	milligram
GLC	-	Gas - liquid chromatography
TLC	-	Thin - layer chromatography
C - M	-	Chloroform - methanol
G-6-P	-	Glucose - 6 - phosphate
F-6-P	-	Fructose - 6 - phosphate
TMS	-	Trimethylsilyl
P	-	Phosphorus
CHO	-	Carbohydrate
ECTEOLA cellulose	-	Epichlorotriethanolamine cellulose
TCA	-	Trichloroacetic acid
CETAB	-	Cetyltrimethyl ammonium bromide
EGA	-	Ethylene glycol adipate
g.	-	Gram

NOTE: All temperatures are expressed in degrees centigrade.

STUDIES ON PLANT METABOLITES

GENERAL INTRODUCTION

This thesis describes investigations carried out on two related aspects of the bracken plant (Pteridium aquilinum). Part 1, gives an account of work performed on the composition, in terms of its component sugars, of a mucilagenous polysaccharide present in quantity in this plant. Part 2, which comprises the major part of the work, is concerned with studies on the nature of the glycolipid components of the plant.

Various studies, to be referred to later in this introduction, carried out in the last few years in several laboratories on the biosynthesis of polysaccharides have led to conjectures that glycolipids may conceivably be involved in glycosyl transfer reactions of direct relevance to these processes. One such study (1, 2) carried out in this laboratory upon the biosynthesis of starch showed that whole starch grains contained at least one glucose containing glycolipid as well as the nucleotide adenosine diphosphate glucose and therefore established that in this morphological entity the only lipid-bound sugar common to both the nucleotide and polysaccharide fractions was glucose. Whilst this did not comprise evidence of a direct connection or an involvement of glycolipid in starch grain biosynthesis it encouraged further investigations of a more complex system, especially when viewed in the light of work done elsewhere.

Such a system was provided by the bracken plant, which as already mentioned contains a complex mucilagenous polysaccharide as well as cell wall polysaccharide and starch grains in the rhizome.

Additionally, earlier results obtained in this laboratory on the nucleotide constituents of bracken (76) were available, and some similar connections between the component sugars of the various polysaccharides, the glycolipid and the nucleotides might thus conceivably emerge here also.

The need for information upon the biosynthetic pathways in the bracken plant is in any case urgent on other grounds. This plant has successfully defied attempts at eradication by conventional techniques of husbandry, as well as by relatively sophisticated methods involving selective herbicides (3, 4, 5, 6, 7) and knowledge of the means by which important components of the plants cellular material (e.g. the mucilagenous polysaccharide) are synthesized could perhaps suggest ways of intervening selectively in these processes.

The first system discovered leading to the synthesis of a polysaccharide was that involving muscle phosphorylase (8). It was shown that this enzyme in the presence of inorganic phosphate would catalyse the degradation, in part, of glycogen to form glucose - 1 - phosphate (G - 1 - P). This reaction also proceeds in a reverse-wise manner, under the appropriate conditions, during which the glucose from the G - 1 - P is transferred to a primer molecule resulting in the synthesis of an amylose-type polysaccharide, with concomitant release of inorganic phosphate.

Phosphorylase systems very similar to the muscle one were later shown by Hanes to be present in plants as well (9).

Because of its reversible nature it was not certain what the exact role of this phosphorylase system <u>in vivo</u> was. For many years attempts to synthesise polysaccharides other than the amylose type using similar enzyme systems were carried out and it was not until ten years later, following the discovery by Leloir of UDP glucose (10) that a different system was considered.

UDP-glucose is structurally related to G - 1 - P in that it contains the latter structure linked by a pyrophosphate bond to the nucleotide uridine-5-phosphate. This connection led many workers to the belief that UDP-glucose could be directly involved in the formation of glycosidic bonds. This was later borne out by many successful experiments.

From the numerous examples to choose from (11, 12, 13, 14) the following serve to demonstrate this involvement. The synthesis of the disaccharide trehalose was demonstrated by Leloir and his co-workers, involving UDP-glucose and an enzyme source from Sacchoromyces fragilis (15). Sucrose was also synthesised, by two different routes, both involving UDP-glucose (16, 17). The plant glycoside arbutin was also formed using UDP-glucose and an enzyme from wheat germ.

Soon after the discovery of UDP-glucose, Leloir also demonstrated the presence of two other nucleotides in yeast: GDP - mannose and UDP - N - acetyl glucosamine (19, 20). This was followed by the discovery of many other nucleotides in a wide variety of tissue, both plant and animal (21, 22, 23, 24).

Many researchers with this evidence that nucleoside diphosphate sugars are involved in the formation of di and trisaccharides logically argued that they may also play an important part in the synthesis of polysaccharides. The attention of workers in this field was therefore directed to this problem.

An account is given in the following pages of the various attempts to synthesise sugar polymers using nucleotides as substrates. The experiments performed are described briefly and the conclusions drawn from the results criticised in an attempt to establish some kind of order in a complex and confused situation.

One of the first claims to success was put forward by Glaser in 1957 (25) who said that synthesis of cellulose had been achieved using radioactive UDP - glucose, a primer cellodextrin and an enzyme preparation from Acetobacter xylinum. His sole criterion for the success of the experiment was the incorporation of between 1 - 2% radioactivity into the product. As no further proof to support his claim was provided and with such a low radioactive incorporation it is doubtful if this could be accepted as conclusive evidence of cellulose synthesis by this system.

A similar experiment was carried out by Glaser in the same year (26) in which he achieved a low incorporation of radioactive N - acetyl glucosamine into a primer of chitin using UDP - N - acetyl glucosamine and an enzyme system from Neurospora crassa. This work can be criticised on the same grounds as above.

However a series of experiments carried out by Elbein, Barber and Hassid (27) concerning cellulose synthesis in which GDP - glucose was the donor and the enzyme was a particulate one from mung beans, enabled sufficient of the product to be synthesised to allow both a chemical and an enzymic identification. No primer was required as the low concentration of cellulose present in the enzyme system was sufficient. One interesting point from this experiment was that when the enzyme preparation was washed it lost its activity which was partly restored by adding a yeast extract. This could point to the participation of other unknown factors in the reaction.

The synthesis of two 1:3 linked glucans have been reported. A particulate enzyme preparation from mung beans was shown by Hassid (28) to catalyse the transfer of glucose to form the β 1:3 glucan callose. The other α 1:3 polymer is paramylon, which is found in Euglena and whose synthesis, similar to callose, has been claimed (29).

Hassid (30) also showed an increase in chain length by one unit of the primer xylan using UDP - xylose and a particulate enzyme from asparagus shoots.

The synthesis of hyaluronic acid has also been claimed using a particulate enzyme from Rous sarcoma cells and radioactive UDP - N - acetyl glucosamine. The identification of the product was confirmed both by radioactive incorporation experiments and also by the use of a specific hyaluronidase (31, 32, 33, 34).

Studies were carried out by Duncan (35) in which he tried to show a direct link between nucleoside diphosphate sugars and polysaccharide synthesis. He prepared a large number of these substrates and using enzyme preparations from wheat scutella, dried peas, and potatoes, a series of experiments using primers of glucose, maltose, maltotriose, cellobiose, sucrose, G - 6 - P and F - 6 - P, were performed. After incubation the digests were assayed for an increase in chain length of the primers. In almost all of the experiments no significant increase in chain length was detected.

It can be seen from the examples cited, of work done in this and other laboratories that nucleoside diphosphate sugars appear to be connected in some definite way with the synthesis of polysaccharides. However, many experiments to illustrate this have not always been successful, and where an increase in chain length has been achieved the systems used lacked clear definition in that the enzyme preparation involved in the majority of the cases were particulate rather than soluble.

It could be suggested from this that because of the very complex structure of particulate systems they may contain not only the correct enzyme, but also other important factors necessary for the reaction. Therefore polysaccharide synthesis may be a more complex series of reactions rather than a direct nucleotide-polysaccharide link.

The synthesis of glycogen was investigated by Leloir in 1957 (36). He extracted an enzyme from rat liver and showed that with glycogen as a primer it catalysed the further synthesis of the glycogen by transferring glucose from UDP - glucose to the primer . This enzyme is referred to as glycogen synthetase. Leloir's work on glycogen stimulated greater effort into the examination of starch synthesis. As a result, during the last ten years, a great deal of information has accumulated and many ideas put forward, concerning not only the biochemical synthesis of starch but also the physiological processes involved in forming the grain. This complex problem is of particular interest in the present context and points arising from it will be discussed in the following pages.

Leloir (37), succeeded in incorporating glucose from UDP - glucose into starch grains using an enzyme preparation which appeared to be attached to the actual starch grain itself, and which could not be removed without loss of activity. This possibility that the enzyme was directly attached to the starch in some way, was borne out by Pottinger and Oliver (39) who, using a modified isolation technique increased the activity of the isolated starch grains by some two thousand fold.

However in 1964 Frydman and Cardini (40) suggested that the enzyme might not be attached irreversibly to the starch grain and that the starch was only necessary as a primer. This assumption was based on an experiment in which the transfer of radioactive glucose from ADP - glucose into starch was achieved using a soluble enzyme extract from potatoes.

Results of preliminary experiments on starch synthesis in this laboratory (41), when maltodextrins were included in the digest, were contrary to those of Leloir in that no higher polymers were detected.

In further investigations (41) in which digests containing whole starch grains as an enzyme source, UDP - glucose, 'primer' starch, G - 6 - P and

magnesium sulphate were incubated and assayed by the method of Pottinger and Oliver (39). No significant increase in chain length could be found.

The synthesis of starch therefore appears to be a particularly complex process and so far the experimental evidence as to how it is carried out is contradictory in nature.

Badenhuizen (42) in an attempt to clarify the starch synthesising problem, put forward the theory that the de novo synthesis of starch is carried out by the phosphorylase / G - 1 - P system acting in the presence of sucrose as a primer to form amylose. Q enzyme then converts this to amylopectin and there is "crystallisation" of the starch from coacervate droplets to form the grain. Leloir's starch synthetase system appears to be responsible only for extending starch already formed.

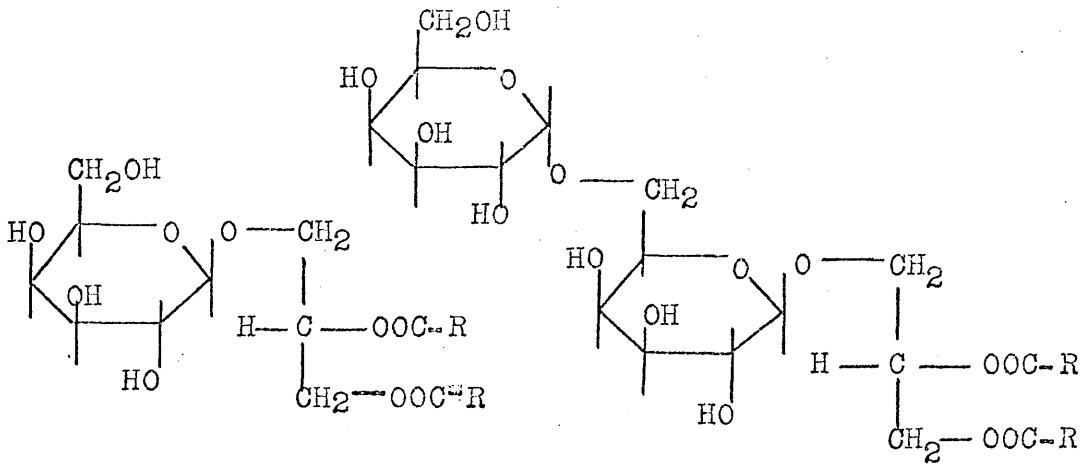
From Badenhuizen's theory that starch grains are built up in layers by the continual formation of coacervate droplets followed by "crystallisation" out of solution by the starch (42) Duncan (44) has implied that for this to be successful an interface is necessary otherwise it would be impossible for such a droplet to form in a wholly aqueous medium.

Taking this thinking a stage further he suggested the need for material of a lipoidal nature to be present in the environment.

The extraction and identification of two galactoglycerides from wheat flour by Carter et al in 1956 (45) (FIG. 1, Nos. (i) and (ii)), followed by the knowledge that such compounds, as well as other newly discovered glycolipids, are widespread throughout the plant world, has led various workers to surmise that they may play an important, but as yet unknown, role in plant metabolism.

From FIG. 1, Nos. (i) and (ii) it can be seen that the structural properties of such compounds would endow them with detergent qualities and hence it could be

Fig. 1



(i)

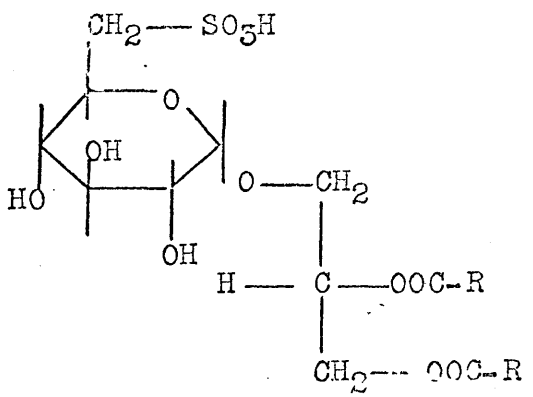
Monogalactosyl diglyceride.

(2,3-diacyl-1-(β-D-galacto-pyranosyl)-D-glycerol.)

(ii)

Digalactosyl diglyceride.

(2,3-diacyl-1-(α-D-galacto-pyranosyl-1,6-β-D-galacto-pyranosyl)-D-glycerol.)



(iii)

Plant sulpholipid.

(2,3-diacyl-1-(6-sulpho-α-D-quinovo-pyranosyl)-D-glycerol.)

envisaged that they would be most suitable in bridging the interface between aqueous and nonaqueous environments. It would not, therefore, be outrageous to postulate that such compounds as glycolipids could play an important role in the scheme outlined previously concerning starch synthesis, as they could span the gap between a lipoidal droplet and the final formation of the starch grain in an aqueous medium.

Northcote's work on cell wall biosynthesis is also of interest in the present context (46). He states that the endoplasmic reticulum, along with other cell organelles, is responsible for the transport and synthesis of material for the cell plate. He also showed, by electron mic roscopy, that the cell walls of plants are composed of microfibrils of polysaccharide of the α-cellulose type set in a continous matrix of hemicellulose and lipoprotein (47, 48, 49). Hassid, Neufeld and Feingold (50) have stated that the sugars of these cell wall polysaccharides are derived from uridine diphosphate compounds of the corresponding sugars. The mechanism, as yet unknown, by which these cell wall polysaccharides are synthesised is therefore of considerable interest.

Benson (51) discovered a compound in plants similar in general to those found by Carter. It is a glycosyl diglyceride : the sugar moiety being identified as a 6 - sulphonated glucose (FIG. 1, No. (iii)). Benson also realised that in the problem concerning the lipoprotein structure of plant cell membranes, glycolipid compounds of the above types described could act as surfactant reagents on the plant membrane, facilitating the transport of ions through the membrane, and thus enhancing the aqueous solubility of hydrophobic substances (52).

Duncan took this reckoning a stage further (53) within the present context, by saying that the glycolipids themselves may act, under the above conditions, as acceptors or donors in glycosyl transfer reactions between the different phases. As a result of this reasoning Duncan (54) investigated starch for the presence of glycolipid compounds. Small, but distinct, amounts of two glycolipids were found. One contained galactose and behaved in a similar manner to Carter's galactoglycerides, while the other contained glucose and appeared to be resistant to saponification with N. aqueous KOH at 37° for 24 hours.

Further evidence of a possible involvement of glycolipid compounds in the formation of glycosidic bonds and hence in the synthesis of polysaccharides is cited on the following pages.

Of particular interest here is the work of Colvin (55). He showed that when 80% alcoholic extracts from Acetobacter xylinum cells were incubated with an enzyme preparation obtained from the ultrafiltered supernatant of washed A. xylinum cells, cellulose fibrils were rapidly formed. Their presence was confirmed by physical methods, mainly electron microscopy. When samples of this ethanolic extract were dried, hydrolysed with 5N. H_2SO_4 and the hydrolysate examined by paper chromatography, a spot was detected which ran at the same rate as glucose. Later he discovered a similar cellulose synthesising system in plants, using Avena coleoptiles and Pisum seedlings. Here the enzyme source was again from A. xylinum (56).

In 1964 Lennarz discovered the presence of a dimanno lipid together with two other mannose containing lipids in the bacterium M. lysodeckticus (57). Also when uniformly labelled GDP - mannose C^{14} was incubated with an extract from the cells of the above bacteria it resulted in the formation of labelled mannose containing lipids. However the nature of the lipid acceptor or acceptors has not yet been identified.

Also of interest is the work done by Neufeld and Hall (58) who using spinach leaf chloroplasts demonstrated the enzymic synthesis of a series of labelled galactolipids from radioactive UDP - galactose. Labelled mono, di, tri and tetragalactosyl glycerol were identified as reaction products.

Because of these observations and because of the undefined and contradictory nature of the work done on polysaccharide synthesis previously mentioned, it was thought that the question of sugar-lipid involvement warranted further investigation. Duncan (59) constructed a table (TABLE 1) in which he compares the sugar content of both the nucleotide and polysaccharide fractions from the same source. This table, thought not attempting to prove any definate connection between the two fractions, far less proof of the involvement of nucleotides in polysaccharide synthesis, cannot but convey this line of thought. It was therefore deemed to be useful if a similar table could be composed in which a comparison is made between the sugar content of the nucleotide, polysaccharide and glycolipid fractions from the same source.

The rhizomes of the bracken plant were chosen for this work for the following reasons.

1. On preliminary investigations it appeared to contain a complex polysaccharide composed of several sugars. One of these sugars, L-fucose, was present in fairly large quantities. This is a sugar not normally found to any large degree in the polysaccharides of land plants. Hence if it could be shown that this sugar was present in the polysaccharide, the nucleotide and the glycolipid fractions this would indicate a more direct connection between them than would finding D-glucose or any other more common sugar in all three fractions.

2. The nucleotide content of the rhizomes has already been studied by

TABLE 1

SOURCE	NUCLEOTIDES FOUND	KNOWN POLYSACCHARIDES AND CONSTITUENT SUGARS
Yeast. (Saccharomyces fragilis)	UDP-glucose(10), GDP-mannose (20), UDP-N-acetylglucosamine (19), and GDP-mannoheptulose (60).	Glycogen (glucose), glucan (glucose), chitin (N-acetylglucosamine) and mannan (mannose) (61).
Liver. (mainly chicken).	UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine and UDP-glucuronic acid (62).	Glycogen (glucose), hyaluronic acid (N-acetylglucosamine and glucuronic acid) and heparin (N-acetylglucosamine and glucuronic acid) (63).
Hen oviduct.	UDP-glucose, UDP-galactose, GDP-mannose, UDP-N-acetylgalactosamine, UDP-N-acetylgalactosamine sulphate and UDP-N-acetylglucosamine-6-phosphoro-β-galactoside (64) (22).	Egg membrane (galactose, N-acetylglucosamine and phosphate) (22), hyaluronic acid (glucuronic acid and N-acetylglucosamine) and chondroitin sulph- (glucuronic acid, N-acetylgalactosamine and sulphate (63).
Red Algae.	UDP-glucose, UDP-galactose, UDP-glucuronic acid, GDP-L-galactose, GDP-mannose and adenosine 3',5' diphosphate (65).	Starch (glucose), cellulose (glucose), mannan (mannose) (66), galactan sulphate (D and L-galactose and sulphate (65).
Pneumococcii 1	UDP-galacturonic acid (67).	Polymer of galacturonic acid and N-acetylglucosamine (68).

TABLE 1 (contd.)

SOURCE.	NUCLEOTIDES FOUND.	KNOWN POLYSACCHARIDES AND CONSTITUENT SUGARS.
Pneumococcii 2 and 3.	UDP-glucose, UDP- glucuronic acid and UDP-N-acetylglucosamine (69).	Polymer of glucose and glucuronic acid (68).
Milk. (cow)	UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, GDP-mannose, GDP-glucose, GDP-L-fucose, UDP-N-acetyllactosamine and UDP-N-acetyllactosamine fucoside (70).	Lactose (glucose and galactose), N-acetyllactosamine (galactose and N-acetylglucosamine) and N-acetyllactosamine fucoside (N-acetylglucosamine, galactose and fucose (71).
Penicillin.	UDP-glucose, UDP-N-acetylglucosamine, UDP-galactose and GDP-mannose (72).	Dextran (glucose), mannan (mannose), galactan (galactose) and chitin (N-acetylglucosamine) (61).
Salmonella.	UDP-glucose, UDP-galactose, TDP-rhamnose, CDP-tyvelose, and CDP-abequose (73).	Polymer of glucose mannose, rhamnose, galactose and dideoxyhexoses (68).
Mung beans.	UDP-glucose, UDP-galactose, UDP-xylose, UDP-L-arabinose and UDP-glucuronic acid (31).	Callose (glucose), cellulose (glucose)and hemicellulose (galactose, xylose, arabinose, and glucuronic acid) (74).
Staphylococcus aureus.	UDP-N-acetylglucosamine, UDP-N-acetylmuramic acid and CDP-ribitol (75).	Chitin (N-acetylglucosamine), teichoic acid (ribitol and phosphate) and a muramic acid polymer (68, 74).

Weir (76) thus facilitating a comparison between the three fractions.

3. It was thought that because bracken was such an important agricultural plant, proving very difficult to eradicate effectively, an investigation in depth of part of its metabolism would be useful.

Previous research into the nature of the bracken plant has followed three main lines of investigation, all basically non-biochemical. Its composition has been intensively examined for elemental, ash, moisture and free sugar content (77, 78, 79, 80, 81, 82, 83, 84, 85). The toxicity of bracken to domestic animals has stimulated attempts to determine the toxic reagent and the precise nature of its effect on the animal. Finally, the possibility of controlling the weed by chemical herbicides has been investigated. The following are a few of the chemicals tested for this purpose; sodium chlorate, dalapon, pichloram and aminotriazole (4, 5, 6, 7, 86). This work however, has been on the whole empirically based and to date no expedient method has been found which has a lasting effect on the subterranean parts of the plant as well as destroying the ariel parts. In consequence, such herbicides may be successful in the short term but are unsatisfactory in the long term.

SCOPE OF THE PRESENT WORK

The work reported in this thesis was performed to ascertain if there was any similarity between the sugar content of the mucilagenous polysaccharide and that of the glycolipid fraction of the mature bracken plant.

PART 1.

This part is concerned with the mucilagenous polysaccharide. It gives an account of its extraction, purification and analysis. It pays particular attention to the certain identification of L - fucose as a component sugar of the polysaccharide.

PART 2.

This part describes the extraction, purification and resolution of the lipids from the rhizomes, firstly into classes, and finally into individual glycolipids. Attempts were made to determine what sugars were present in the glycolipid fraction. A comparison was then made between the sugars found in the nucleotide, polysaccharide and glycolipid fractions of the rhizomes.

A short account of an investigation into the fatty acid content, of the lipid extract as a whole, and also of the individual glycolipids is given.

PART 1.

THE ANALYSIS OF BRACKEN POLYSACCHARIDE

1. General comments on the extraction, purification and fractionation of plant polysaccharides.

a) Extraction.

Plant gums and mucilages are polysaccharide materials of high molecular weight. Some are completely soluble in water forming viscous solutions, while others absorb water and swell, but do not dissolve. Gums can usually be differentiated from mucilages, in appearance, in that they are normally plant exudates, often produced as a result of infection, while mucilages are obtained by extraction with a suitable reagent.

The criterion of a good method of extraction of a plant polysaccharide, as is the case in the extraction of all biological material, could be stated as follows:- it must extract as much of the designed material as possible, free from contamination, and as far as possible in the native state. Any method of extraction which is chosen therefore must prevent the chemical and enzyme degration of the material being extracted.

The technique used for the isolation of plant mucilages are of necessity diverse and their use, though normally of an empirical nature, can be tailored to suit certain characteristics of the polymer under investigation.

At the turn of the century the most commonly used methods involved the use of alkaline extracting reagents, such as 2% Na_2CO_3, K_2CO_3 and NaOH. Use of these solutions compensated for the fall in pH during extraction. However it was found that when some of the above reagents were used the yields obtained were not very high, and there was still the possibility of enzymic degradation.

If hot or cold water is used as an extracting medium, other water soluble compounds, such as amino acids and proteins are extracted. Hot water extraction has the added disadvantage of rupturing starch grains present in the plant, resulting in contamination of some of the desired polysaccharide.

One of the major changes to be avoided during extracting is degradative enzymic action. This can often be avoided by the addition of inhibitors, such as mercuric chloride or lead acetate, to the extracting medium.

It was found in the extraction of the mucilagenous polysaccharide from the rhizomes, after trials using 2.0% Na_2CO_3 and hot water, that a cold 5.0% aqueous solution of trichloroacetic acid was not only effective in preventing enzyme action but also avoided the extraction of starch as well.

b) Purification.

One of the major problems in polysaccharide research is the assessment of purity. Purity with respect to inorganic and protein contamination is readily established but there is no general, quickly applied, method for testing purity with respect to freedom from contamination by other polysaccharides. The effectiveness of a purification step can only be monitored by measuring some property of the polysaccharide in question, such as its optical rotation, molecular weight, the products of hydrolysis or ash content, until limiting purification is achieved.

Various methods have been used to remove proteins and other non-carbohydrates from polysaccharide extracts. One such technique involves the denaturation of the proteinatious material by shaking the extract with a 1:3 v/v mixture of amyl alcohol and chloroform (87, 88). 40% trichloroacetic acid has also been used in a similar manner (89).

Another method used involves the precipitation of crude polysaccharide extracts by pouring into cold absolute alcohol. While this effectively removes

amino acids, sugars and other low molecular weight contaminants, proteins can be co-precipitated with the polysaccharide.

A similar criticism applies to dialysis as a purification step.

Protein absorbents such as Fuller's Earth have also been used (90). However here a certain amount of carbohydrate material may also be absorbed by the reagent along with the protein contaminants.

Protein contamination can be largely avoided by using solutions of TCA as the polysaccharide extracting medium. This precipitates proteins by denaturation and consequently they are not extracted in the first place.

c) Fractionation.

One technique used for the separation of a mixture of polysaccharides is fractional precipitation from alcoholic solutions of varying concentrations in the presence of various metal cations (91). This method depends upon the dehydration of the polysaccharide molecules causing them to precipitate out of solution. The concentration of alcohol at which this takes place, varies from polysaccharide to polysaccharide enabling a fractionation to be obtained. The presence of cations facilitates precipitation by neutralising any charge present. However, using this method can often result in cross contamination of the various fractions.

In the case of neutral polysaccharides there are certain cases where specific fractionation techniques are applicable. Starch can be precipitated from solution in the form of an iodine complex, amylose separated from amylopectin by the formation of insoluble complexes with a range of compounds (92, 93) and yeast mannan can be precipitated as its copper complex (94).

The fractionation of mixtures of neutral polysaccharides has been achieved using electrophoresis on supports of filter paper, silk and glass-fibre paper (96).

Voltages of up to 2,000 are used and a charge induced on the polysaccharides by the formation of borate complexes. Mixtures containing mannan, galactan, glycogen and inulin have been separated by this method. However if the polysaccharide to be studied already contains ionisable groups then electrophoretic separations can be achieved without the formation of a complex (95). Electrophoretic separations using cellulose columns have also been applied and found to give good results (97).

Ion-exchange chromatography of charged polysaccharides on columns of ECTEOLA cellulose has been used by Ringertz and Riechard (98, 99). This ion-exchange material is obtained by reacting cellulose with epichlorohydrin and triethanolamine resulting in the introduction of basic groups to the structure. The separation is carried out at an acid pH using either a stepwise or gradient elution with 0.1 to 3.0M NaCl - HCl, 1:1 v/v.

The fractionation of mixtures of acidic polysaccharides on columns of DEAE cellulose can also be accomplished (204). The column material is used either in the basic or phosphate form. The polysaccharides are eluted from the column using buffers of increasing strength at a neutral pH or buffers of increasing acidity or alkalinity. Using DEAE cellulose, Neri fractionated a mixture of pectic substances into its individual components (205, 206).

Schmidt (101) obtained a good separation of acidic mucopolysaccharides from each other by ion-exchange chromatography on DEAE Sephadex-A-25. Thus hyaluronic acid, heparin, heparitin sulphate and chondroitin sulphate were completely separated by this method.

Resolution by gel filtration techniques is accomplished as a result of differences in molecular size. Polysaccharides, structurally identical from the standpoint of composition and integrity of repetition of the unit structure, may nevertheless exist in a range of molecular sizes.

Hence gel filtration will not give rise to fractionation into distinct species and the method is thus limited. Ringertz (102) however separated the polysaccharide extract from mice tumours, using gel filtration, into fractions containing different sulphate content, anti-coagulant activity and type of amino sugar present. Gel filtration using Sephadex can be used successfully for removing low molecular weight material, such as salts, sugars, and amino acids from higher polymers (103, 104, 105).

Scott (106, 107) made use of the fact that acidic polysaccharides when titrated with a quaternary ammonium detergent, such as cetyl trimethyl ammonium bromide, form insoluble complexes which precipitate out of solution (FIG. 2). Neutral polymers, under normal circumstances, do not form this complex and hence remain in solution. A separation of neutral from acidic polysaccharides can therefore be obtained.

Fig. 2

$$P^{n-}nM^{+} \quad + \quad nQN^{+}A^{-} \longrightarrow \quad P^{n-}nQN^{+} \quad + \quad nM^{+}A^{-}$$

$P^{n-}nM^{+}$ is the salt of the cation M^{+} with the polyanion of valency n.
$nQN^{+}A^{-}$ is the quaternary ammonium salt where QN^{+} is the cetyl trimethyl ammonium cation and A^{-} is the anion, e.g., Br^{-}.

By adjusting the pH it is possible to separate polysaccharides by inducing varying degrees of dissociation. Thus a polysaccharide which fails to precipitate at pH 2 may be induced to do so by raising the pH to 4 or 5. These QN^{+} polyanion complexes are soluble in varying concentrations of salt solutions e.g. sodium chloride or lithium chloride. If lithium chloride is used the polysaccharide can

be reprecipitated as its lithium salt by pouring the salt solution of the complex into cold absolute alcohol, the excess lithium chloride remaining in solution (207).

This last method of fractionation was the one chosen in an attempt to resolve the polysaccharide extract from the bracken as it was found to be of an acidic nature and hence suitable for this particular technique.

2. The extraction of a mucilagenous polysaccharide from bracken rhizomes.

Fresh samples of bracken rhizomes were collected during the dormant season, November to February 1963-64, from Milngavie Moor, on the outskirts of Glasgow. The rhizomes were washed, and chopped into lengths of approximately one inch.

When 1.0kg. of these rhizomes were extracted by blending with 2.0% $Na_2 CO_3$ the yield of polysaccharide obtained was 2.5% of the dry weight of the bracken (bracken containing 80% moisture). This method of extraction was abandoned because of the low yield and also because of the possibility that enzyme action might still be taking place during extraction.

A second trial extraction was carried out in which 1.0kg. chopped rhizomes were firstly boiled in water to inactivate degradative enzymes. The rhizomes then extracted by blending with cold water yielded material accounting for 12.5% of the dry weight of the bracken. However this method was also discarded when it was found that the increased yield was due to the extraction of starch as well as the mucilagenous polysaccharide.

The method finally chosen involved blending 1.0kg. chopped rhizomes with 5.0% cold trichloroacetic acid in a high speed Waring Blendor (Waring Products, Winstead, Conn., U.S.A.) (Exp. 1), passing the viscous solution obtained through muslin and re-extracting the residue with cold water. The two extracts were combined, centrifuged and the supernatant immediately extracted with ether to

remove the TCA and prevent the chemical breakdown of the polysaccharide.

3. Purification of crude polysaccharide extract.

An initial purification of the crude extract was carried out by repeated precipitation in cold absolute alcohol (Exp. 1). The white fibrous polysaccharide was collected by centrifugation, washed twice with alcohol, then twice with ether and allowed to dry in vacuo over paraffin wax. The dried polysaccharide obtained could be re-dissolved in water by initially wetting the powder with a small amount of benzene and then adding small quantities of water and blending until the material was in solution. The above alcoholic precipitation step was repeated, finally to yield a dry white fibrous material weighing 7.5g. (3.75% of the dry weight of the bracken). The absence of starch was established by testing with an I_2 potassium iodide reagent.

Although the yield of polysaccharide was not very high in terms of dry weight, its mucilagenous properties allow it to absorb large quantities of water, many times its own weight, resulting in considerable swelling.

4. Attempted fractionation of polysaccharide extract.

In order to ascertain if this extract was composed of a single polysaccharide or contained two or more different polymers, it was decided to attempt its fractionation using the quaternary ammonium complex technique of Scott (106, 107) (Exp. 2). This method was chosen, as it was found after passing a solution of the polysaccharide through an Amberlite IR 120 ion-exchange column in the hydrogen form (G.M.2) that the polysaccharide solution obtained had a pH of 3 to 3.5. The fact therefore that the polysaccharide was acidic made it suitable for this method of fractionation.

A 0.25% solution of the polysaccharide was passed through an Amberlite IR 120 H^+ column (G.M.2) and the effluent titrated to a slight excess with a 10% solution

of cetyl trimethyl ammonium bromide. A thick precipitate was obtained which was collected by centrifugation. The pH of the supernatant was slowly raised to 7.5 with N NaOH. Upon the addition of more CETAB a second precipitate was formed and this was also collected by centrifugation. Both complexes were dissolved in a 10% solution of lithium chloride and the presumed lithium salt of the polysaccharides re-precipitated by pouring into three volumes of cold absolute alcohol. Of the 2.5g. polysaccharide applied to the ion-exchange column approximately 40% was present in Fraction A and 60% in Fraction B.

Samples from both fractions were hydrolysed with N H_2SO_4 (G.M.3) and examined for monosaccharide content by paper chromatography using a butanol-pyridine-water solvent mixture (G.M.4). The spots were located with alkaline silver nitrate (G.M.5).

The results of the chromatogram showed that, qualitatively, both Fraction A and Fraction B were composed of exactly the same sugars. At least six spots were detected in each case including the characteristic fish tail effect normally given by a uronic acid. No significant degree of purification from other minor polysaccharide components was evident from the chromatogram.

In case the extract contained not a mixture of acidic and neutral polysaccharides but a mixture of acidic polymers, e.g. one component polysaccharide containing uronic acid residues, the other sulphated sugar residues possibly, the experiment was repeated at pH 2.0 instead of pH 3.5. The fact that a solution of the free acid of the polysaccharide was only pH 3 - 3.5 was thought not sufficient to rule out the possibility of sulphate groups being present. However the results from this second experiment were similar to those from the first. Two fractions were again obtained: the first precipitating at pH 2.5

and the second at pH 7.0. No difference in the qualitative sugar content of each fraction was found.

The polysaccharide extract, therefore, could not be fractionated by this method and appeared to consist of a single acidic polysaccharide component containing several constituent sugars.

Because Fraction A and Fraction B were, using paper chromatography, similar in sugar content, they were combined and this combined sample referred to as "CETAB treated polysaccharide", samples of it being used in further experiments. Otherwise the polysaccharide samples used were obtained after repeated alcohol precipitation only (Exp. 1).

Since this experiment showed the polysaccharide to be composed of several different sugars it was decided to carry out a detailed examination by paper chromatography and gas-liquid chromatography to identify each one conclusively.

5. Qualitative paper chromatographic analysis of polysaccharide.

One dimensional paper chromatography was carried out (G.M.4) by hydrolysing 1.0g of polysaccharide with 2N H_2SO_4 (G.M.3) and analysing the hydrolysate using the following solvent mixtures (G.M.4).

1) Butanol - pyridine - water (108)

2) Phenol - water (109)

3) Ethyl acetate - pyridine - water (109)

4) Formic acid - methyl ethyl ketone - butanol - water (110)

Unknown spots and standards were located by dipping or spraying with the following reagents (G.M.5) :

Ammoniacal silver nitrate (111)

Benzidine trichloroacetic acid (112)

p-anisidine - HCl in n-butanol (113)

α-naphthol/phosphoric acid (114)

TABLE 2

Standard sugars	Solvents							
	1		2		3		4	
	Rg std.	Rg hyd.	Rg std.	Rg hyd.	Rg std.	Rg hyd.	Rg std.	Rg hyd.
D-glucose	100	101*	100	100*	100	99*	100	
L-arabinose	117	116	143	143	115	114	143	141
D-mannose	115		112		112		128	129
D-galactose		91	114	115	87	86	93	93
D-xylose	127	130	120	121	142	144	158	156
L-fucose	130		170	167	135	137	182	181
D-fructose			138					
L-rhamnose					179		218	
Uronic acid		+		+		+		+

Rg values for standard sugars and unknowns from bracken polysaccharide after hydrolysis with 2N H_2SO_4. + denotes presence of uronic acid. * denotes a trace.

Solvents: 1. butanol-pyridine-water (3:1:1.5 v/v) 2. phenol-water (160:40 w/v)

2. ethyl acetate-pyridine-water (120:50:40) 3. formic acid-methyl ethyl ketone-butanol-water (15:30:40:15 v/v).

TABLE 2 summarises the Rg. values obtained for both the unknown sugars and the standards. The Rg. values for uronic acid spots are not given due to the difficulty of ascertaining the meaningful centre of the spot due to the tailing effect. Instead, the presence of a uronic spot is denoted by a + sign.

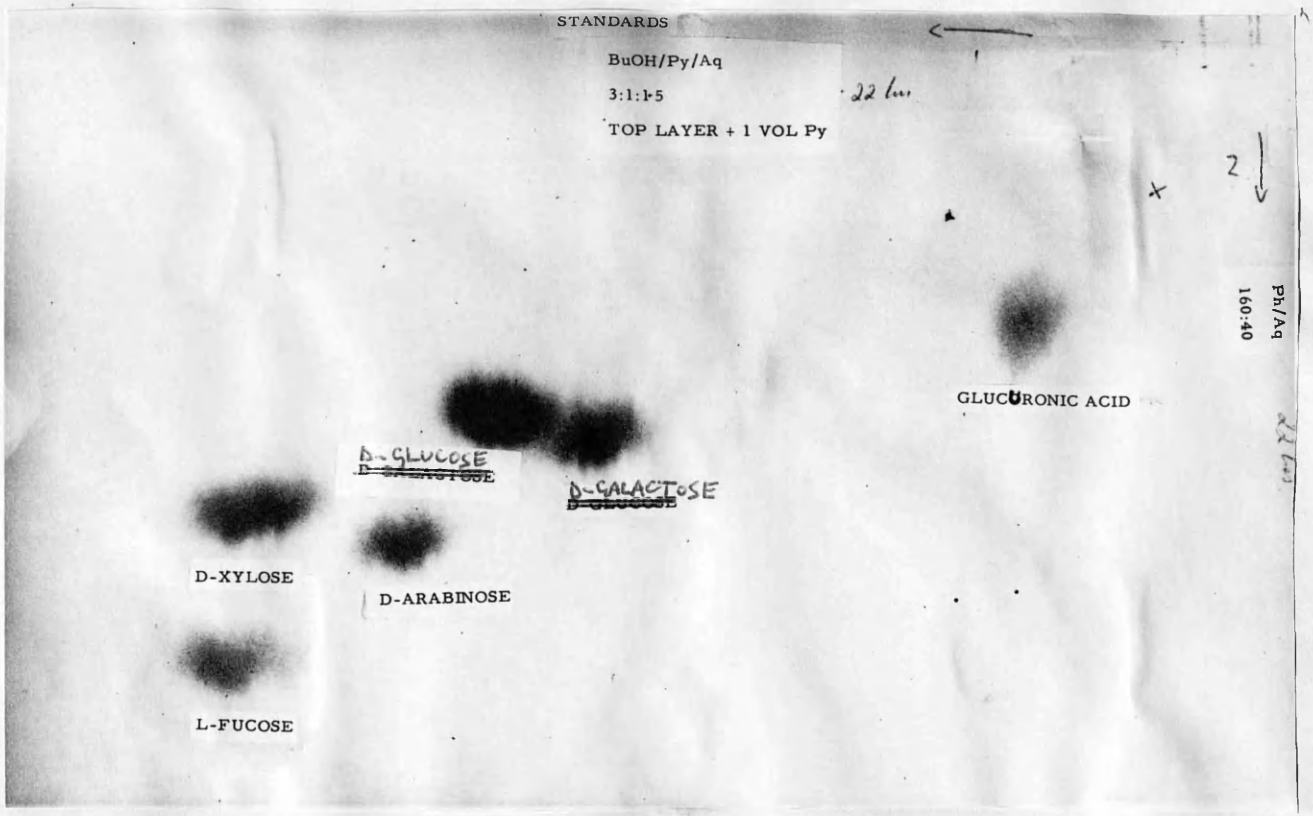
From TABLE 2 it can be seen that D-galactose, D-mannose, L-arabinose, D-xylose, L-fucose, D-glucose and a uronic acid sugar were all present as components of the polysaccharide. Glucose was, however, shown by all four chromatograms to be present only in trace amounts in the sample used, and may have arisen as a result of residual contamination by some other component incompletely removed and may not in fact be a structural part of the mucilagenous polysaccharide. This result is supported by literature evidence in that glucose has been found only in very few plant mucilages (203). Ketoses were tested for by spraying with α-naphthol/phosphoric acid reagent (G.M.5).

Traces of slow running sugar components were also noted on the one dimensional paper chromatograms. These spots were not unexpected and were probably due to incomplete hydrolysis of the polysaccharide leading to the formation of small amounts of di and trisaccharides.

Two dimensional paper chromatography was also performed (G.M.3). A standard chromatogram was run under exactly the same conditions as the unknown sample. The chromatograms were developed firstly with butanol-pyridine-water, followed by phenol-water at right angles to the first solvent. The spots were located using aniline-oxalate reagent (G.M.5) (115).

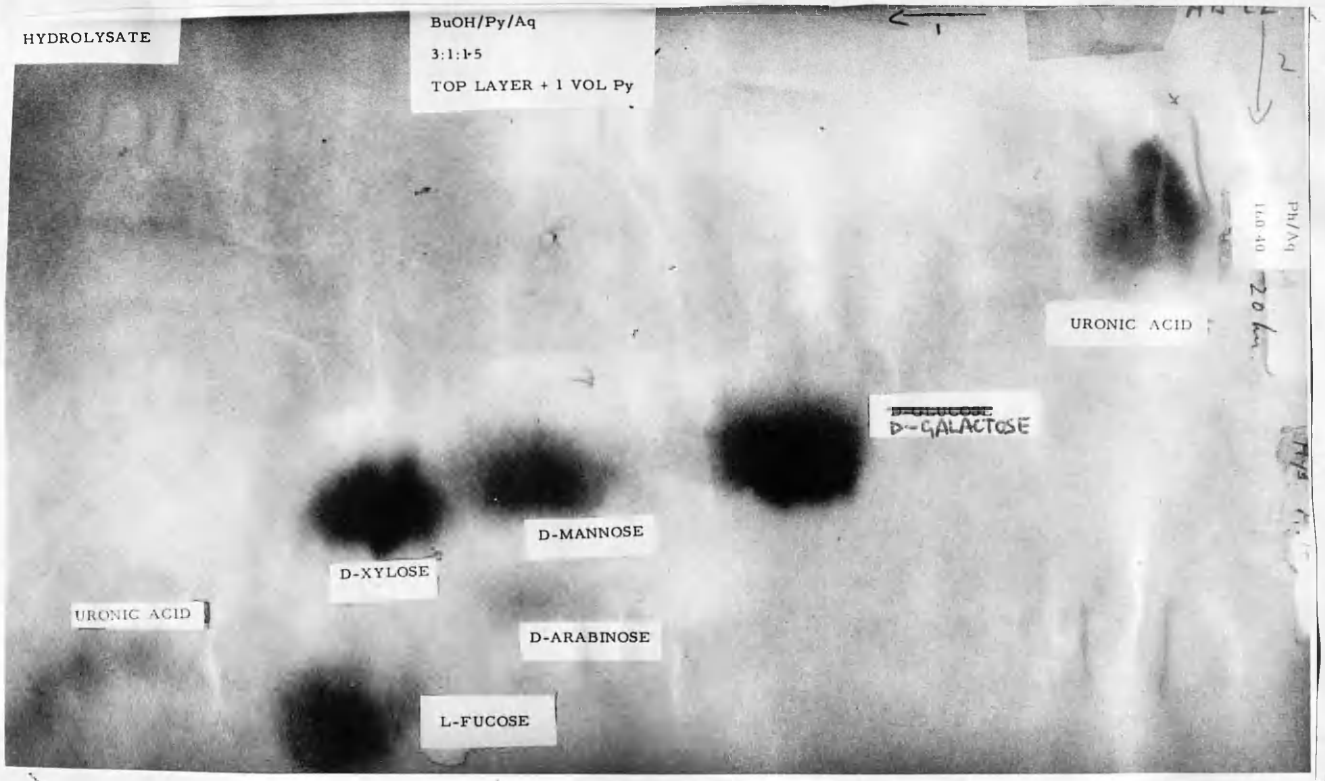
Comparison of the spots on the unknown chromatogram with those on the standard (FIG. 3) confirmed the results obtained from TABLE 2. However here no glucose at all was located. Galactose, mannose, xylose, fucose and uronic acid all gave strong spots whereas the L-arabinose spot was more faint.

Fig. 3 (1).



Two dimensional paper chromatography of standard sugars.

Fig. 3 (2).



Two dimensional paper chromatography of unknown sugars obtained from bracken polusaccharide after hydrolysis with sulphuric acid. The chromatogram was run under exactly the same conditions as for the known sugars (Fig. 3 (1)).

6. Qualitative gas-liquid chromatographic analysis of bracken polysaccharide.

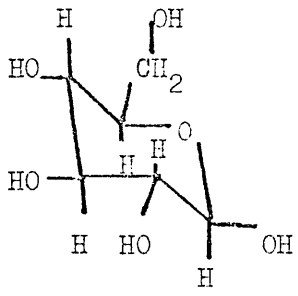
GLC of carbohydrates is rapidly becoming a standard technique. However, the difficulty in devising volatile derivatives suitable for use in GLC delayed the application of this very desirable technique to sugar analysis.

Initially workers made use of the O-methyl ether or acetyl derivatives (116). Disaccharides were resolved as their octoacetates or methylated disaccharides (117, 116).

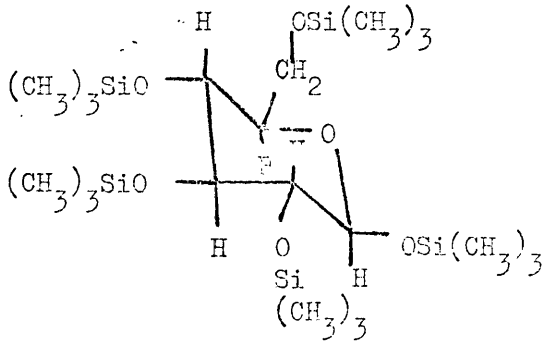
The requirement that the sugar derivative be volatile is obvious. However it must also be prepared readily and quantitatively, the method must be applicable to a wide variety of carbohydrate compounds, and the derivative must be stable at the high temperatures likely to be employed during its analysis. Also it should not require extensive purification prior to chromatography and it should be possible to degrade the derivative to the starting material in order that preparative GLC can be performed.

The above requirements were fulfilled to a great extent when Bentley, Sweeley, Makita and Wells (118, 119) demonstrated the use of trimethyl silyl ether (TMS) derivatives of polydroxy compounds as suitable volatile compounds for use in GLC (FIG. 4). They showed that silyl compounds were easily

FIG. 4



Hexose



TMS derivative

prepared in quantitative yields, were stable at high temperatures and could be applied directly from the reaction mixture to the column. The formation of the TMS derivatives is quick and the reaction goes to completion at room temperature or with gentle warming. As a result multiple peaks are not observed as was the case with previous methods, when only partial methylation and acetylation was often achieved.

It was decided therefore to use this technique to confirm the results from the paper chromatography analysis of the polysaccharide components.

Previous to an examination of the components present in the polysaccharide by this method, a number of preliminary experiments using known sugars were performed in order to gain experience in operating a gas-liquid chromatogram, to determine suitable conditions for the separation of a mixture of sugars and to compare the results obtained with those given in the literature. In this way it was possible to gain a degree of familiarity with the technique.

The TMS derivatives of these known sugars were prepared by dissolving approximately 10mg. of each in dry pyridine (G.M.6) and adding to this a freshly prepared 2:1 v/v mixture of hexamethyldisilazane and trimethylchlorosilane (119) (G.M.15). A mixture of known sugars composed of D-glucose, D-galactose, D-xylose, D-mannose, L-arabinose and L-fucose was also prepared in a similar manner. Initially the single sugar derivatives were analysed and their retention times calculated relative to α-glucose. This was followed by an anlysis of the mixture of sugars and the various peaks obtained identified by comparison with the single runs. In this way the conditions leading to the best separation of the sugars were determined. Similar conditions were used in the analysis of the sugar components from the polysaccharide (see below). Comparison of the results obtained from

these initial exploratory studies compared sufficiently well with those from the literature (119, 120, 121, 122) that it was thought permissable to proceed with an investigation, using this technique, into the sugar composition of the bracken polysaccharide.

Gas-liquid chromatography of the monosaccharide content of the polysaccharide took the following form: 0.5g. polysaccharide were hydrolysed with 2N H_2SO_4 (G.M.3) and the hydrolysate reduced to dryness using a rotary evaporator at a temperature not more than 60°. The residue in the flask was thoroughly dried by shaking with dry methanol (G.M.6) and reducing again to dryness. This was repeated.

The TMS derivatives of the sugars in this mixture were formed by dissolving it in dry pyridine (G.M.6) and adding, with shaking, a 2:1 v/v mixture of hexamethyldisilazane and trimethylchlorosilane (G.M.15).

A commercial Pye 104 dual flame ionisation detector chromatograph was used for the analysis (G.M.16). Samples of the reaction mixture (1 - 10µl) were injected into a glass column 152.0 x 0.4cm. in dimension packed with 2.5% S.E. 30 (a non-polar silicone gum coated on an inert support) (G.M.7). The column packing was prepared using pre-treated material (G.M.13). The separation was carried out under temperature programmed conditions of 130 - 160° with a linear rise in temperature of 2°/minute.

The fractionated components were recorded automatically as peaks using a Smiths Servoscribe recorder (Kelvin Electronics Company, Wembley, Middlesex, England).

The resolution of the TMS derivatives of sugars by this method is so sensitive that the anomers of individual sugars, such as α and β-D-glucopyranose are separated and appear as individual peaks (119). Because of this it was

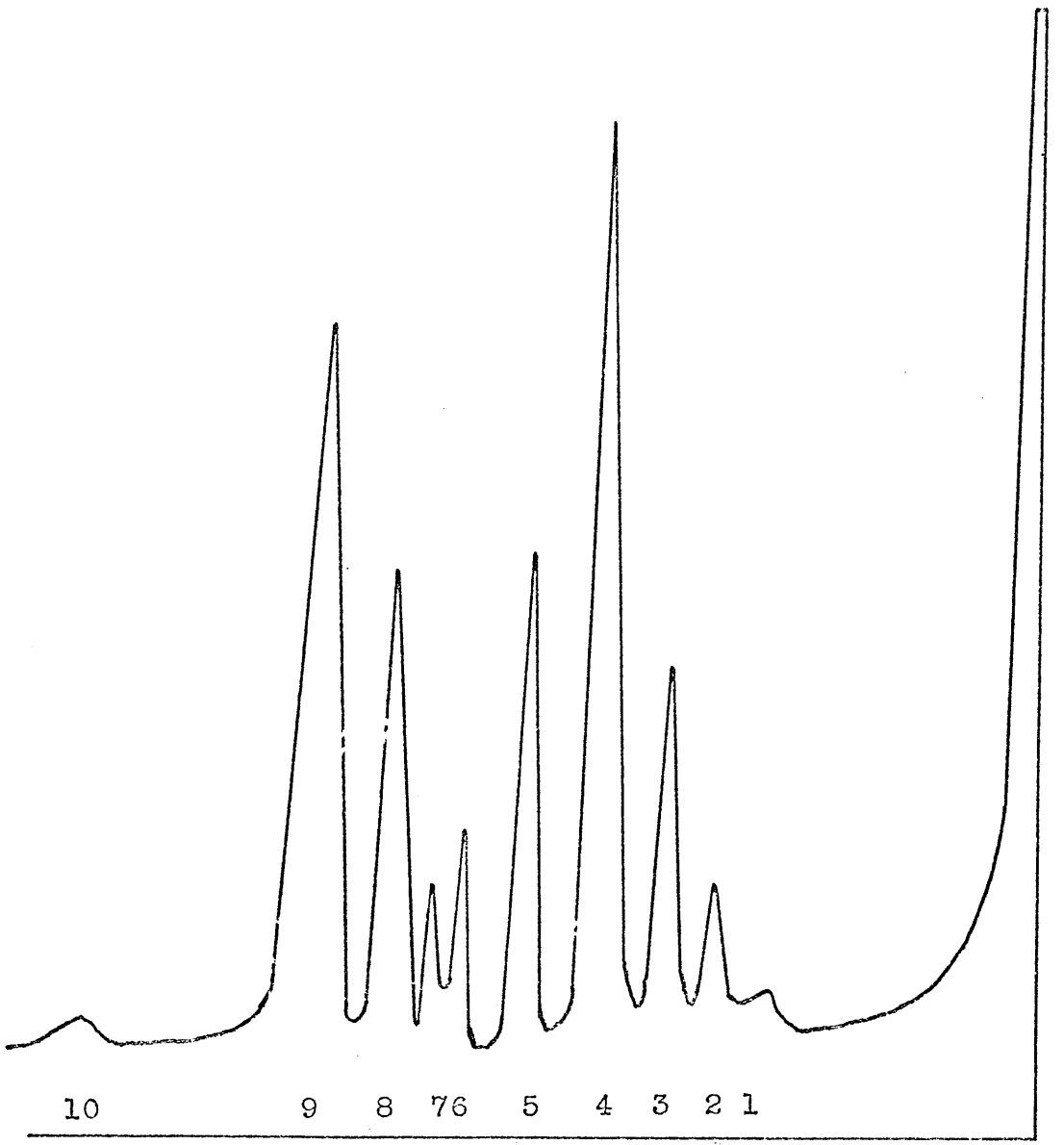
expected that the sugars present in the aqueous hydrolysate would have formed equilibriated mixtures of their respective anomers, and as a result several peaks representing these different anomers of the same sugar would be recorded. Because of this the standard sugars used for comparison were also allowed to form equilibriated mixtures of the anomers by dissolving them in water and standing overnight. Their TMS derivations were then prepared (G.M.15) and run under exactly the same conditions as the unknown mixture.

The separation of the TMS derivatives of the unknown sugar mixture is shown in FIG. 5. The Rt. values of the ten peaks were calculated, relative to the α-glucose peak present in the standard, and compared with the values found for the standard sugars (TABLE 3). The various peaks obtained for each standard sugar, representing the different anomers, were identified by reference to the literature (119, 120, 121, 122) and by comparison with results from the preliminary experiments. From TABLE 3 the following conclusions can be drawn: peaks one and two are L-arabinose, peak three is L-fucose, peaks four and five are D-xylose, peaks six and nine are D-mannose, peaks seven and eight and nine are D-galactose and the trace peak ten is β-D-glucose.

These results confirm those already found by paper chromatography, including the fact that glucose is present only in very small amounts suggesting as previously mentioned that it may not be a genuine component of the polysaccharide.

The only sugar not identified by this method was the uronic acid component. This was probably due to the fact that a charged sugar such as a uronic acid would have a very large Rt. value when run on a barely non-polar S.E. 30 column. As a result the uronic acid peak may have been so slow at the temperature used for the analysis that it did not have a sufficiently long time to appear. So

Fig. 5



GLC of sugars (TMS derivatives) obtained from bracken polysaccharide after hydrolysis with 2N H_2SO_4. The mixture was separated on an SE-30 column using temperature programmed conditions of 130-160° with a rise of 2°/min. (TABLE 3).

TABLE 3

Standards	Anomers	(Stds.) Rt. rel. to α-glucose	(Hyd.) Rt. rel. to α-glucose	Peak No.
Arabinose	β	0.42	0.419	1
	α	0.46	0.456	2
Fucose	α	0.503	0.506	3
Xylose	α	0.579	0.574	4
	β	0.698	0.69	5
Mannose	α	0.83	0.823	6
	β	1.045	1.05	9
Galactose	Y?	0.875	0.868*	7
	α	0.943	0.943	8
	β	1.05	1.05	9
Glucose	α	1.0	/	/
	β	1.3	1.3 *	10

Rt. values for standard sugars and unknowns from bracken polysaccharide after hydrolysis with 2N H_2SO_4, followed by GLC analysis using a column of 2.5% SE-30.

* trace.

far there is not a great deal of information with regard to the GLC analysis of uronic acids and time did not allow for such an investigation to be performed here.

The absence of a large glucose peak was further confirmed by rechromatographing a sample of the polysaccharide hydrolysate containing an internal standard of D-glucose. L-fucose was also confirmed as being present in a similar manner.

This GLC analysis has therefore confirmed the paper chromatographic investigation in showing that the monosaccharides present in the polysaccharide are galactose, mannose, xylose, fucose, a smaller amount of arabinose, a uronic acid component and also a trace amount of glucose (which may be an artifact).

L-fucose was first shown to be a major component of a plant polysaccharide in fucoidin, obtained from the Brown Algae (123, 124). Fucoidin was later found to contain up to 48% L-fucose and up to 30% sulphate (125, 126).

The traganthic acid fraction of gum tragacanth is also reported to contain ca 10% L-fucose (127) but apart from these two sources significant amounts of this sugar do not appear to be present in any other polysaccharides.

That L-fucose was shown to be present in this polysaccharide from bracken and on appearance in large quantities was therefore thought to be of considerable interest. Also because of the reasons stated in the introduction (page11) with regard to the importance of L-fucose as a basis for comparison between the polysaccharide and glycolipid fractions, it was decided that its presence in the polysaccharide must be confirmed without doubt. The following experiment was performed to do this.

7. Cellulose column chromatography of the components of the polysaccharide.

This experiment was performed to isolate an adequate amount of a pure sample of the L-fucose from the polysaccharide hydrolysate to enable certain identification by means of specific optical rotation measurement, and derivative formation.

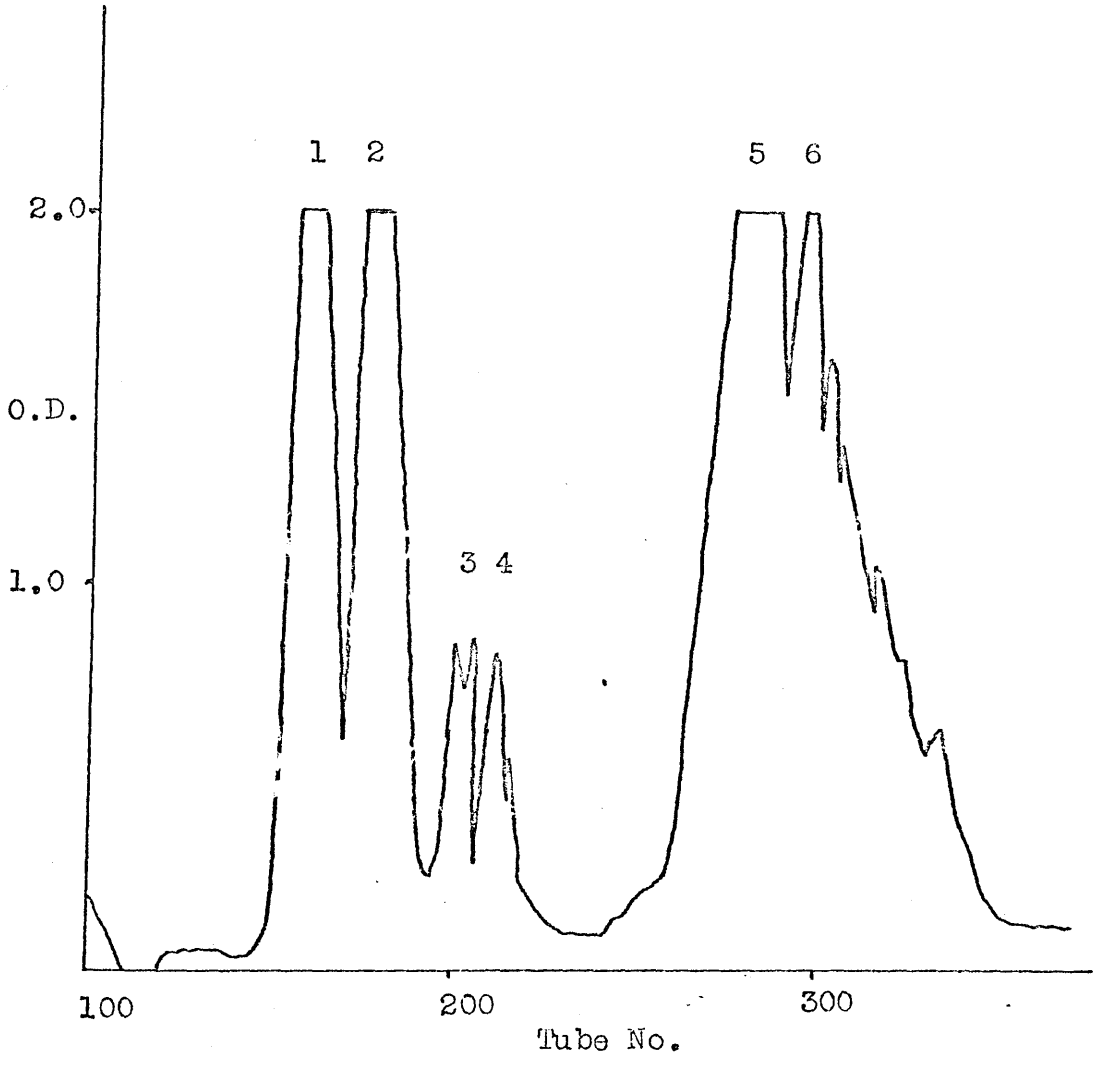
To this end a powdered cellulose column after Hough, Jones and Wadman was employed (128) (Exp. 3). A column 3.5cm. in diameter was packed uniformly to a height of 100cm. with powdered cellulose in a slurry of water saturated n-butanol (G.M.7). 1.0g. polysaccharide was hydrolysed with 2N. H_2SO_4 (G.M.3), the hydrolysate reduced to dryness in vacuo using a rotary evaporator, re-dissolved in 3 to 4ml. deionised water and applied to the column which was then eluted with water saturated n-butanol. 10ml. fractions were collected automatically and each one analysed for carbohydrate content by the method of Sven Gardell (129) (G.M.11).

A complete separation according to carbohydrate content was not achieved, but when the appropriate tubes were bulked and the peaks obtained (FIG. 6) analysed by paper chromatography, using a solvent mixture of phenol-water (G.M.4) to determine what sugars were present in each one, it was possible to obtain a pure sample (102 mg.) of the suspected L-fucose from Peak 1.

The specific rotation of the compound present in Peak 1. was calculated to be $-73.5 \pm 3.1°$ (C 5.1 in water) (Exp. 3). The specific rotation of L-fucose is cited in the literature as being -75.9 (130).

The constant melting point of the osazone derivative of the compound present in Peak 1. was 174 - 176° (Exp. 3). The published value for the osazone derivative of L-fucose is 178° (131).

Fig. 6



Fractionation of sugars obtained from bracken polysaccharide after hydrolysis with 2N H_2SO_4, using a column of powdered cellulose eluted with water saturated n-butanol. (Exp. 3).

These results appear to establish beyond doubt that the sugar in question is indeed L-fucose.

8. Investigation into the acidic component present in the polysaccharide.

Further work into the nature of the acidic component present in the polysaccharide was carried out.

As mentioned earlier the results obtained from the paper chromatographic investigation (page 25) indicated a uronic acid residue but there still remained the possibility that the polysaccharide might also be sulphated to some extent.

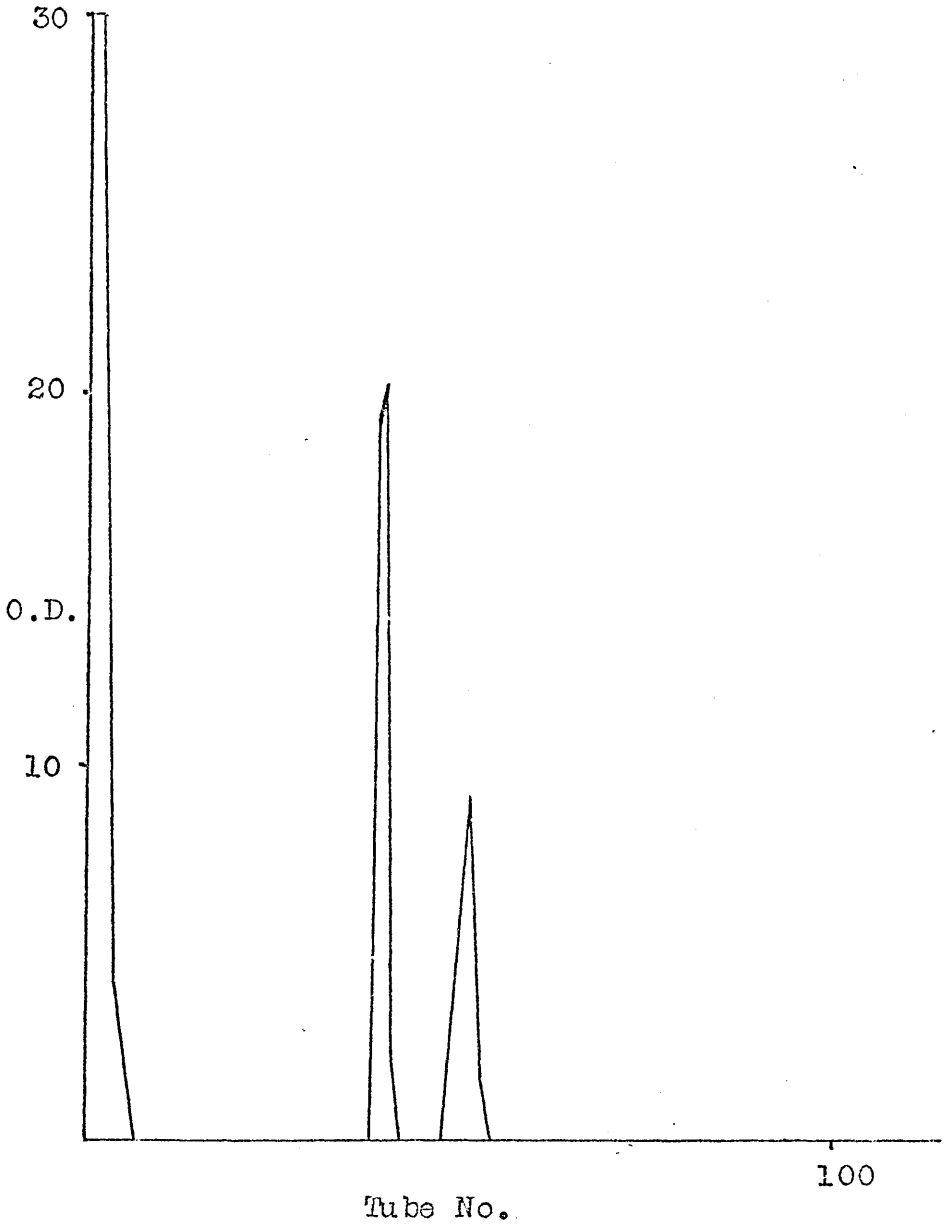
An attempted fractionation of the charged groups present was performed on a column of Dowex ion-exchange resin in the acetate from (G.M.2). This was done (Exp. 4) by eluting a sample of the acid hydrolysate (G.M.3a) of the polysaccharide with a linear gradient of 0 to 1.0M acetic acid, after the method of Khym and Doherty (132). 25ml. fractions were collected and analysed by the Somogyi/Nelson reaction (133) (G.M.11).

A complete separation of the hydrolysate into three fractions was achieved (FIG. 7). The first fraction was eluted without retention and would be expected to contain neutral sugars, while the other two fractions would be expected to contain acidic components.

Examination of the three fractions by paper chromatography using a solvent mixture of phenol - water and benzidine TCA to locate the spots, showed that fraction one contained D-galactose, D-mannose, L-arabinose, D-xylose and L-fucose while the other two fractions both gave characteristic uronic acid streaks, indistinguishable from each other.

The presence of two differently charged moieties in the polysaccharide hydrolysate was confirmed by paper electrophoresis of the hydrolysate (G.M.9).

Fig. 7



Fractionation of acidic components from bracken polysaccharide after hydrolysis with 2N H_2SO_4, using acolumn of Dowex 1, CH_3COO^-, eluted with a linear gradient of 0-1M acetic acid. (Exp. 4).

The electrophoresis was carried out in a citrate buffer at pH 5. The separated bands were located using benzidine TCA reagent (G.M.5), showing clearly a large stationary band at the origin and two charged bands, quite separate from each other, towards the anode. When the pH of the buffer was lowered to 2.5 and the experiment repeated both charged bands were suppressed equally in their migration. At pH 5.0 the two charged bands moved 9.5 and 6.5cm. respectively from the origin towards the anode. Electrophoresis at pH 2.5 at the same voltage and for the same length of time reduced this movement by~2.5cm. in each case.

Repetition of the electrophoresis at pH 5.0 and elution of all three bands from the electrophoretogram with water, followed by paper chromatography of the concentrated eluants using a solvent mixture of phenol - water (G.M.4) and benzidine TCA to locate the spots, confirmed the results from the ion-exchange column. The large stationary band containing D-galactose, D-mannose, L-arabinose, D-xylose, and L-fucose, while the two charged bands both gave pink coloured 'fish tail' effects on the paper.

It could reasonably be concluded from these investigations that the two acidic groups were both uronic acids : because of the characteristic fish tail effect given on paper chromatography and also because both were equally suppressed in their migration when the pH was lowered from 5 to 2.5 during electrophoresis. It may be that one is a monouronic acid while the other is an aldobiuronic acid. Both could arise from the conditions used during hydrolysis of the polysaccharide.

This is supported by evidence from the literature. Hulyalkar, Ingle and Bhide (134) reported the production of an aldobiuronic acid, 6-β-D-glucuronosyl-D-galactose, upon the hydrolysis of a gum from *A. catechu* with 10% (~2N) sulphuric acid. Also, Falconer and Adams (135) upon the hydrolysis of hemicellulose B from

oat hulls with 4% (~N) sulphuric acid obtained two aldobiuronic acids, 2-O-(4-O-methyl-α-D-glucopyruronosyl)-D-xylose and 2-O-(α-D-glucopyruronosyl)-D-xylose. Aldobiuronic acids have been obtained on many other occasions by the graded hydrolysis of plant gums and mucilages (136, 137, 138, 139, 140).

Therefore since the hydrolysis of the polysaccharide from bracken was performed using 1.5-2N (5-10%) sulphuric acid, the formation of an aldobiuronic acid would not be too surprising.

9. Quantitative analysis of the polysaccharide.

A complete analysis was carried out, mainly to determine in what percentage L-fucose was present but also to confirm, if possible, whether sulphate was absent as indicated by the previous experiment.

In the ash and sulphate determinations two polysaccharide samples were used: one being the polysaccharide after alcoholic precipitation (Exp. 1, page 22), and the other being 'CETAB treated polysaccharide' (Exp. 2, page 24). Otherwise, for the remaining analyses only the polysaccharide after alcoholic purification was used.

Moisture was determined using a heating pistol under vacuum, at a temperature of 85° and containing phosphorus pentoxide (Exp. 5).

Ashing was performed firstly by charring the sample over a bunsen then heating in a muffle furnace at 500° (Exp. 5).

The method of Treon and Crutchfield was used to determine sulphate content (141). This is a turbidimetric method involving the precipitation of sulphate as barium sulphate. The turbidity produced is measured using a spectrophotometer at 370mμ. The percentage sulphate is calculated by reference to a standard graph (Exp. 5).

Uronic acid was determined by titrating a solution of the free acid of the polysaccharide with 0.01N NaOH and taking the molecular weight of a hexuronic acid (anhydro) to be 176 (Exp. 5).

The estimation of hexose and methyl hexose in the presence of other sugars obviously poses problems of accuracy. However the L-cysteine/sulphuric acid method of Dische (142, 143) (G.M.11) claims to be able to determine both hexose and methyl hexose separately in solutions also containing pentoses and uronic acids.

Dische's method involves heating a solution of the unknown hexose or methyl hexose with sulphuric acid (86%), cooling and adding L-cysteine HCl and measuring the intensity of the colour produced using a spectrophotometer. However the intensity is not read at the wavelength at which it is at a maximum as this would entail and include interference from other sugars present. To eliminate this interference what is known as dichromate readings are taken. For example, the absorption maximum for hexoses in this reaction is at 414mμ. The interference from pentoses, which is small, and methyl hexoses can be eliminated by taking advantage of their absorption maxima. As methyl hexoses have their maximum at 400mμ, at 380mμ the optical density of methyl hexoses is equal or very nearly equal to the optical density at 414mμ. Hence the difference, 414mμ/380mμ is proportional to the concentration of hexose in solution. While being zero for methyl hexoses. Interference from pentoses and hexuronic acids in these dichromate readings is negligible.

The maximum for methyl hexoses is at 400mμ. Pentoses and uronic acids do not interfere significantly at this wavelength but hexoses do. However hexose readings can be eliminated by dichromate readings at 396mμ and 426 - 428mμ. Thus Δ O.D. 396mμ/426 - 428mμ is proportional to the concentration of methyl

hexose in solution while being zero for hexoses.

Using this technique, therefore, the hexose and methyl hexose content of the polysaccharide was determined using the same sample (Exp. 5).

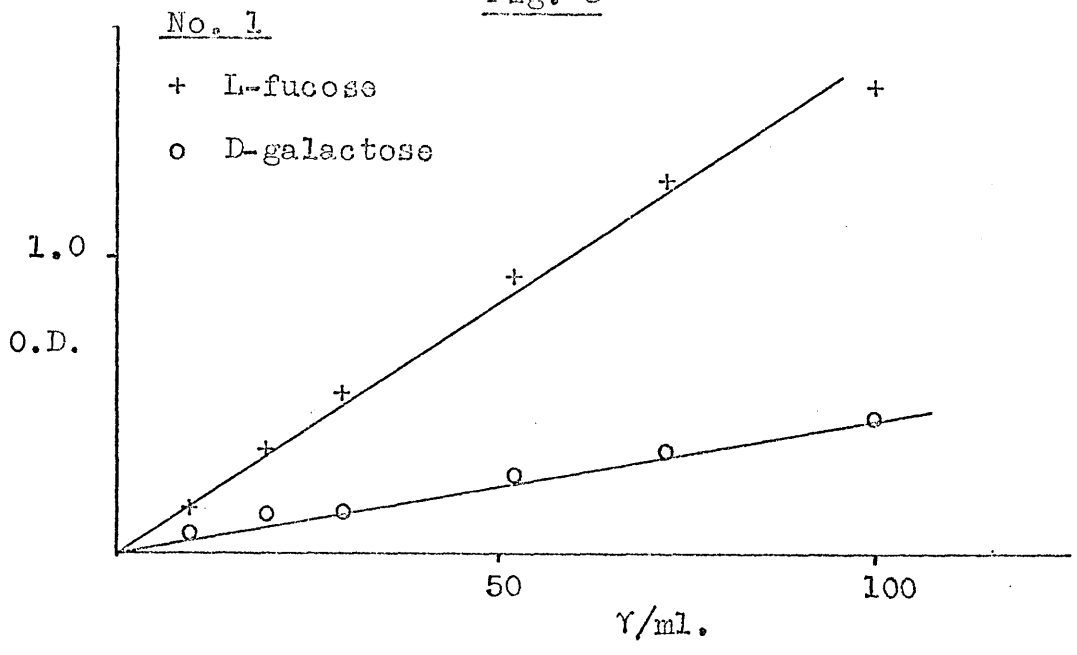
Firstly standard graphs were prepared by two methods. Since the qualitative analysis showed that glucose was present in trace amounts and that galactose and mannose were the major hexose sugars present in the polysaccharides, D-galactose was chosen as the standard sugar for the hexose determination. The relative extinction coefficients of D-galactose and D-mannose at 414mu are almost the same, 0.5 and0.4 respectively. L-fucose was chosen as the standard for the methyl hexose determination. The first standard curve was prepared using known concentrations of D-galactose and L-fucose in separate solutions and the second was drawn up using them in the same solution (G.M.8) (FIG. 8).

Comparison of the two differently prepared graphs showed that they were both identical in the case of D-galactose and almost identical in the case of L-fucose. It was assumed from this that the galactose had not interfered with the determination of the fucose and vice versa.

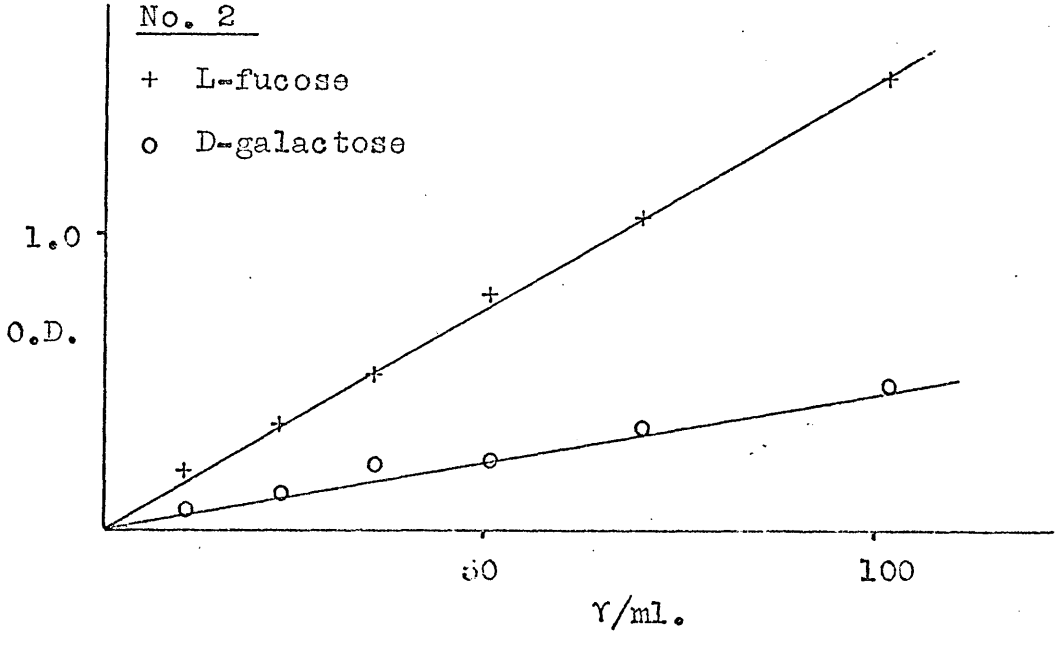
Duplicate samples of different known concentrations of the unhydrolysed polysaccharide were analysed for hexose and methyl hexose content using this L-cysteine/sulphuric acid method of Dische (142, 143). The readings obtained were converted to micrograms/millilitre using the data from the standard graph (FIG. 8 No. 2). This was then used to calculate the percentage hexose and methyl hexose present in the polysaccharide (Table 4).

Pentose was determined using the colorimetric method of Dische and Borenfreund (144,) (G.M.11) in which phloroglucinol, acetic acid, D-glucose and HCl are the component reagents. The unknown sample is heated in a boiling water bath with the above reagents and the colour intensity produced measured using a

Fig. 8



Determination of hexose and methyl hexose in separate solutions by the L-cysteine/H_2SO_4 method of Dische.



Determination of hexose and methyl hexose in the same solution by the L-cysteine/H_2SO_4 method of Dische.

spectrophotometer. This method also depends on dichromate readings to rid any interference from other sugars present. The absorption maximum for pentoses in this reaction is at 552mμ. Uronic acids react to give an absorption at this wavelength but their extinction coefficient is much lower than pentoses and uronic acids produce a different type of absorption curve (145). Hexoses do contribute to the absorption at 552mμ but this can be eliminated by dichromate readings at 552mμ and 510mμ; Δ O.D. 552mμ/510mμ being insignificant for hexoses but a linear function of the pentose concentration. All four aldopentoses have extinction coefficients at 552mμ which are almost exactly the same.

A standard graph using a D-xylose as the standard was prepared using concentrations of 0-125γ/ml. (G.M.17).

Pentose determination was performed using duplicate samples from different known concentrations of the unhydrolysed polysaccharide solution (EXP. 6) The percentage pentose present in the polysaccharide was calculated in a similar manner to that for hexose and methyl hexose (Table 4).

Table 4 summarises the results obtained for this complete analysis. The most unusual feature of this table is that methyl hexose in the form of L-fucose comprises as much as 20% of the sugar content of the polysaccharide. The other interesting result was the sulphate content. The amount found after alcoholic precipitation of the polysaccharide represents 15.0mg/g. or 1.5% which is very low and would be equivalent to only one sulphate group for every thirty units in the polysaccharide chain. After treatment of the polysaccharide with CETAB, the value fell to as little as 2.0mg/g. or 0.2%. This is a considerable drop on the first result and represent one sulphate every two hundred units. The fact

TABLE 4

Sugars	Analysis	Percentage dry weight	Percentage wet weight
galactose mannose	Hexose	36.5	28.5
fucose	Methyl hexose	25.5	19.5
xylose arabinose	Pentose	20.0	15.5
Uronic acid	Titration	11.5	9.0
	Moisture		22.0
	Ash (EtOH pptn.)	1.8	1.4
	Ash (CETAB pptn.)	0.5	0.4
	$SO_4^=$ (EtOH pptn.)	1.9	1.5
	$SO_4^=$ (CETAB pptn.)	0.3	0.2
	TOTAL	94.3	95.1

Summary of analysis of mucilagenous polysaccharide from bracken rhizomes (Exp. 5).

that the sulphate content drops after treatment of the polysaccharide with CETAB would tend to indicate that it was present as a contaminant rather than as an integral part of the structure of the major polysaccharide fraction.

Table 4 also summarises the sugar content of the polysaccharide found by qualitative chromatography.

PART II

STUDIES ON THE GLYCOLIPID FRACTION FROM BRACKEN RHIZOMES

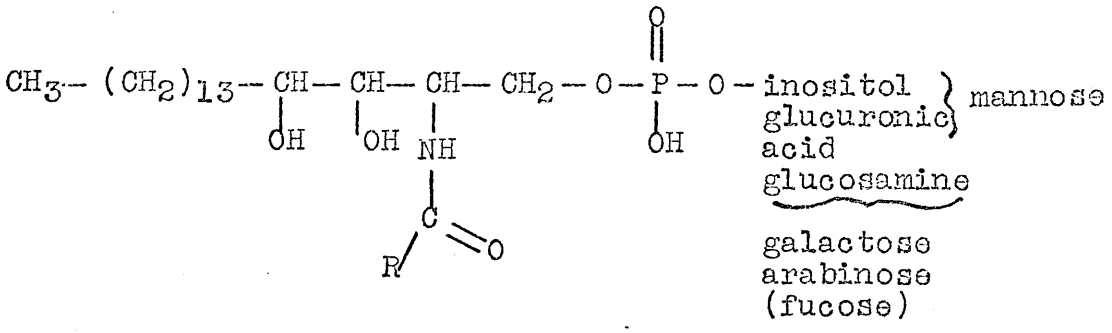
Introduction. In recent years many comprehensive reviews on glycolipids have been written by Law (146), Carter, Johnson and Weber (147). A short review on plant lipids, written by Allen and Good, has also appeared (148). In the following pages a brief account of the various glycolipids so far identified from plant tissue is given.

The importance and widespread distribution of plant galactolipids was first demonstrated by Carter (45). He identified, isolated and characterised both mono and digalactoglyceride from wheat flour (Fig. 1, Nos.(i)&(ii)). This discovery was followed by others which showed that both these glycolipids were present in many different plants and algae (149, 150, 151, 152, 153, 154, 155). It was found that their concentration was particularly high in chloroplasts and it is now believed that they are probably universal constituents of photosynthetic tissue.

The discovery of the plant sulpholipid (Fig.1, No(iii))by Benson (51) in 1959 followed closely the discovery of the galactolipids by Carter. This sulpholipid was later shown to be composed of a sugar moiety; 6-sulpho - 6-deoxy - α - D glucopyranose, a D-glycerol configuration and two acyl groups (156, 157, 158). The sulpholipid was also shown to be present in all photosynthetic tissues examined (156).

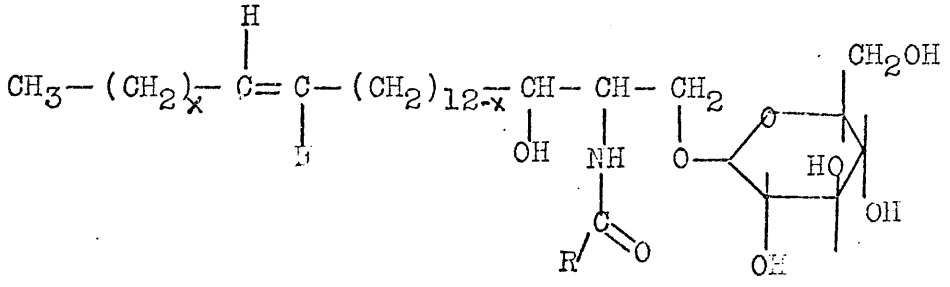
At about the same time the presence of phytoglycolipid (Fig.9 No.i) in a variety of oil seeds including soybean, corn, wheat, cotton, peanut, flax and sunflower, was demonstrated by Carter(159). Figure 9 No.i gives a partial structure for the sphingosine analogue

Fig. 9



No. i

Phytoglycolipid.



No. ii

Glucocerebroside

(containing an isomer of sphingosine).

of phytoglycolipid. The term 'phytoglycolipid' is used by Carter (159) for a group of inositol sphingolipids. The point of attachment of the hexose units remains uncertain, but it appears that the oligosaccharide is derived from a common glucosamido - glucuronido - inositol structure. Depending on the plant source, fucose may sometimes be found. The mannose is thought to be attached through the inositol or glucuronic acid. The function of the phytoglycolipid is as yet unknown.

Again, it was Carter who confirmed the presence of cerebrosides in plant tissue (160, 161). Using wheat flour he isolated a compound shown to be composed of glucose, α-hydroxystearic acid and four long chain bases; phytosphingosine, dehydrophytosphingosine, dihydrosphingosine and an isomer of sphingosine (Fig. 9, No.ii). A glucocerebroside has also been isolated from runner beans by Sastry and Kates (162). A less conventional cerebroside which is thought to be a trimannoside has been detected in wheat (153).

More recently glycolipids of another type have been found in plants. The occurence of free steryl glycosides in various plants has been known for some time and is described in several reports (163, 164, 165, 166, 153). However, in 1964, Lepage isolated and characterised an esterified steryl glucoside from potato tuber lipids and soybean phosphatides (167). It has been identified in peas, alfalfa and wheat (168). It was shown to be composed of sterol, glucose and a fatty acid in the ration 1:1:1. Four sterol components were detected including β-sitosterol and stigmasterol. The fatty acids identified were palmitic, stearic, oleic, linoleic, and linolenic. The sterol appears to be attached, glycosidically, to carbon one of the glucose, whereas

the fatty acid is esterified to carbon six of the sugar. From this, Lepage assigned a steryl - 6 - acyl - D - glucoside structure to the new glycolipid class

Notes on the extraction, purification and fractionation of plant lipids.

a) Extraction. The term 'lipids' refers to a heterogeneous collection of biochemical compounds which have in common the property of being variably soluble in organic solvents, such as ether, petroleum ether, chloroform and methanol. Lipids therefore include compounds not necessarily related to each other structurally, and range from nonpolar compounds such as hydrocarbons, pigments, sterols, as well as free fatty acids, triglycerides, mixed glycerides to the more polar glycolipids and phospholipids.

Initially lipids were extracted from plant material using solvents which were purely organic in nature, such as ether or petroleum ether. However with the discovery of lipids containing polar groups these older extracting solvents have been, on the whole, replaced by ones designed to extract a wider spectrum of lipoidal substances.

Glycolipids, by nature of their structure contain not only a lyophilic fatty acid part but also a lyophobic sugar moiety. When undertaking an extraction of such a group of compounds a purely nonpolar solvent would not be the most desirable choice. The introduction of a more polar element into the extracting medium has therefore resulted. 90% methanol has been used to extract the glycolipids from potato starch (169). Here it is likely that virtually all types of glycolipids, no matter how polar, will be extracted to a greater or lesser extent. The main disadvantage of methanol is that it probably

dissolves much nonlipid material, such as free sugars, and amino acids from the tissue as well.

The most commonly used solvent mixture for the extraction of lipids is chloroform-methanol, 2:1 v/v, introduced by Folch (170). With its use, the risk of nonlipid contamination is greatly reduced. It may be argued that since the solvent is less polar than methanol by itself some of the more polar lipids will not be isolated. However the fact that the plant material under investigation probably has a high moisture content, in the case of bracken rhizomes up to 80%, means that the chloroform-methanol extractant will become more aqueous and hence more polar during the isolation process, consequently reducing the risk of incomplete extraction. This at the same time increases the risk of nonlipid contamination but this should still be less than using methanol alone.

b) Purification. It is obvious that in the study of glycolipid material from plant tissue all traces of nonlipid carbohydrates must be completely removed before analysis can commence. This at first sight may appear to be a relatively simple process but it is complicated by the difficulty in distinguishing what is lipid bound and what is not lipid bound carbohydrate. This complication arises from the following situation. If a lipid extract is shaken with water in a separating funnel to remove nonlipid material, it is more than likely that an emulsion will be formed which cannot easily be broken. Also during the isolation of lipids from the plant source micelle formation can take place in the organic extracting medium. Micelles as opposed to emulsions are not visible, as the water droplets which they are composed of are so small that they can be present in an apparently

homogeneous organic phase. These micelles being aqueous can themselves trap nonlipids such as free sugars and amino acids. The formation of micelles is believed to be due to a converse detergent effect in which the polar lipids orientate around the surface of the micelle offering a lyophilic chain to the organic medium and a lyophobic part to the aqueous phase. In this fashion the micelle is stabilised (171) For these reasons, therefore, purification by the most obvious method of shaking with water in a separating funnel is of little or no use. Any successful method adopted must succeed in either preventing the formation of both emulsions and micelles or, if they are formed, must be able to break them down.

Most workers have concentrated on both these aspects. That is to say, during extraction a certain amount of micelle formation inevitably takes place, but in the purification of the extract, various methods have been devised to prevent further formation, while at the same time attempting to breakdown those micelles already formed during the extraction procedure.

In the Folch technique (170), the lipid extract is treated with 5-20 volumes of water by a diffusion technique between two beakers. This is performed by placing a small beaker containing the chloroform-methanolic lipid extract, inside a larger one. The larger beaker is then slowly filled with water completely covering the small one. This prevents micelle formation while at the same time allows nonlipid contaminants and those micelles already formed to diffuse from the organic phase into the aqueous layer in the outside beaker. This diffusion takes place, in part, due to the gradual pull of the methanol as it moves from the organic phase in the small beaker into the aqueous

layer, in an attempt to equilibriate between the two phases.

In the second method of Folch(172, 173) which is basically the same as the first, a much smaller amount of water is used in the washing step. As a result the aqueous layer eventually contains a much higher concentration of methanol and chloroform and as a result is a fair lipid solvent itself. This can result in the loss of some lipid material.

In connection with the above technique of Folch, Svennerholm proposed (174) that if a salt solution, 0.1% NaCl or $CaCl_2$, was used in place of water as the washing medium, it would help to prevent the loss, during purification, of some water soluble lipids, such as sulphatides and gangliosides. However no glycolipids of the above types have so far been found in lipid extracts from plant tissue and as a result it was decided that during the purification of the lipid extract from bracken using this technique of Folch, water and not salt was quite suitable as the washing medium.

A further modification of the Folch technique is to dialyse the chloroform-methanol extract against a large volume of water (175). The mechanism of breakdown of the micelles and removal of nonlipid contaminants from the organic phase is similar in this modification to that of the original Folch technique. The methanol diffuses into the aqueous phase taking with it the micelles and other nonlipids. The main disadvantage of this modification is that high molecular weight lipoprotein, soluble in the original chloroform-methanol extract but insoluble in either chloroform or water, cannot pass through the dialysis sac but remains as a white oily mass at the interface between the chloroform and water inside the bag.

A preliminary treatment prior to dialysis can ease this situation. If the crude extract is reduced to dryness several times, under vacuum and at a low temperature, and redissolved in increasingly smaller volumes of chloroform-methanol, 2: 1 v/v, this results in the denaturation of a substantial amount of the lipoprotein as well as getting rid of some of the nonlipid carbohydrate. If this is then followed by dialysis, lipoprotein precipitation at the interface is greatly reduced.

The other main disadvantage of dialysis, which is common to all the methods based on the Folch technique, is that it is time consuming.

Cellulose column chromatography has been used by both Rouser and Svennerholm (176, 177) as a means of purifying lipid extracts. The procedures they employ are different, and both appear to be difficult to operate in a reproducible manner. In the method used by Rouser involving elution of the column with chloroform-methanol, 9:1 v/v, to remove the major lipid classes, followed by methanol-water, 9: 1 v/v, to elute nonlipids, a good separation between the lipid material and the nonlipid material was not obtained when this procedure was tried (Exp.10, page 98). Because of the difficulty of reproducibility, the use of cellulose column chromatography in the present context was not pursued.

More recently Wells and Dittmer (178) have suggested that the cross-linked dextran gel, Sephadex, could be a useful column chromatographic technique for the separation of lipids from nonlipid contaminants. Following this in 1965 Siakotos and Rouser (179) described a procedure for the analytical separation of nonlipid water soluble compounds from other lipids by dextran gel chromatography. Lipids, other than gangliosides, are eluted with chloroform-methanol saturated with water, gangliosides are eluted with a mixture of chloroform, methanol, water, containing acetic

acid, and finally water soluble nonlipids are removed with a mixture of methanol and water.

The Folch technique was finally chosen for the purification of the lipid extract from bracken, after other methods had been tried and found unsucessful. However the more recent procedure employing Sephadex column chromatography would appear to be very promising. This method was not used as the major portion of the work had been performed before the method was published.

c) Fractionation. Originally the techniques employed in the fractionation of lipid extracts depended on the partition of the different classes between immiscible solvents. Phospholipids were originally thought to be insoluble in acetone and hence if a lipid mixture was shaken up with hot acetone then all the lipids except the phospholipids would be extracted. This was, however, later shown not to be the case, in that certain phospholipids are in fact dissolved.

Carter in his experiments concerned with the isolation of galactolipids from wheat flour employed this system of liquid partition (45). He used methanol and heptane in the belief that polar lipids would preferentially dissolve in the methanol while less polar lipids would prefer the heptane. While this system does work it has the disadvantage that the phases take some time in separating and also that lipoprotein material collects at the interface making a clean separation more difficult.

Liquid partition techniques have now almost been completely replaced by the discovery of several solid materials, which can effect the fractionation of lipid mixtures into their various classes by means of column chromatography based on the principles of adsorption and ionexchange. These more novel techniques can be used by themselves but are more effective when used in conjunction with other analytical tools such as thin-

layer and gas-liquid chromatography. In combining all three techniques the fractionation of lipid mixtures can be acheived not only into different classes but also into individual lipid compounds. Thin-layer and gas-liquid chromatography can be further employed in the investigation of the structure of the individual lipid compounds obtained.

The following pages give an account of the three main column chromatographic media used to effect a separation of lipid mixtures. They are:-

1. Silicic acid
2. Magnesium silicate
3. DEAE cellulose

1. Silicic acid, sometimes called silica gel, has been the most widely used material for the column fractionation of lipids (153, 180). Its main use has been in its effectiveness in separating neutral from acidic and other polar lipids. However, commercial preparations of silicic acid from different sources vary considerably in their ability to perform this separation. The most important variation is the presence of contaminants such as sodium silicate and water. Rouser states (180) that the ionic state of the lipids, fatty acids in particular, on a silicic acid column depends to a great extent on the amount of silicate and other salts present in the preparation. The more silicate or salt present, the less acidic is the column and as a result the fatty acids applied to it become more ionised, and as there is also an ion-exchange effect as well as an adsorption one (181, 176) these fatty acids become more difficult to remove from the column. The converse is also true the less silicate there is present. Silicic acid preparations, therefore, were always pretreated (G.M.13) to remove any sodium silicate present, ensuring that fatty acids were not retained by the column.

This pretreatment also ensured that the preparation was completely dry before being used, as the presence of small amounts of water in the column is also associated with increased binding of the lipids (181, 176, 182).

Although it is possible to separate neutral from acidic and other polar lipids on silicic acid columns, it is also possible to fractionate the polar lipids into classes by eluting stepwise with increasing concentrations of methanol in chloroform (Fig.10) or mixtures of hexane, ether and chloroform (167).

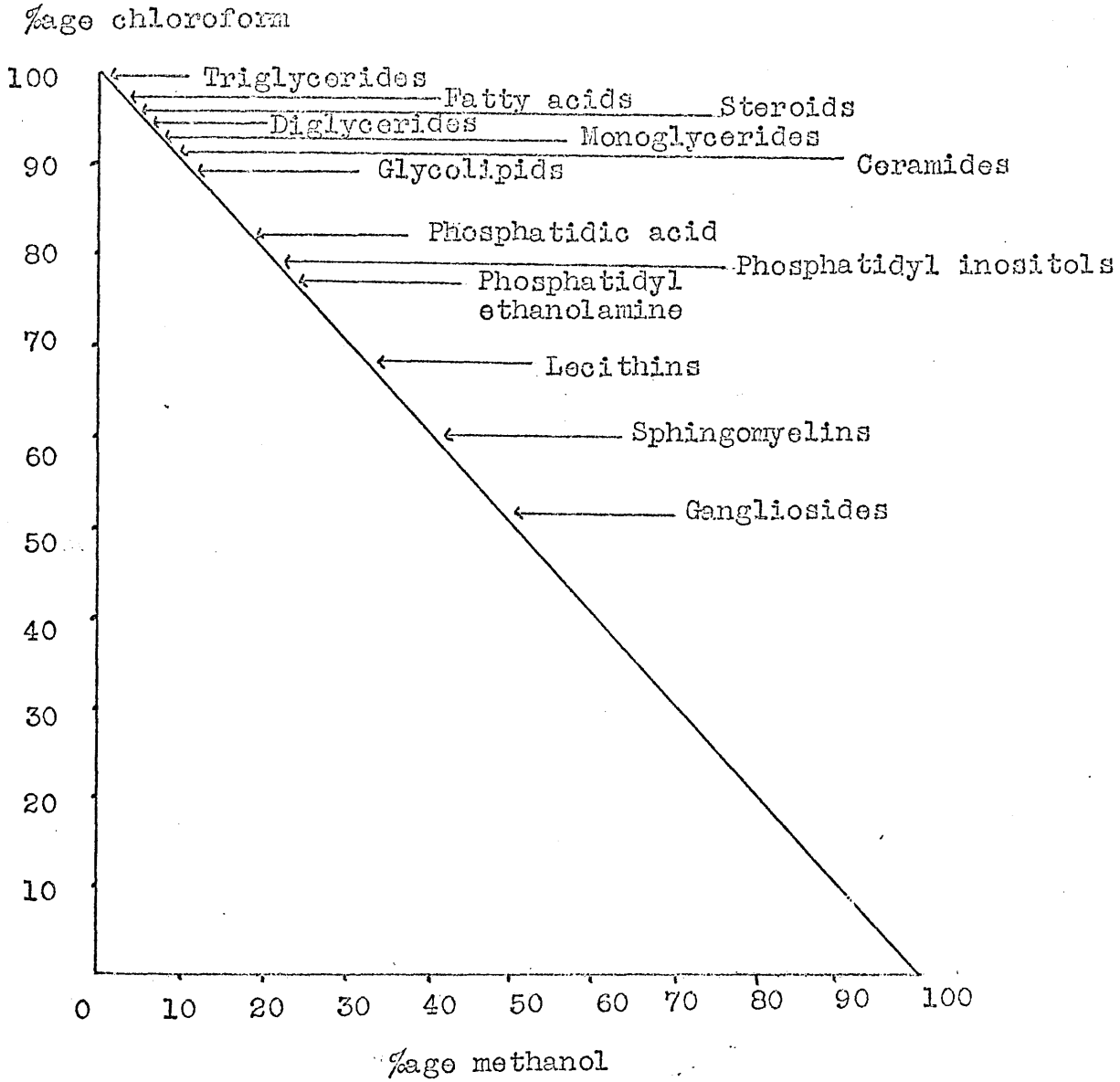
The degree of unsaturation of the fatty acids of lipids from the same class has been shown to have little effect upon their order of elution from silicic acid columns (183).

During the present investigation it was found that the silicic acid preparation supplied by the Mallinckrodt Chemical Works was quite suitable and is in any case generally used by others. It is a uniform mesh size with a low content of sodium silicate.

2. Magnesium silicate columns (Florisil) have also been used in the fractionation of lipids. It was first used for this purpose by Hadin (184). It has been employed in the fractionation of neutral lipids, the resolution of individual neutral lipid classes (185) and the fractionation of individual polar lipid classes (181, 176). Magnesium silicate is a much stronger adsorbant of lipids than silicic acid and must be heat activated and kept in a thoroughly dry state before and during use.

Benson (186) employed Florisil with increasing concentration of methanol in chloroform to fractionate a lipid extract from alfafa leaves into neutral lipids, followed by glycolipids and finally acidic lipids composed mainly of phospholipids. He found that the plant sulpholipid (fig.1, No.[illegible]) was eluted mainly in the second fraction. This is contrary

Fig. 10



The order of elution of lipids from silicic acid columns with chloroform-methanol mixtures (103).

to the results found in a similar separation performed on the lipid extract from bracken which showed that the majority of the sulpholipid was retained by the column along with the phospholipids (Exp.14).

3. The third material extensively used is diethylaminoethyl (DEAE) cellulose. It was first used for the fractionation of lipids by Rouser in 1961 (187). It can be employed either on a complex unfractionated mixture or on a partially fractionated one. It is mainly used to separate acidic lipids as a group from nonacidic lipids.

Both Rouser (181, 176) and Svennerholm (188) have used DEAE cellulose. However their procedures differ somewhat although both are based on the principle of ion-exchange. In Rouser's method the DEAE cellulose is firstly washed with acid and alkali then converted into the acetate form with glacial acetic acid before being packed into the column. The lipid mixture applied to the column is washed with a series of up to seven eluting agents, ranging from chloroform-methanol, 9:1 v/v, to remove neutral lipids, to chloroform-methanol, 4:1 v/v, containing aqueous ammonia and ammonium acetate to remove acidic lipids. (181, 176). More recently (180) Rouser has also devised a system whereby the column is washed with a series of solvents prior to the lipid mixture being applied, to prevent the formation of channels in the column due to a sudden change of solvent from a nonpolar to a polar one.

In contrast to this rather complex system, Svennerholm's method is relatively simple in that the DEAE cellulose is used in the hydroxyl form in which it is provided. Also conveniently, only three eluting steps are used. Chloroform-methanol, 2:1 v/v, is used to remove neutral lipids and zwitter-ions such as phosphatidyl ethanolamine, chloroform-methanol, 2:1 v/v, plus 5% glacial acetic acid to remove any altered lipid material and nonlipid contaminants, and finally chloroform-methanol, 2:1 v/v, plus

5% 0.5N. aqueous lithium chloride is used to remove acidic lipids.

DEAE cellulose does not have a very large capacity and as a result must not be overloaded with sample. Rouser (181, 180) recommends that no more than 200 mgs. of lipid mixture should be applied to a column containing 15g. cellulose.

It was found after several trials that the procedure of Svennerholm gave the best results in the present work. The procedure was much simpler, the cellulose not requiring any elaborate pretreatment, and the results obtained were as satisfactory as those obtained by the method of Rouser.

Thus to summarise; the results of preliminary trials with these various techniques led finally to the following procedures being adopted.

a) When it was desired to separate neutral lipids from acidic and glycolipids, silicic acid was used.

b) Magnesium silicate was used to fractionate glycolipids from phospholipids.

c) DEAE cellulose was used to separate acidic glycolipids from neutral glycolipids.

In all of the above column separations, thin-layer chromatography on silicic acid (189) (S.M.4) was used to monitor the fractions obtained. TLC was also used in preparative analysis of the various fractions from the columns.

SECTION A

The extraction of the lipid fraction from bracken rhizomes

Because of the possibility of oxidative degradation of certain lipids the following precautions were taken:-

a) Lipid samples were handled as much as possible in solution, e.g., chloroform-methanol, 2:1 v/v.

b) High temperatures were avoided.

c) Samples were stored in solution, in the dark, in a deep-freeze when not being used.

d) Where possible samples were kept in an atmosphere of nitrogen.

Solvents used in the extraction of the lipid fraction were not redistilled but in the purification or fractionation of the extract all solvents were redistilled or dried and stored over a suitable drying agent before being used (G.M. 6).

In the initial work done on the glycolipids from the bracken various methods of extraction, purification and fractionation were tried to determine which were the most effective and practical. The work at this stage was mainly of a qualitative nature.

Throughout the whole investigation, alkaline silver nitrate was the detecting reagent used to locate sugars on paper chromatograms.

In these preliminary studies the following bracken preparations were used:-

1. Freshly dug rhizomes which were washed and chopped into lengths of approximately one inch.

2. Freshly dug rhizomes which were washed, dried in an air blown oven at 110° for twenty four hours, then reduced to a fine powder in a hammer mill.

3. Freshly dug rhizomes which were washed, chopped into lengths of one inch, then immediately freeze-dried.

4. Freshly dug bracken, washed, chopped, freeze-dried, then powdered finely in a hammer mill.

I. First method of extraction.

This was carried out by blending 3kg. freshly dug rhizomes, which were chopped into lengths of one inch, with methanol at room temperature

(G.M. 1a). It was assumed that degradative enzymes released during the blending would be inactivated by the methanol. On reducing the volume of the filtered extract to dryness using a large scale vacuum distillation apparatus followed by a rotary evaporator, it was obvious that a large quantity of nonlipid material, mainly carbohydrate, had also been extracted. This crude lipid extract was purified (G.M.1a.) by refluxing it with chloroform-methanol, 2:1 v/v, leaving the majority of the nonlipid material undissolved. This was filtered and the filtrate purified by diffusion against approximately twenty volumes of water (G.M.1a), according to the method of Folch (170). Any interfacial lipoprotein which appeared was filtered off leaving 4.7 g. of lipid mixture.

The presence of nonlipid carbohydrate was tested for by thin-layer chromatography on powdered cellulose using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4). It was expected that any lipid material present would be carried with the solvent front while free sugars would be left further behind. A special alkaline silver nitrate dip was used to locate the spots (G.M. 5). Using this monitoring system no free sugars could be detected in the lipid mixture.

This extraction and purification procedure appeared to be quite satisfactory. However there was a risk of extracting a very high proportion of non-lipid contaminant using a polar solvent such as methanol as the extracting agent. This situation was aggravated by the fact that fresh bracken rhizomes contain up to 80% moisture. Thus if 3 kg. are extracted with 8 litres methanol, the final extracting solution eventually contains as much as 30% water. This would also result in the methanol being too aqueous to dissolve the more hydrophobic lipids from the bracken.

To determine if any lipid-bound sugars were present in the lipid

mixture, an initial qualitive study was carried out.

A sample, 1.5g, of the lipid mixture was submitted to a procedure designed to separate the saponifiable and nonsaponifiable fractions. Thus after refluxing with 10% methanolic KOH (G.M.10), the resultant reaction mixture was acidified with sulphuric acid and the nonsaponifiable fraction removed by extraction with ether. The water soluble products of the saponification step were hydrolysed with 2N H_2 SO_4 (G.M. 3), the hydrolysate filtered and the filtrate examined for the presence of sugars by both thin-layer chromatography and paper chromatography, using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4). Using standard sugars as a reference and alkaline silver nitrate to locate the spots (G.M. 5) the presence of glycerol, galactose and a slow running spot was confirmed. A fourth very faint spot with an Rg. value similar to that of glucose was also observed. These results are consistant with the possibility that the galactose was present in the form of the mono and digalactoglycerides first discovered by Carter in wheat flour (45) (Fig. 1, Nos. i and ii). On the basis of comparisons with information from the literature (51, 186, 190) the unidentified slow running spot could possibly be in the form of a sulpholipid similar to that discovered by Benson (51) (Fig. 1, No.iii). The origin of the faint spot corresponding to glucose is, however, more obscure.

It therefore appeared certain that at least three, and possibly more lipid-bound sugars were present in the mixture.

To investigate the possibility of other lipid-bound sugars being present, the lipid mixture was further purified by column chromatography (Exp.6). Neutral lipids, including fatty acids, were removed with the aid of a silicic acid column, which was prepared (G.M. 7) using pretreated material (G.M. 13). To the column was applied 3.5g. lipid mixture,

followed by the elution of neutral lipids with chloroform, the remaining polar lipids being eluted directly with methanol. The first fraction was discarded. The second weighing 1.5g. after solvent removal, should now contain all the glycolipids present in the lipid mixture, together with phospholipids and other polar compounds.

To separate this mixture further (Exp.6) a DEAE cellulose column with effective dimensions 20.0 x 3.2 cm. was prepared (G.M. 7). The elution pattern used was that of Svennerholm (138). The mixture was applied to the DEAE column in a solution of chloroform-methanol, 2: 1 v/v. Three separate fractions were then eluted off, using chloroform-methanol, 2: 1 v/v, chloroform-methanol, 2: 1 v/v, containing 5% glacial acetic acid, and finally chloroform-methanol, 2: 1 v/v, containing 5% 0.5N aqueous lithium chloride.

All three fractions were examined by thin-layer chromatography on silicic acid (G.M. 4). The lipid spots were located using iodine vapour (G.M. 5). This analysis confirmed the belief that a good separation of the mixture had not been achieved.

Half of each of the three fractions were saponified with 10% methanolic KOH (G.M. 10) and the reaction mixtures extracted with ether after acidification with sulphuric acid. The three aqueous soluble fractions from the saponification were hydrolysed with sulphuric acid (G.M. 3a) and the hydrolysates examined for sugar content by thin-layer chromatography on powdered cellulose using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4). The Rg. values of the unknown spots were compared with those from standard sugars (Table 5).

All three fractions were found to contain glycerol, D - galactose and D - glucose as expected, as a result of the poor separation on the DEAE cellulose column earlier referred to. Only the slow running spot

TABLE 5

Standards	Rg (stds)	Rg (Fr 1)	Rg (Fr 2)	Rg (Fr 3)
Glycerol	144	144	144	144
D-glucose	100	100	100	97
D-galactose	88	88	86	87
				38

Rg values for standards and unknown sugars obtained from the aqueous extracts of fractions 1, 2 and 3 from the DEAE cellulose column (Exp.6) after saponification with 10% KOH. Solvent: ethyl acetate-pyridine-water, 120:50:40 v/v.

(Rg. 38) was confined to one fraction, namely the third. Its presence in the acidic fraction supports the view that it is a charged sugar, probably derived from a sulpholipid. (186) Also when it was run on a paper chromatogram a round concise spot was obtained unlike the characteristic fish tail appearance given by a uronic acid. Similarly, hydrolysis with sulphuric acid of this charged glycolipid did not appear to remove the acidic group present on the sugar (if it had the sugar would have run much faster on the chromatogram). This is also characteristic of an acid resistant sulphonate group, as in sulpholipid (190).

The fact that the other three compounds were present in all the fractions from the DEAE cellulose column was probably due mainly to gross overloading of the column with sample. However it may be that the separation is dependent to some extent upon polarity, as well as ion-exchange. For example fatty acid constituents which have polar side chains, may have some effect. Similarly glycolipids containing two or more sugar units may be more strongly held than those containing only one sugar molecule.

The overloading of the DEAE cellulose column was not thought to be of prime importance in these initial investigations, as the main purpose was to remove nonglycolipid material and not effect an accurate or quantitative fractionation of the glycolipids themselves.

This experiment confirmed the results from the previous paper chromatographic investigation. No more lipid-bound sugars were detected.

So far the results obtained with the glycolipids from the saponifiable fraction of the lipid mixture only have been described. The nonsaponifiable lipids present in the three fractions from the DEAE cellulose column (Exp.6) were therefore examined for the presence of combined sugars (Exp. 7). The remaining halves of Fractions 1 and 2 were combined, as it was thought that no useful purpose would be gained by treating them separately.

This combined fraction and the remaining half of Fraction 3 were both saponified with methanolic KOH (G.M.10). After acidification, the two mixtures were extracted with ether. The residues of both ether extracts, after removal of the ether, were further purified by passage through a small silicic acid column, washing it firstly with chloroform to remove any neutral lipids, followed by methanol to remove the remaining lipids (183). A fresh column was used for each extract (G.M. 7). The first fraction in each case was discarded as these consisted of nonglycolipid material. The two second fractions were retained, reduced to dryness, and hydrolysed with 2N H_2SO_4 (G.M. 3). The hydrolysates were examined for the presence of sugars by paper chromatography using the solvent systems, ethyl acetate - pyridine - water and phenol - water (G.M. 4).

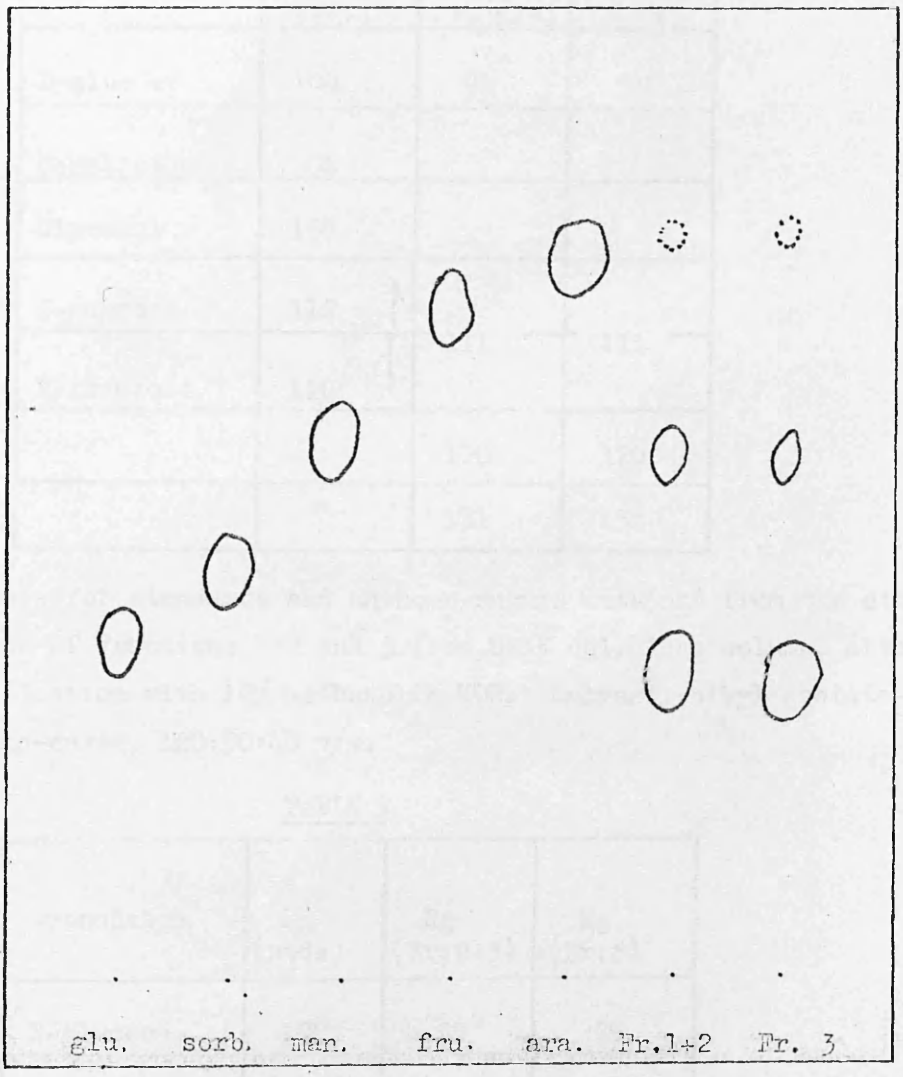
Both fractions contained a similar sugar pattern (Fig.11, Tables 6 and 7), each containing four possible sugars, two of which were in fairly large amounts and behaved identically with the D - glucose and D - mannose standards, in both solvent systems. The other two spots were present in trace amounts, one of which chromatographed as L - arabinose, but the other faster running one, was unidentified.

This experiment demonstrated the presence of lipid-bound sugars in the nonaponifiable fraction of the lipid mixture from bracken rhizomes. Although no useful fractionation of the glycolipids was achieved using this combination of silicic acid and DEAE cellulose column chromatography, their combined use served to rid the lipid mixture of the vast majority of the other lipids which could have masked any small amounts of glycolipid material present in the nonsaponifiable fraction of the mixture.

2. Second method of extraction.

The second method of extraction of the lipid material from bracken rhizomes made use of material dried at a temperature of 110° for twenty

Fig. 11



Paper chromatography of standard sugars and unknowns obtained from the etherial extracts of Fractions 1+2 and 3 from DEAE cellulose column, after saponification with 10% methanolic KOH (Exp.7). Solvent: phenol-water, 160:40 w/v.

TABLE 6

Standards	Rg (stds)	Rg (Fr.1+2)	Rg (Fr.3)
D-glucose	100	98	98
D-galactose	84		
Glycerol	158		
D-mannose	112	111	111
D-fructose	110		
		120	120
		131	131

Rg values for standards and unknown sugars obtained from the etherial extracts of Fractions 1+2 and 3 from DEAE cellulose column, after saponification with 10% methanolic KOH. Solvent, ethyl acetate-pyridine-water, 120:50:40 v/v.

TABLE 7

Standards	Rg (stds)	Rg (Fr.2+3)	Rg (Fr.3)
D-glucose	100	99	99
scrbose	107		
D-mannose	118	117	117
D-fructose	133		
L-arabinose	138	139	139

Rg values for standard sugars and unknowns obtained from the etherial extracts of Fractions 1+2 and 3 from DEAE cellulose column, after saponification with 10% methanolic KOH. Solvent: phenol-water, 160:40 w/v.

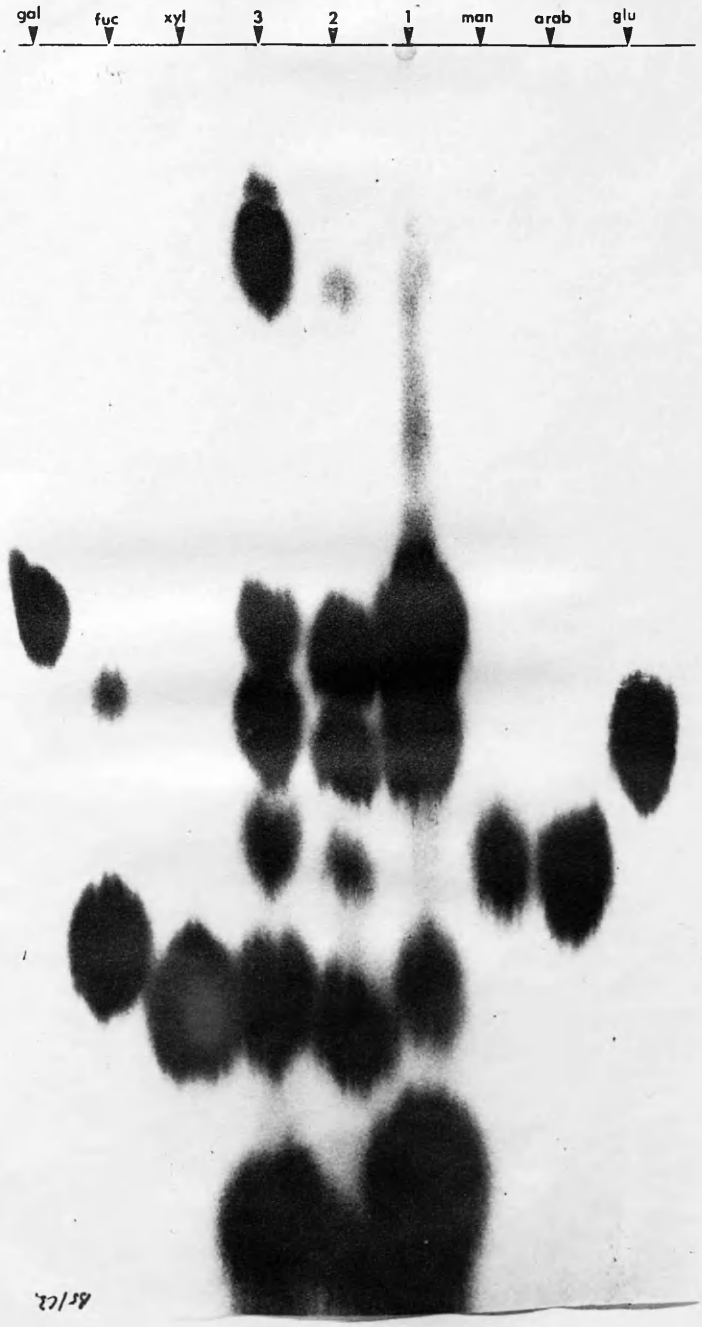
four hours. 5.0 Kg. of freshly dug bracken thus treated had a moisture content of 77%. The dry rhizomes were reduced to a powder in a hammer mill and the lipids extracted by elution of this powder, packed in a large glass column, 16 cm. wide and 60 cm. long, with hot chloroform-methanol, 2: 1v/v (C.M. 1b). The yellow coloured extract was reduced to an oil under vacuum. This crude extract was purified (C.M. 1b) by re-extraction of the oil with chloroform-methanol, 2: 1v/v, followed by dialysis of the chloroform-methanol solution against a large volume (35-40 litres) of water for sixty hours (175). After this period of time the water was a distinct yellow-brown colour. The two phases inside the sac were filtered to remove lipoprotein, and the biphasial filtrate made homogeneous by the addition of methanol. This process yielded a purified lipid mixture of total weight 10.0g.

This extracting process has certain advantages and disadvantages over the previous one in which the rhizomes were blended with methanol. One of the main reasons for trying out different methods of extraction was the necessity to prevent enzymic activity as much as possible. However it could be argued that, in the first method, not all enzymes involved in lipid metabolism would be denatured with methanol; their substrates being of a fatty nature implies that they must be able to function, to some extent, within a nonpolar enviroment. Similarly in the second extraction the heat treatment may initially promote enzymic action prior to suppressing it.

An advantage in the use of chloroform-methanol, 2:1 v/v, instead of methanol only as the extracting agent is that less nonlipid material will be extracted along with the lipids.

2.0g. of lipid mixture from this second extraction were subjected to a similar preliminary examination as was the first. The sample was

Fig. 12



Photograph showing the paper chromatographic separation of the components of the aqueous extracts from Fractions 1, 2 and 3 from the DEAE cellulose column after saponification with 10% methanolic KOH, (Exp. 8) cf TABLE 8.

TABLE 8

Standards	Rg (stds)	Rg (Fr.1)	Rg (Fr.2)	Rg (Fr.3)
D-glucose	100	99	98	98
D-galactose	84	84	85	83
D-mannose	112	110	111	111
L-arabinose	114			
L-fucose	122			
D-xylose	129	131	132	130
		160	160	160
				38

Rg values for standard sugars and unknowns obtained from the aqueous extracts of Fractions 1, 2 and 3 from DEAE cellulose column, after saponification with 10% methanolic KOH. Solvent: ethyl acetate-pyridine-water, 120:50:40 v/v. (Exp. 8).

applied to a silicic acid column (Exp. 8) which was then eluted with chloroform to remove neutral lipids followed by chloroform-methanol, 4:1 v/v, to remove the remaining lipids held by the column. The first fraction was discarded and the second, after solvent removal was applied to a DEAE cellulose column (Exp.8). Three fractions were eluted off using chloroform-methanol, 2:1 v/v, chloroform-methanol, 2:1 v/v, containing 5% glacial acetic acid and finally chloroform-methanol, 2:1 v/v, plus 5% 0.5N aqueous lithium chloride (188).

All three fractions were saponified with 10% methanolic KOH (G.M.10), acidified to pH5, water (50ml.) added to each and this extracted with chloroform to remove the nonsaponifiable fractions.

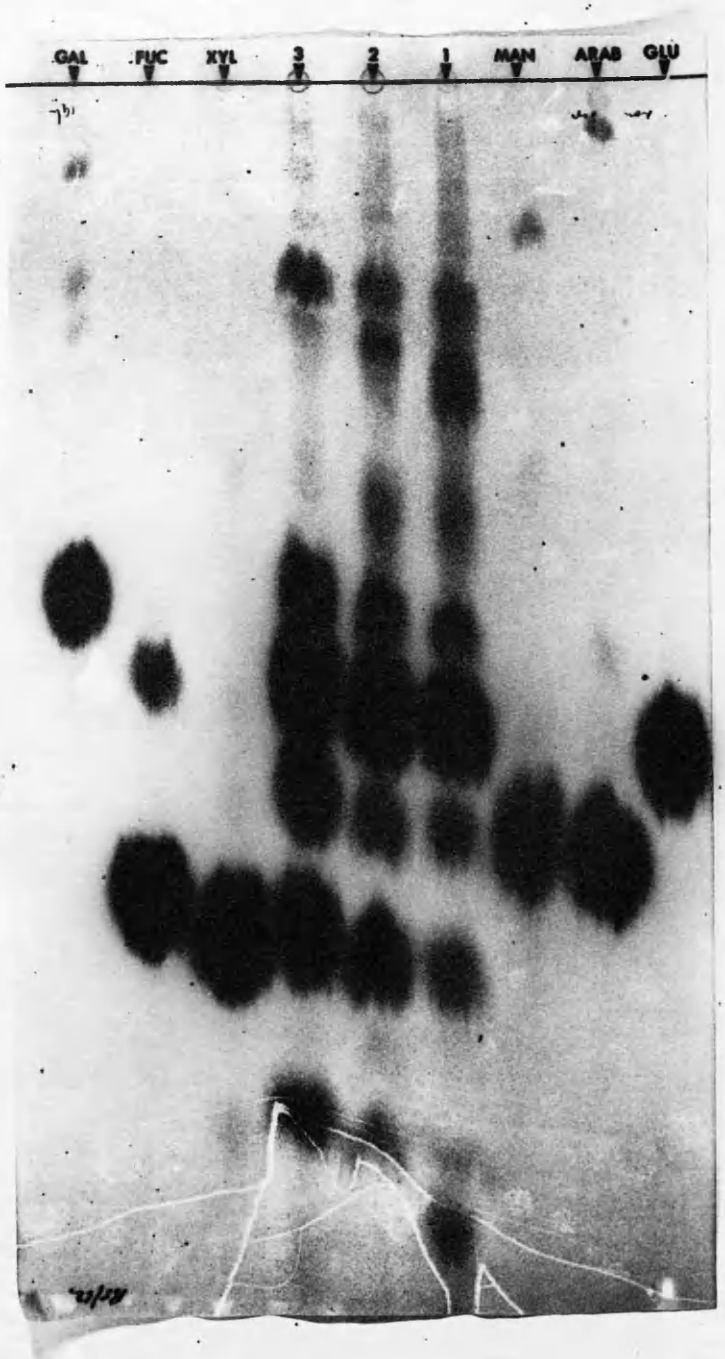
The three aqueous extracts were hydrolysed with sulphuric acid (G.M. 3a) and examined for sugar content by paper chromatography (G.M. 4) (Fig. 12, Table 8).

The three chloroform extracts were also hydrolysed with a mixture of ethanol, chloroform and 5N HCl (191) (G.M. 3c). This system was employed to obtain a homogeneous mixture. The three hydrolysates obtained were also examined for the presence of sugars by paper chromatography (Fig.13, Table 9).

The results confirmed previous conclusions concerning the lipidbound sugars present in the lipid mixture. In the water extracts a total of six spots were located, five of them corresponded to glycerol, D - glucose, D - galactose, D - xylose, D - mannose and / or L - arabinose and were spread throughout all three fractions from the DEAE cellulose column, but the sixth (Rg.38) was almost totally confined to Fraction 3. The chloroform extracts contained the same five spots in all three fractions but did not contain spot Rg.38.

No fucose was detected in either the water or chloroform extracts.

Fig. 13



Photograph showing the paper chromatographic separation of the components of the chloroform extracts from Fractions 1, 2 and 3 from the DEAE cellulose column after saponification with 10% methanolic KOH, (Exp. 8) cf TABLE 9. Solvent: ethyl acetate-pyridine-water, 120:50:40 v/v.

TABLE 9

Standards	Rg (stds)	Rg (Fr.1)	Rg (Fr.2)	Rg (Fr.3)
D-glucose	100	97	95	96
D-galactose	80	82	79	80
D-mannose	113			
L-arabinose	115	112	110	111
L-fucose	122			
D-xylose	128	130	130	131
		160	159	160

Rg values for standard sugars and unknowns obtained from the chloroform extracts of Fractions 1, 2 and 3 from DEAE cellulose column, after saponification with 10% methanolic KOH.
Solvent: ethyl acetate-pyridine-water, 120:50:40 v/v. (Exp. 8).

Again no useful fractionation, apart from the slow running sugar (Rg.38), was obtained. Overloading of the DEAE cellulose column was again thought to be the main reason for this, even though only 0.7g. were applied this time compared with 1.5g. in Exp.6. However as previously stated, these were preliminary experiments to judge techniques and to remove nonglycolipid material from the mixture. To get a complete fractionation of the glycolipids themselves was not of vital importance at this stage.

The fact that the three chloroform extracts, after saponification, contained a very small amount of glycerol (Table 9, Fig. 13), was thought to be due to incomplete saponification and/or incomplete extraction of the saponification reaction with water. This must, therefore, also cast some doubt as to the effectiveness of the saponification methods used in separating the glycolipids in the saponifiable fraction from those in the nonsaponifiable fraction of the lipid mixture. It was thought, therefore, that with regard to the distribution of the glycolipids between the saponifiable and nonsaponifiable fractions, the results were not, so far, reproducible enough to draw any definite conclusions. Particular care was taken when performing any future saponification reactions to try and ensure that a complete separation was achieved.

Finally this second extraction procedure appeared to have produced the two fast running trace spots, identified here as xylose and mannose and/or arabinose, which were first observed in Exp.7, in much greater amounts. This may have been due to an improved extraction technique or to enzymic action promoted by the heat treatment prior to extraction.

3. Third method of extraction.

The difficulty of finding reliable means of preventing possible enzymic action before and during the extraction process is obvious.

One further technique employed was the use of freeze-dried starting material.

Approximately 16 Kg. of freshly dug rhizomes were washed and chopped into lengths of one inch. They were then immediately frozen and loaded onto a large industrial freeze-drier. After drying the weight obtained was 3.2 Kg. The dry down ratio, i.e., the weight loaded onto the drier divided by the weight after drying, was thus 4.9 :1. From this the moisture content of the bracken was calculated to be almost 80%. The vacuum in the drying cabinet was released by feeding nitrogen in and the dried rhizomes were packed, under nitrogen, in sealed cans. The bracken was stored in this fashion until required. (I would like to thank Unilever Ltd., who kindly performed the freeze-drying process).

1.5 Kg. of this freeze-dried material (7Kg. wet weight) were reduced to a powder in a hammer mill. The powder was then eluted in a glass column with hot chloroform-methanol, 2: 1 v/v (G.M. 1b), the eluent being reduced to an oily residue under vacuum. This crude extract was purified by re-extraction with chloroform-methanol, 2: 1 v/v, followed by dialysis of the extract against a large volume of water (G.M. 1b). The total weight of purified lipid was 17.0g.

Examination of this mixture for lipid-bound sugar was carried out by the saponification of a 2.0g. sample with 10% methanolic KOH (G.M.10), followed by the extraction of the acidified mixture with water (30 ml.) and chloroform (50 ml.). Hydrolysis of the residue from the water extract with H_2SO_4 (G.M. 3a) and the residue from the chloroform extract with ethanol-chloroform - 5N HCl (G.M. 3c), followed by the examination for the presence of sugars in each hydrolysate using paper chromatography (G.M. 4), showed the same sugars to be present in this lipid mixture as were detected in the lipid sample obtained from rhizomes heated

at 110° (Exp. 8). However the amount in which they were present differed considerably from that found in Exp. 8. The two pentoses, arabinose and xylose, were located only in trace amounts while in Exp.8, they were present in some cases in amounts almost as great as galactose and glucose.

The difficulty in reproducing a pattern of sugars not only similar in content but also similar in the relative proportion of each sugar, was a continual problem throughout these initial investigations. The main difficulty in this respect lay with the faster running pentose spots which did not always appear. When they did appear they often varied in their relative amounts compared with the other sugars present. As explained, this variability was largely dependent upon the way in which the starting material was treated prior to extraction and the actual extraction procedure itself.

Because of these varying results further experiments were carried out in an attempt to define more clearly their cause.

4. Comparison of the lipid-bound sugars present in lipid mixtures from different bracken preparations

The following bracken rhizome preparations were extracted:-

1. 2 Kg. fresh bracken rhizomes were heated to 110° for twenty-four hours, then ground to a powder and extracted in a large glass column by eluting with hot chloroform-methanol, 2: 1 v/v, (C.M. 1b). The effluent was concentrated under vacuum and the oily residue re-extracted with chloroform-methanol, 2: 1 v/v, and then dialysed against water for forty-eight hours (C.M. 1b). The weight of the lipid mixture obtained was 6.5g.

2. 0.5 Kg. of freeze-dried rhizomes (2.2 kg. wet weight) were extracted (C.M. 1b), after heating at 110° for twenty-four hours, in the same manner as the heated fresh bracken above. The weight of lipid mixture obtained was 4.0 g.

3. 0.5 Kg. of freeze-dried rhizomes were milled and extracted (G.M.1b) without prior heating at 110° in the same way as the previous two preparations. The weight of the lipid extract was 5.3g.

Samples from the above three lipid mixtures were saponified with 10% methanolic KOH (G.M. 10). Ether extraction, after acidification, enabled the isolation of the respective three unsaponifiable fractions, the aqueous phases containing the products of saponification. All six fractions were then hydrolysed. Because of their aqueous insolubility the three unsaponifiable fractions were refluxed with ethanol-chloroform - 5N HCl (G.M. 3), while the aqueous fractions were hydrolysed with 2N H_2SO_4 (G.M. 3). The six hydrolysates were examined for sugar content by paper chromatography using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4).

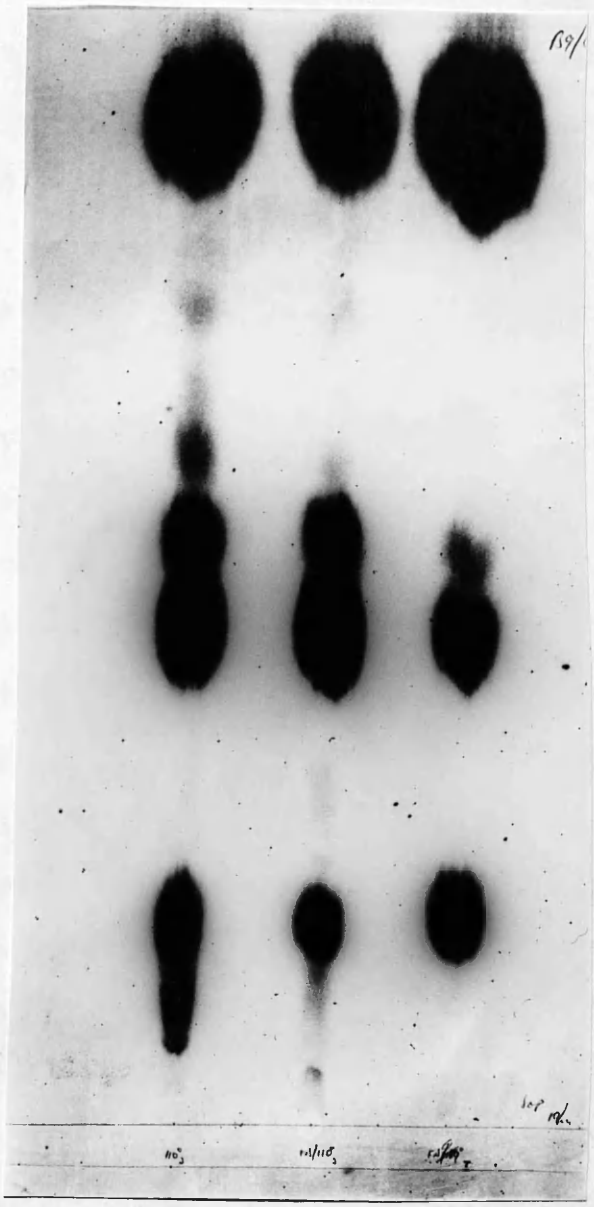
From the chromatogram showing the sugars from the three saponifiable fractions a marked difference was observed (Fig.14). Glycerol, glucose, galactose and the slow running spot (Rg.38) were present in all three samples, but the three faster running spots, while clearly visible in the sample from the fresh rhizomes heated to 110°, were fainter in the sample from the freeze-dried sample heated to 110°, and in the rhizomes which were not heated, they were only visible on very close inspection.

In the chromatograms containing the sugars from the three nonsaponifiable extracts, such a clear distinction was not so evident; all three having a somewhat similar pattern showing glucose and galactose and faint traces of faster running spots.

If the results from the heated rhizomes are compared with the results from the sample which was not heated, then two effects can be considered.-

1. The effect of the heating on the extraction process used.
2. The effect of the heating on producing more sugars in the sample

Fig. 14



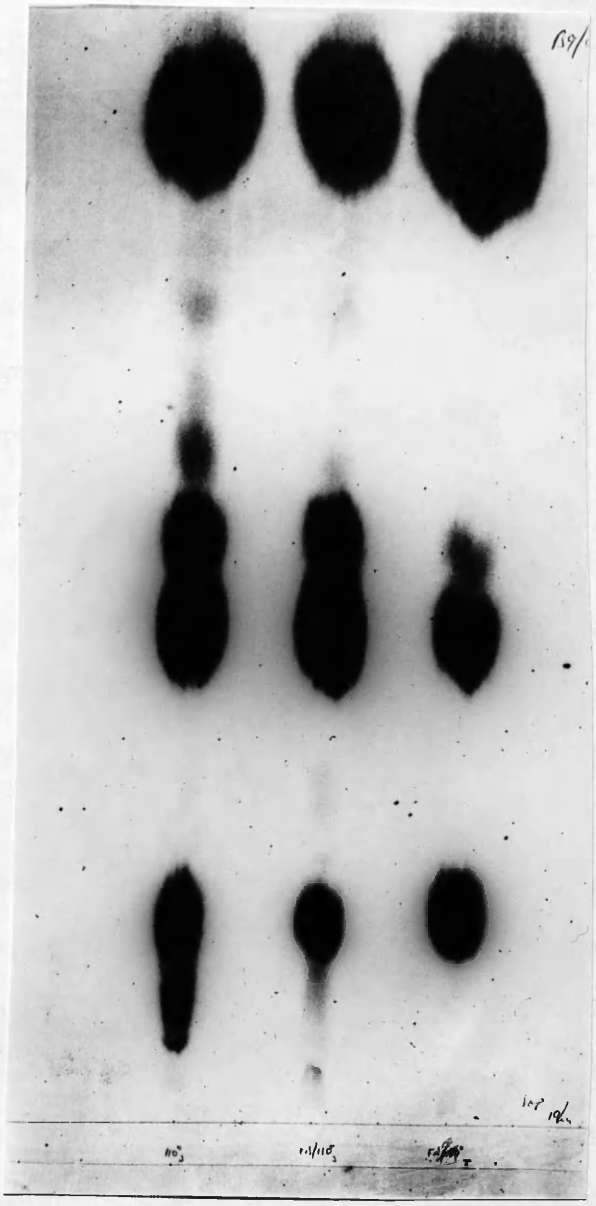
A comparison of the sugars found in the aqueous extracts from lipid mixtures obtained from three differently treated bracken rhizome samples, after saponification with 10% methanolic KOH.

1 fresh bracken heated to 110°,

2 freeze-dried bracken heated to 110°,

3 freeze-dried bracken unheated.

Fig. 14



A comparison of the sugars found in the aqueous extracts from lipid mixtures obtained from three differently treated bracken rhizome samples, after saponification with 10% methanolic KOH.

1 fresh bracken heated to 110°,

2 freeze-dried bracken heated to 110°,

3 freeze-dried bracken unheated.

during the actual heating procedure.

The first effect was considered to be less important as if this happened the sugar patterns obtained from the two heated brackensamples would be expected to be the same. As can be seen from Fig.14 this was not so, while the slower moving sugars were similar the faster ones varied in quantity. It was thought that the second effect was predominant and that the heat accelerated some enzymic process prior to suppressing it.

The appearance of a third fast running spot (Fig.14), which, by its chromatographic behaviour in the solvent used, might be another pentose sugar, was observed during this investigation.

Although the three different bracken samples used in this study were extracted and the extracts obtained purified in exactly the same manner, there were other variables which could have had an effect. These included the moisture content of the samples, the time of year when the samples were collected (the freeze-dried bracken was collected in mid-December, while the fresh rhizomes were collected at the beginning of February), and also the location where the samples were collected (the freeze-dried near Aberdeen, the fresh bracken on the outskirts of Glasgow).

A further experiment was performed, in an attempt to eliminate some of these variables, such as water content, etc.

A comparison was made between the lipid-bound sugars found in the lipid mixtures extracted from:-

1. freeze-dried bracken soaked in water, then heated to 110°,
2. freeze-dried bracken soaked in 0.5% mercuric chloride, then heated to 110°,
3. freeze-dried bracken soaked in 1.0% mercuric chloride, then heated to 110°.

It was reasoned that if the sugars present in the extract from the water soaked rhizomes varied considerably from those present in the other

two, this would suggest an effect caused by enzymic activity taking place in the water soaked bracken prior to extraction.

The investigation took the following form (Exp.9). 0.5 Kg. freeze-dried rhizomes were divided into three equal amounts. One portion was soaked in water, another in 0.5% $HgCl_2$ and the third in 1.0% $HgCl_2$, for a total of twelve hours. Each sample was then pressed through muslin and the residue dried at 110° for twenty-four hours. The samples of dried rhizomes were extracted by blending with chloroform-methanol, 2: 1 v/v (G.M. 1 c), filtering and re-extracting the residue with chloroform-methanol, 2: 1 v/v (G.M. 1 c). The two extracts in each case were combined and reduced to an oily residue under vacuum. This crude lipid mixture was purified by extraction with chloroform-methanol, 2: 1 v/v, followed by dialysis against water (G.M. 1 c). The weight of each lipid mixture was 1.6g, 1.3g., and 1.4g., from the water, 0.5% $HgCl_2$ and 1.0% $HgCl_2$ treated rhizomes respectively.

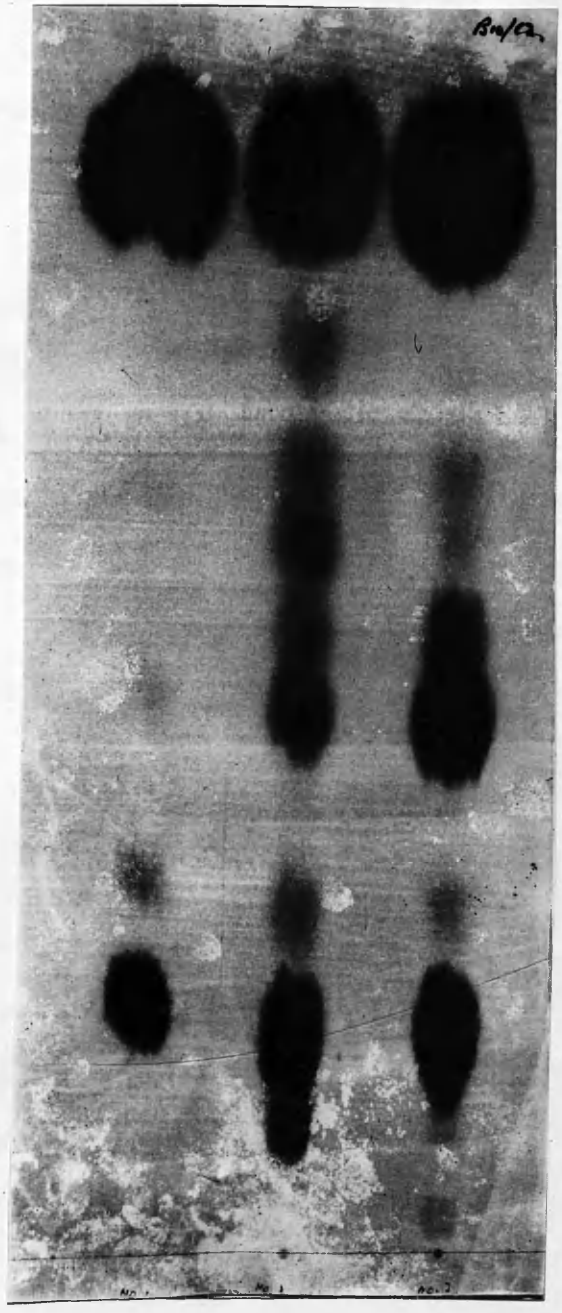
Each sample was hydrolysed directly with 2N H_2SO_4 (G.M. 3) and equivalent amounts of the hydrolysates analysed for sugar content by paper chromatography using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4).

From the chromatogram (Fig.15) it can be seen that the lipid mixtures from the bracken samples soaked in murcuric chloride contain a much greater quantity of sugars than was found in the lipid mixture from the rhizomes soaked in water..

These results indicate enzymic activity of some kind taking place in the bracken soaked in water, prior to extraction. However, despite the presence of $HgCl_2$ the lipid-bound sugars in 2 and 3 have increased.

This is in contradiction to what was previously believed to happen (page 80) which was that any enzymic activity present in the rhizomes

Fig. 15



1 2 3

A comparison of the sugars found in the lipid extracts from freeze-dried bracken rhizomes after hydrolysis with N H_2SO_4.

1 freeze-dried rhizomes soaked in water,

2 freeze-dried rhizomes soaked in 0.5% $HgCl_2$,

3 freeze-dried rhizomes soaked in 1.0% $HgCl_2$.

increased the amount of lipid-bound sugars.

An explanation to reconcile this contradiction may be that the enzymes involved are hydrolytic in nature and also that heating at 110° does in fact suppress enzymic activity completely. If this is correct then the enzymic processes in the previous investigation, in which the bracken was heated at 110°, would be stopped before becoming active, while in the bracken rhizomes soaked in water, enzymic activity would have more than sufficient time to become effective, thus reducing the amount of the sugars found in the lipid mixture.

The results from Exp. 9 do not support the idea that the heat treatment had purely chemical effect on the sugar content of the lipid extracts. If this was true, all three samples would have been expected to contain a similar pattern. This, however, was not indicated by the results.

It must be remembered that the above suggestions are put forward only to try and explain the observations noted during the different extraction procedures and subsequent investigations, in particular the results from the previous two studies. Before they could be verified, more work would have to be carried out into the various factors involved. Lack of time, however, prevented this work being pursued.

The appearance of a third fast running spot (Rg.128) was again detected during Experiment 9 (Fig.15). It ran between the xylose and the arabinose spots.

It was concluded from these preliminary extraction procedures that the methods involving the use of methanol as an extracting reagent, and heating as a method of suppressing enzymic activity, were not suitable. The results described in this section make it clear that these procedures cannot be well controlled and may lead to the production of spurious products or alternatively to the breakdown and disappearance of naturally

occurring metabolities.

It was decided that the procedure likely to give rise to the least amount of such undesirable changes was the freeze-drying technique and in the work that follows, this material was used exclusively where stored material was required. Otherwise during favourable seasons freshly dug rhizomes, immediately chopped and extracted with chloroform-methanol, 2: 1 v/v, were used. It was thought that using the above materials and procedures, a glycolipid extract more representative of that found in the plant in its natural state would be obtained.

5. A quantitative comparison of the carbohydrate and phosphorus content of lipid extracts from freeze-dried and fresh rhizomes.

In order to obtain results for this comparison 0.5 Kg. freeze-dried rhizomes (~ 2.2 Kg. wet weight) were blended with chloroform-methanol, 2: 1 v/v, (G.M. 1 c), and the crude extract, after reduction to an oily residue under vacuum, was purified by extraction with chloroform-methanol, 2: 1 v/v, followed by dialysis against water (G.M. 1 c). The weight of lipid mixture obtained was 7.5g.

1.0g of this mixture was hydrolysed with 2N H_2SO_4 (G.M. 3) and the hydrolysate analysed by paper chromatography for sugar content (G.M. 4). The following sugars were detected; D - glucose, D - galactose, slow running spot (Rg.38), D - xylose, L - arabinose and/or D - mannose, and the fast running spot (Rg.128). Glycerol was also present.

Carbohydrate and phosphorus analysis was carried out by hydrolysing 0.25g. of the lipid mixture with ethanol-chloroform - 5N HCl (G.M. 3c). The hydrolysate was reduced to dryness under vacuum and the residue then extracted with a water-chloroform mixture. Total carbohydrate was estimated on suitable aliquots of the aqueous phase by the phenol /H_2SO_4

method of Dubois et al (192) (G.M. 11 c) and phosphorus by the method of King (193) (G.M. 11 c). The results are shown in Table 10.

In order to compare the above results with those from fresh bracken 2.0kg. freshly dug rhizomes were washed, chopped into lengths of one inch and extracted with chloroform-methanol, 2: 1 v/v, in a blender (G.M. 1c). The crude extract, after removal of the solvent by vacuum distillation, was purified by extraction with chloroform-methanol, 2: 1 v/v, and dialysis against water (G.M. 1 c). The weight of lipid mixture was 9.4g.

0.46g. of this mixture were hydrolysed (G.M. 3c) in the same manner as the freeze-dried extract. The water phase was removed and made up to a known volume. Samples of this were then taken and used for the carbohydrate and phosphorus analysis (G.M. 11c, 12c) (Table 10).

It can be seen from Table 10 that the sugar content of both extracts were approximately equivalent. However there was a wider variation in the phosphorus content, in that the lipid mixture from the fresh rhizomes contained almost four times that of the freeze-dried material.

Qualitative analysis of the sugar content of the lipid mixtures by paper chromatography showed that both had the same sugars present.

The yields of lipid mixture obtained by the two extractions were 3.4g/kg. and 4.7g/kg. from the freeze-dried rhizomes and fresh rhizomes respectively. These yields could possibly explain the much higher phosphorus content in the lipid mixture from the fresh rhizomes. As indicated earlier in this account, the moisture content of a tissue contributes, as the extraction proceeds, to making the extracting solvent (chloroform-methanol) more polar. Hence the most polar entities in a lipid fraction, such as the phospholipids will be extracted more efficiently as the polarity of the extracting medium inevitably increases with time. This of course cannot occur to the same degree with the freeze-dried material and hence

TABLE 10

Sample	Carbohydrate content, mg/g extract	Phosphorus content, mg/g extract	Weight of extract, g/kg bracken
Freeze-dried	50.2	3.1	3.4
Fresh	54.4	12.0	4.7

A comparison of the carbohydrate and phosphorus content of the lipid extracts from freeze-dried and fresh bracken rhizomes. Column 4 gives the weights of lipid mixtures obtained.

this may account for the much dimished amount of phosphorus compounds extracted. This explanation may not be entirely valid however since there was no great difference in the amount of glycolipids extracted in each case.

From this analysis the glycolipid content of the two lipid extracts from the different bracken preparations appear similar. However because of the difference in phosphorus content it was thought that a more detailed comparison of the contents of the two lipid extracts was required.

6. Two dimensional thin-layer chromatographic analysis of the lipid extracts from fresh and freeze-dried rhizomes.

This analysis was performed using the lipid extracts from freeze-dried rhizomes and fresh rhizomes. The two dimensional technique employed was that derived by Lepage (189) (G.M. 4). This enables the complete or almost complete resolution of the lipid mixture into individual compounds as well as into classes such as neutral lipids etc.

The silicic acid stationary phase (Merck, Dermstadt, Ger.) was pre-treated before being used, to remove impurities and fines (G.M. 13).

The 20 x 20 cm. glass plates were also thoroughly washed to ensure the removal of all grease and dirt from the surface, this facilitating the uniform deposition of the silicic acid layer (G.M. 4).

The silicic acid was spread onto the plates to a uniform thickness of 0.25mm. in a slurry of water and reactivated by a heat treatment as described (G.M. 4).

All the organic solvents used in the development of the plates were redistilled or dried and stored over a suitable drying agent (potassium alumino - silicate) before being used (G.M. 6).

The plates were developed initially in chloroform-methanol-water, allowed to dry and developed subsequently, at right-angles to the first

development, in diisobutyl ketone - water - acetic acid (G.M. 4).

The spots were located using both general and specific spray reagents (G.M. 5).

Iodine vapour	-	General	(194)
20% perchloric acid	-	"	(189)
0.001% rhodamine 6 G.	-	" (visible in u.v. light)	(195)
0.2% ninhydrin	-	Aminolipids	(189)
Modified Dragendorff reagent	-	Choline containing lipids	(196)
Schiff's reagent	-	Phospholipids/Glycolipids	(197)
Molybdenum / $H_2 SO_4$	-	Phospholipids	(198)
0.5% α - naphthol	-	Glycolipids	(199)

Occasionally each plate was sprayed with only one particular reagent. But once the iodine vapour, perchloric acid and Schiff's reagent had been used separately to locate the majority of the spots present, it was then feasable to spray each plate with a series of reagents in sequence. Thus the plate could initially be sprayed with ninhydrin to detect aminolipids and this could be followed by a treatment with the molybdenum/$H_2 SO_4$ reagent to show up any phospholipids. Finally it was possible to spray the plate with 20% perchloric acid to locate all the spots present for confirmation purposes. Alternately the plate could be sprayed with Dragendorff reagent, followed with the molybdenum /$H_2 SO_4$ spray and finally once again with perchloric acid. Another sequence used was 0.5% α - naphthol to locate glycolipids, followed by perchloric acid.

In this way it was possible to determine which of the spots present in the 'total lipid map' prepared using general detecting reagents such as perchloric acid, were aminolipids, glycolipids and phospholipids.

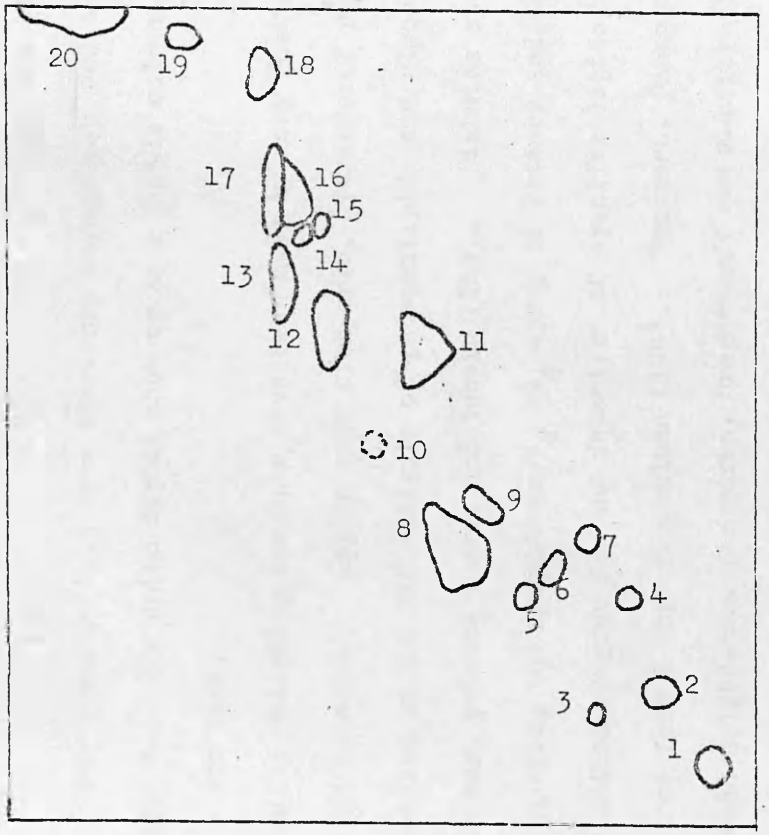
This procedure was carried out on both lipid mixtures.

Fig 16, No.1 is a photograph of the resolution obtained of a sample

Fig. 16

No. 1

Photograph of the two dimensional TLC fractionation obtained of the lipid mixture from bracken rhizomes.



No. 2

Diagram of two dimensional TLC fractionation of lipid mixture from bracken (see Fig. 16, No. 1). The spots are numbered to give a "LIPID MAP".

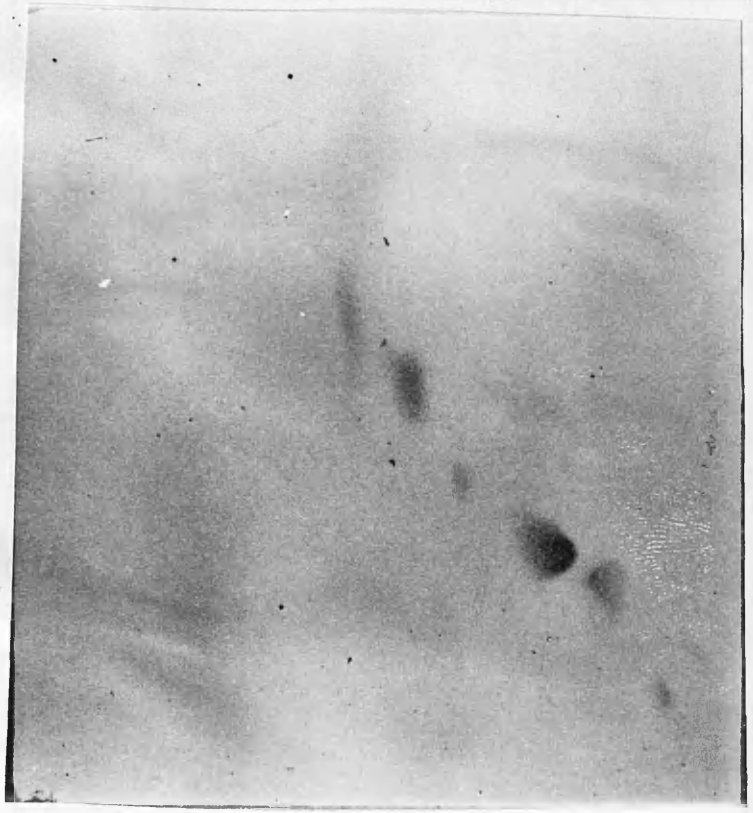
of the lipid extract from fresh rhizomes, using this method. The plate was sprayed with 20% perchloric acid to locate all the lipid spots present. Figure 16, No.2 is a diagram of the same plate in which the spots have been numbered to give a 'total lipid map'. This map was used as a reference for the identification of spots detected using specific spray reagents. The lipid mixture from the fresh rhizomes was resolved into a total of at least twenty spots.

Figure 17, No.1 is an example of the use of a more specific spray on the resolved lipid mixture. In this case phospholipids were located using a specific molybdenum/H_2SO_4 reagent. With this reagent the phospholipids show as bright blue spots. Figure 17, No.2 is a diagram of the same photograph with the spots numbers in accordance with the 'total lipid map' in Fig. 16, No.2.

Figure 17, Nos 3 and 4 are diagrams of the spots detected on thin-layer plates which were sprayed with 0.2% ninhydrin and 0.5% α - naphthol respectively. The spots in No.3 were therefore aminolipids and those in No.4 glycolipids. The amino lipids show up as a purple colour while the glycolipids are blue.

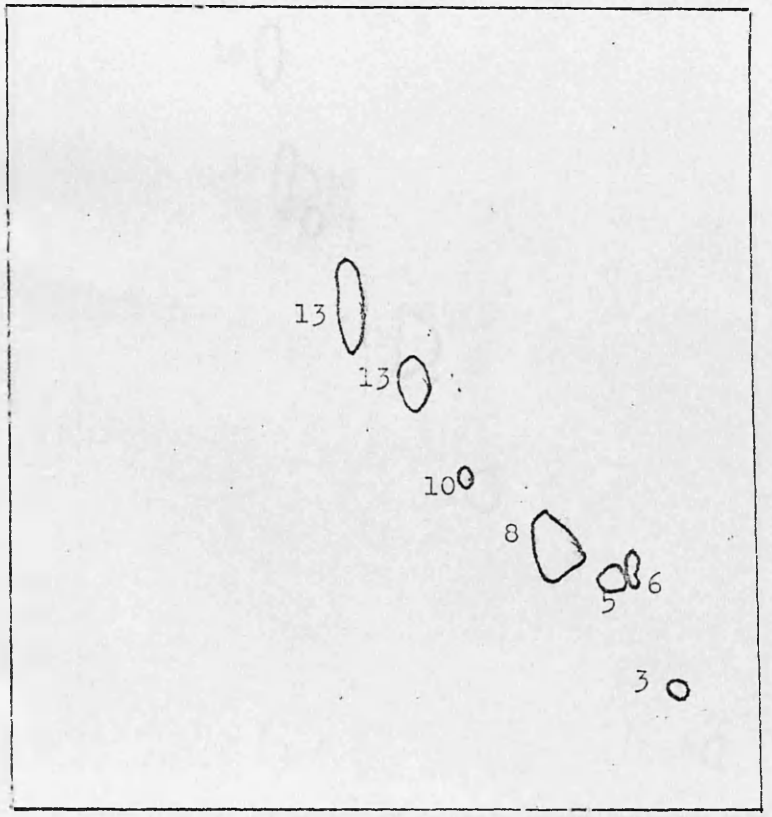
These specific detecting reagents have been used by many workers involved in lipid research. Lepage used ninhydrin, Dragendorff reagent, and Schiff's reagent in the TLC analysis of phospholipids and glycolipids from alfalfa leaves, potatoe leaves and tubers (189). Siakotos and Rouser used ninhydrin, the molybdenum/H_2SO_4 spray of Dittmer and Lester (198) and α-naphthol reagent for the detection of specific lipids in fractions obtained from a Sephadex column (199). Pomeranz, Chung and Robinson also used molybdenum, ninhydrin, Dragendorff and α-naphthol reagents in the TLC analysis of lipids from various classes and varieties

Fig. 17



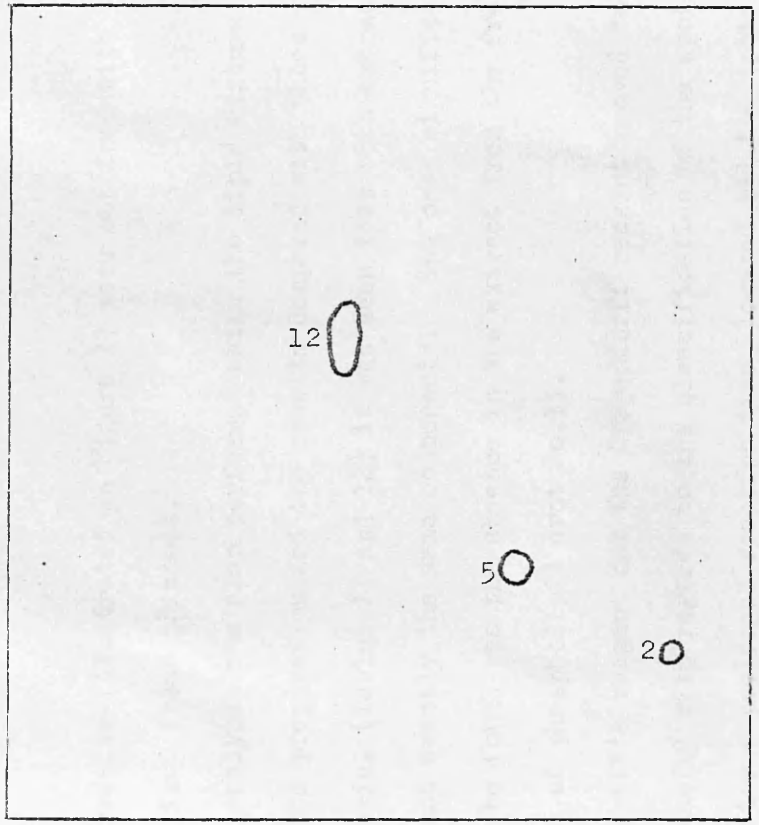
No. 1

Photograph showing the detection of phospholipid compounds after two dimensional TLC fractionation, using molybdenum/H_2SO_4 reagent.



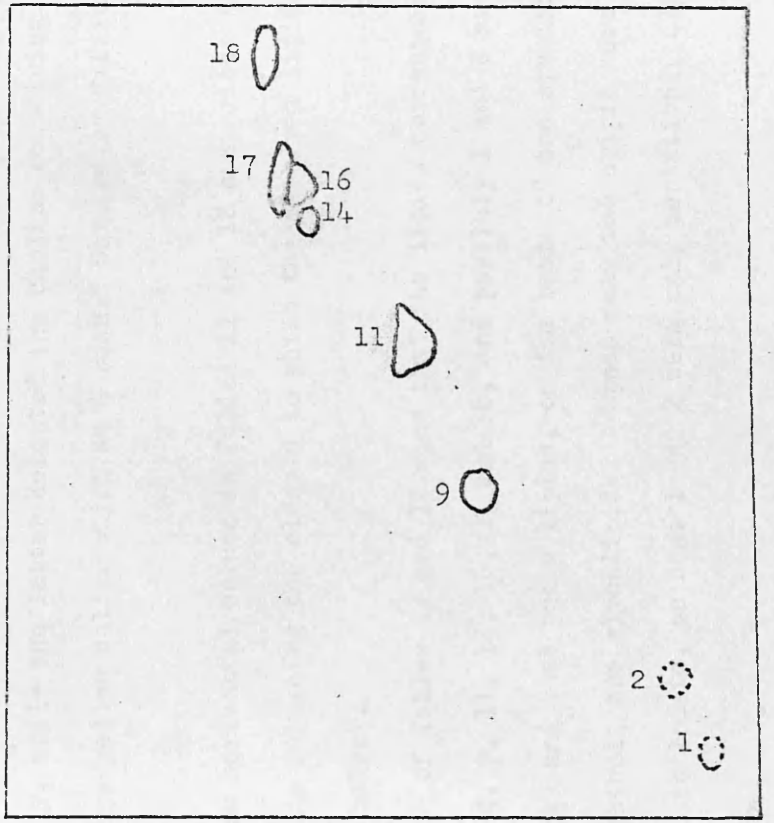
No. 2

Diagram of Fig. 17 No. 1 showing the phospholipid spots detected. The spots are numbered according to the "LIPID MAP" (Fig. 16, No. 2).



No. 3

Two dimensional TLC of lipid mixture. Diagram of aminolipid compounds detected using ninhydrin reagent. The spots are numbered according to the "LIPID MAP" (Fig. 16, No. 2).



No. 4

Two dimensional TLC of lipid mixture. Diagram of glycolipids detected using the specific spray α-naphthol. The spots are numbered according to the "LIPID MAP" (Fig. 16, No. 2).

of wheat (200). These reagents are, therefore, generally recognised as a valid means of identification of different lipid classes by TLC analysis.

All the results illustrated in Figure 17 were obtained with the lipid extract from fresh rhizomes.

When the analysis was also performed using the lipid mixture from the freeze-dried preparation and the results compared with those from the fresh material (Tables 11 and 12) it was seen that both extracts contained almost exactly the same components. The only significant difference to be found was the absence in the extract from the freeze-dried rhizomes of phospholipid spot No.13.

Neither Sciff's reagent nor the Dragendorff reagent proved to be particularly useful with regards to the classification of the spots. The former, after a prolonged reaction time, located all the spots present unspecificially, while the latter detected the choline containing lipids as a pale orange/yellow colour which as a result were often difficult to locate.

The bottom horizontal column in Tables 11 and 12 summarises the conclusions made regarding the classes to which the resolved lipids from both extracts belong.

Comparison of Tables 11 and 12 show that the lipids corresponding to spot Nos. 4, 9, 11, 14, 16, 17 and 18, and possibly 1 and 2 are common to both extracts and that these lipids, on the basis of the specific spray reagent, α-naphthol, are glycolipids. Spots near the origin were much more difficult to locate, so Nos. 1 and 2 were only tentatively classed as glycolipids.

This two dimensional thin-layer chromatographic resolution of the

TABLE 11

Perchloric 20% acid	w 1	w 2	w 3	w 4	w 5	m 6	w 7	s 8	m 9	w 10	s 11	s 12	m 13	m 14	m 15	s 16	s 17	s 18	m 19	s 20
Ninhydrin		w +			w +							s +								
Dragendorff		+m			+w?	yw +?		+s	+m	+w	+s	+m	+w		+m	+m		+m		
Schiff's	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Molybdenum			+s		+w	+s		+s		+m		+s	+s							
α-naphthol	+w ?	+w ?		?					+s		+s			+s		+s	+s	+s		
Conclusion	glycolipid?	amino-glycolipid?	phospholipid	glycolipid?	amino-phospholipid	phospholipid	?	phospholipid	glycolipid	phospholipid	glycolipid	amino-phospholipid	phospholipid	glycolipid	?	glycolipid	glycolipid	glycolipid	?	neutral

2 dimensional TLC analysis of lipid mixture from fresh rhizomes.

KEY:
+ definate spot.
+? definate spot but No. in doubt.
? possible spot but not definate.
vw very weak spot.
w weak spot.
m medium spot.
s strong spot.

Spots are numbered as in "TOTAL LIPID MAP" (Fig.16 No.2)

TABLE 12

20% Perchloric acid	1 w	2 vw	3 w	4 w	5 w	6 w	7 w	8 s	9 m	10 w	11 s	12 s		14 m	15 m	16 s	17 s	18 m	19 m	20 s
Ninhydrin		w +			w +							s +								
Dragendorff					w +?			s +	m +	w +	s +	m +			m +	m +		m +		
Schiff's	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+
Molybdenum			w +		m +	m +		s +		m +		s +								
α-naphthol	w +?	w +?		w +					s +		s +			s +		s +	s +	s +		
Conclusion	glycolipid?	glycolipid?	phospholipid	glycolipid	amino-phospholipid	phospholipid	?	phospholipid	glycolipid	phospholipid	glycolipid	amino-phospholipid		glycolipid	?	glycolipid	glycolipid	glycolipid	?	neutral

2 dimensional TLC analysis of lipid mixture from freeze-dried rhizomes.

KEY:
+ definate spot.
+? definate spot but No. in doubt.
? possible spot but not definate.
vw very weak spot.
w weak spot.
m medium spot.
s strong spot.

Spots are numbered as in "TOTAL LIPID MAP" (Fig.16 No.2)

lipid mixtures was proved to be both successful and reproducible. It was decided therefore to adopt it as a general diagnostic technique for following the separation of lipid mixtures by column chromatography.

7. Investigation into the use of powdered cellulose columns as means of purifying lipid mixtures.

At this stage in the work a final experiment was carried out into suitable purification techniques. Rouser has stated (176, 180) that powdered cellulose columns can be used in the purification of total lipid extracts from animal tissue, by separating nonlipids from the remainder of the lipid mixture. An experiment (Exp.10) was performed to ascertain if this method was applicable to lipid extracts from plant (bracken) as well as animal tissue, and in particular to test its effectiveness in removing free sugars from the crude extracts.

A column with effective dimensions 17.0 x 3.2cm. was prepared using powdered cellulose (Whatman) (G.M. 7). The column was washed, according to Rouser's recommendations, to prevent the formation of channels caused by a sudden change from a polar to a nonpolar eluting agent (180).

A crude lipid mixture obtained by the extraction of fresh bracken in a blender with chloroform-methanol, 2:1 v/v, (G.M. 1c) was partially purified by re-extraction, after removal of the solvent, with chloroform-methanol, 2:1 v/v, (G.M. 1c). 2.5g. of this partially purified mixture was applied to the cellulose column and eluted with water saturated chloroform-methanol, 2:1 v/v, in order to recover all the major lipid classes in one fraction. Methanol-water, 9:1 v/v, was then applied with the aim of recovering nonlipid material. The weights of the two fractions obtained were 2.0g. and 0.29g. respectively.

Both fractions were examined by two dimensional thin -layer chromatography on silicic acid (G.M. 4), 20% perchloric acid being used to locate

TABLE 13

Spots numbered as in " LIPID MAP " (Fig. 16)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 1					+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Fraction 2	+	+	+?	+	+	+	+	+	+		+			+	+	+				

Two dimensional TLC analysis of Fractions 1 and 2 from cellulose column (Exp. 10).

TABLE 14

Fraction	Analysis	Weight (mg)
1	Carbohydrate	71.4
	Phosphorus	20.7
2	Carbohydrate	42.0
	Phosphorus	4.2

Carbohydrate and phosphorus analysis of Fractions 1 and 2 from cellulose column (Exp. 10).

the spots (G.M. 5). The result showed that cross contamination between the two fractions, with regard to the lipid content was quite considerable (Table 13). Neutral lipids were mainly in the first fraction while the polar ones were confined mainly to the second fraction.

Both fractions were, nevertheless, analysed for sugar and phosphorus (G.M. 11c, 12c) after hydrolysis with ethyl alcohol-chloroform - 5 N HCl (G.M. 3 c). The results (Table 14) bear out the TLC analysis in that the polar lipids, phospholipids and glycolipids, were present mainly in Fraction 2.

In conclusion, therefore, a rough separation was achieved between the neutral and polar fractions of the lipid mixture. However, the results did not show a fractionation into lipid and non-lipid material and therefore this technique of purification was not pursued.

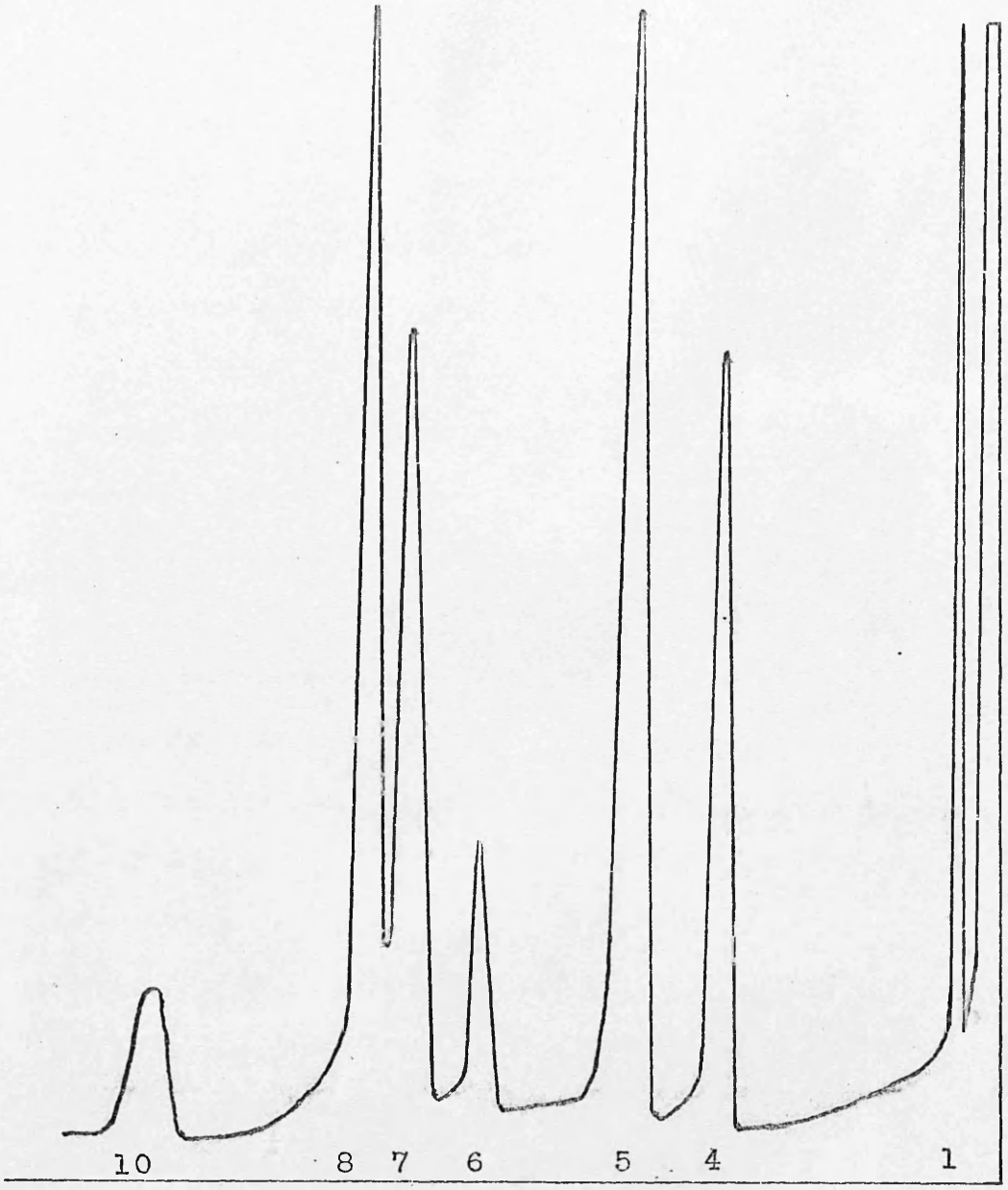
8. Analysis of sugars present in the lipid mixture from bracken by gas-liquid chromatography.

The GLC analysis of sugars as previously stated (page 29) is rapidly becoming standard laboratory procedure. It was thought, therefore, that confirmatory proof of the lipid-bound sugars so far identified by paper chromatography, using this technique, would be useful.

Since this method of analysis resolves the sugar anomers as well as the sugar isomers, it was decided, prior to an investigation of the actual lipid-bound sugars, that a trial experiment using known sugars should be carried out to determine the best conditions for the analysis.

This was done by preparing the trimethlysilyl (TMS) derivatives of the following sugars D - glucose, D - galactose, D - mannose, D - xylose, L - arabinose and L - fucose. This was achieved by dissolving 10 mgs. of each sugar in dry pyridine (G.M. 6) and adding to this, with vigorous shaking, a freshly prepared 2: 1 v/v mixture of hexamethyldisilazane and

Fig. 18



GLC of standard sugars (TMS derivatives). Peak 1 glycerol, peak 4 α-arabinose, peak 5 α-xylose, peak 6 α-mannose, peak 7 α-galactose, peak 8 α-glucose, peak 10 β-glucose. The separation was performed on an SE-30 column with temperature programmed conditions of 130-160°. (TABLE 15).

and trimethlychlorosilane (G.M. 15). A mixture of the TMS derivatives of the above sugars was also prepared in a similar manner (G.M.15).

The analysis was performed (G.M. 16) using a commercial Pye 104 flame ionisation detector chromatograph and the resolution carried out using glass columns 152 x 0.4cm. in dimension containing 2.5% S.E. 30 (G.M. 7) (Applied Science Labs., Penn., U.S.A.). The temperature was programmed between 130 and 160° with a linear increase of 2° /min. At the start of the analysis therefore the temperature is at 130° but rises at 2° /min. until it reaches 160° . This enables a better separation of the faster running components without the slower ones spreading themselves out and coming off the column as flat peaks.

Trials were then conducted by injecting, separately, 1 to 10 γl.samples of both the individual sugars and the standard sugar mixture. The fractionated components were recorded automatically as peaks using a Smiths Servoscribe recorder.

Using the above condition it was possible to obtain an almost complete separation of the sugars in the standard mixture (Fig.18). Only small peaks representing minor anomeric components were not identifiable, when a separation of the mixture was compared with the resolutions obtained for individual sugars. The major 'anomeric peaks' of the various sugars in the mixture were resolved. The retention times of the separated components were calculated (Table 15) and the various peaks identified by comparison with those found in the resolutions obtained for the individual standard sugars and by comparison with the results obtained by other workers (119, 120, 121, 122).

Having decided on the best conditions, an analysis of the lipid bound sugars from bracken was subsequently carried out (G.M. 16), the lipid mixture used was that obtained from freeze-dried rhizomes. Approximately 1g. of

TABLE 15

Standards	Anomers	(stds.) Rt. rel. to α-glucose	(Hyd.) Rt. rel. to α-glucose	Peak No.
Glycerol		0.68	0.68	1
			0.155	2
			0.245	3
Arabinose	α	0.42	0.42	4
Xylose	α	0.58	0.58	5
Mannose	α	0.83	0.84	6
	β		1.1	9
Galactose	α	0.94	0.935	7
Glucose	α	1.0	1.0	8
	β	1.34	1.32	10

GLC analysis of standard sugars and unknowns from the glycolipid fraction of the lipid mixture from bracken. Rt. values are relative to α-glucose (std.). The separation was performed on a 2.5% SE-30 column using temperature programmed conditions of 130-160° with a linear rise of 2°/min.

this mixture was hydrolysed with ethanol-chloroform- 5N HCl (G.M.3), the solution then reduced to dryness and the residue extracted in turn with chloroform and water. The aqueous phase was reduced to dryness under vacuum and the residue obtained thoroughly dried by washing with dry methanol (G.M. 6).

TMS derivatives of the unknown sugar mixture were prepared using the method of Sweeley, Bentley, Makita and Wells (118, 119). The mixture was dissolved in dry pyridine by shaking and the reaction carried out as with the standards (G.M. 15).

1 to 10 γl. samples of this mixture were injected onto the S.E.30 column and the separation performed (G.M. 16) under exactly the same conditions as for the standard sugars. The retention times of the peaks recorded were calculated (Table 15) and compared with those for the standards.

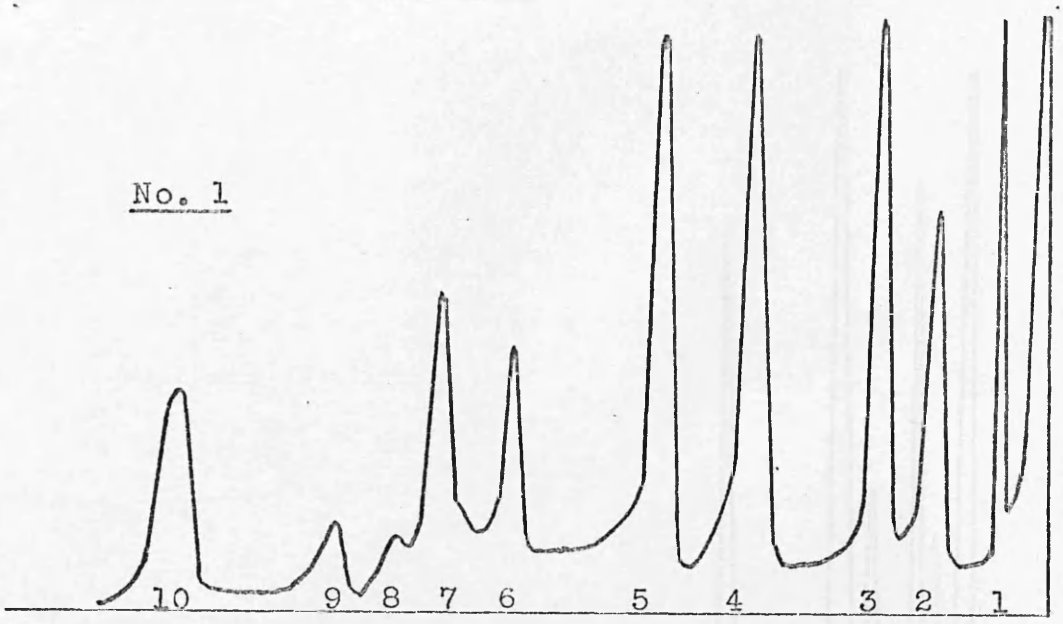
Figure 19, Nos. 1 and 2 are separations obtained for the unknown sugar mixture and the unknown sugar mixture containing internal standards of D - glucose, D - galactose, D - mannose, D - xylose and L - arabinose.

The results obtained confirmed previous conclusions and showed that the lipid extract contained glucose, galactose, mannose, arabinose and xylose. Two unidentified peaks, 2 and 3, were also noted. Either one or both of these peaks could correspond to the anomers of the unknown sugar (Rg.128) previously found (Exp.9).

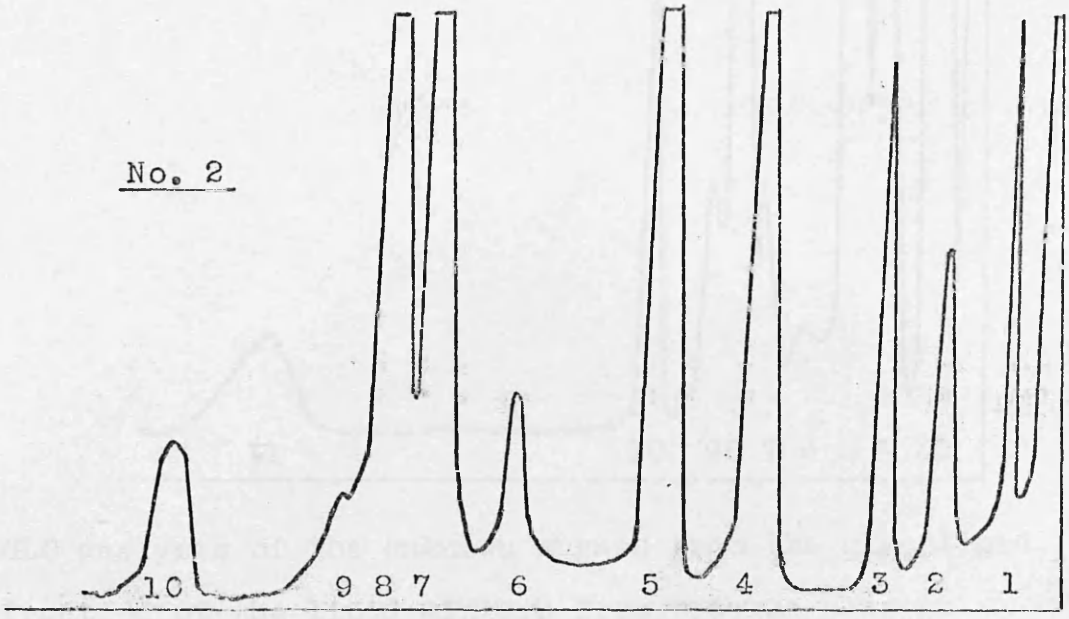
L-fucose was not found to be present in the lipid-bound sugar mixture.

To determine if there were any slow running sugar components in the mixture which did not appear when the chromatograph was programmed at 130-160°, a sample was analysed with the instrument programmed at 160-200° (G.M. 16). The separation obtained (Fig.20) showed one extra peak (No.11)

Fig. 19

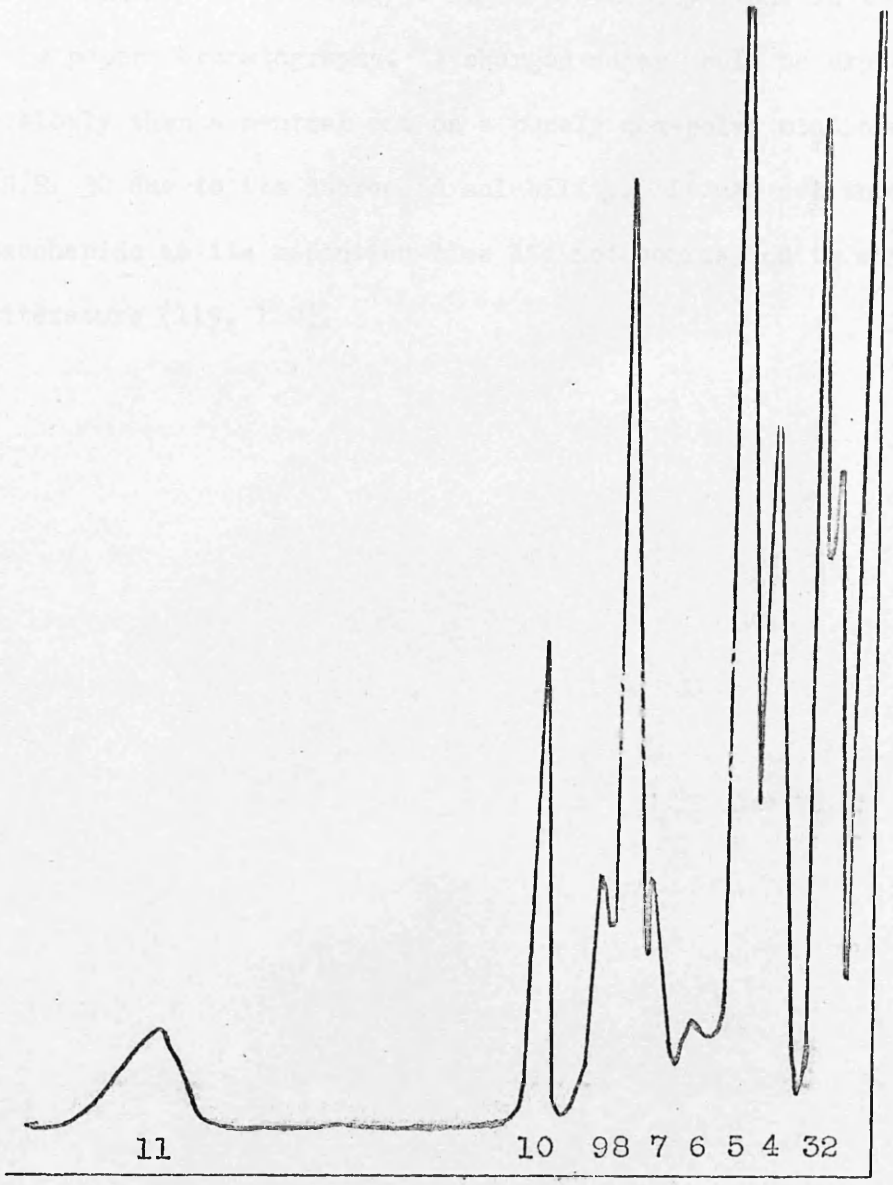


GLC analysis of the unknown sugars from the glycolipid fraction of the lipid mixture from bracken. (TABLE 15).



GLC analysis of the sugars from the glycolipid fraction of the lipid mixture from bracken, plus internal standards of arabinose, xylose, mannose, galactose and glucose.

Fig. 20



GLC analysis of the unknown sugars from the glycolipid fraction of the lipid mixture from bracken, plus an internal standard of α-glucose (8). The separation was performed on an SE-30 column under temperature programmed conditions of 160-200° with a linear rise of 2°/min.

which had a retention time of 2.85. This slow running component could not be identified and did not correspond to any of the known standards. It is possible that it may be the charged sugar previously found in the glycolipid fraction by paper chromatography. A charged sugar would be expected to run more slowly than a neutral one on a purely non-polar stationary phase such as S.E. 30 due to its decreased solubility. It was not thought to be a disaccharide as its retention time did not correspond to any cited in the literature (119, 120).

SECTION B.

The studies so far described have shown that lipid extracts from bracken contain glycolipid components and that the entities in this subfraction contain bound glucose, galactose, mannose, arabinose, xylose and two unknown sugars; one of which is charged and has characteristics inmany ways similar to that derived from plant sulpholipid and the other chromatographs in a manner similar to a pentose.

At this stage it was clear that more definite information could be obtained if methods could be derived for the isolation, in a pure state, of each of the original glycolipids. The sugar bound into each could then be identified with certainty and some other observations concerning the non-sugar portion of the compounds could perhaps be made.

To this end quantitative studies into the effectiveness of various column techniques were carried out. These included the use of silicic acid and magnesium silicate as column media. Decisions as to the effectiveness of the columns used were based on the analysis of the various fractions obtained for carbohydrate and phosphorus content and also upon their analysis by thin-layer chromatography.

I. The evaluation of silicic acid column chromatography as a technique for fractionating lipid mixtures.

This experiment was carried out to find out how efficient silicic acid was in separating neutral lipids from polar lipids (including glycolipids). The system chosen was basically the same as that described in the introduction (page 57) in which chloroform and methanol are the only two eluting agents used. This is the simplest system and was thought a suitable one to start with.

The lipid mixture used was that obtained from fresh rhizomes blended

TABLE 16

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery of CHOαP (%)
1	1.22	70.0	Phosphorus	2.0	13.5
			Carbohydrate	2.9	3.0
2	0.42	23.5	Phosphorus	15.6	75.0
			Carbohydrate	86.0	90.0

Phosphorus and carbohydrate analysis of Fractions 1 and 2 from silicic acid column (Exp. 11).

TABLE 17

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
1750 lipid	1640	93.5
21 P	18.4	88.2
95.4 CHO	88.9	93.0

Percentage recovery of lipid, phosphorus and carbohydrate from silicic acid column (Exp. 11).

TABLE 18

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 1																		+	+	+
Fraction 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

2 dimensional TLC analysis of fractions from silicic acid column. (Exp. 11).

with chloroform-methanol, 2:1 v/v and subsequently purified (page 87).

The silicic acid was pretreated to remove impurities and fines (G.M. 13) and then used to prepare a column with effective demensions 18.0 x 2.5cm. (G.M. 7). To this was applied 1.75g. lipid mixture in a solution of chloroform and the column was then eluted with chloroform followed by methanol (Exp.11).

Both fractions were analysed for carbohydrate and phosphorus content, after hydrolysis with ethanol-chloroform-5N HCl (G.M. 3c), by the phenol /H_2SO_4 method (G.M. 11 c) and King's method (G.M. 12 c) respectively.

Both fractions were also analysed by two dimensional T L C on silicic acid (G.M. 4). 20% perchloric acid reagent was used to detect the spots (G.M. 5) (Table 18). This analysis showed that an almost complete separation was achieved between the neutral and polar lipids applied to the column in the mixture, spot No.18 being the only one which appeared in both fractions.

The results of the TLC analysis were supported by the carbohydrate and phosphorus determinations (Table 16) which showed that Fraction 2 contained by far the majority of the carbohydrate and phosphorus applied to the column.

Data concerning the recoveries of lipid, carbohydrate and phosphorus are given in Table 17.

This system proved, therefore, to be very efficient. However, it may be worth investigating a more complex elution system in which the neutral and polar lipids are not only separated but in which the polar lipids themselves, the glycolipids in particular, undergo further fractionation.

2. The evaluation of magnesium silicate chromatography as a technique for fractionating lipid mixtures.

The ability of magnesium silicate to fractionate the lipid mixture from bracken into a neutral fraction, a glycolipid fraction and acidic fraction was studied (Exp.12).

The lipid mixture used was that obtained by the elution in a glass column of 1.5 kg. freeze-dried rhizomes, ground to a powder in a hammer mill, with hot chloroform-methanol, 2: 1 v/v (G.M. 1b), and purified by re-extraction and dialysis (G.M. 1b).

A preliminary analysis of the lipid mixture for total phosphorus and carbohydrate content was performed. The phosphorus analysis (G.M. 12c) was performed on the aqueous extracts of three differently prepared samples.

1. 0.9g. lipid mixture saponified with 10% methanolic KOH (G.M.10).
2. 1.02g. lipid mixture hydrolysed with ethanol-chloroform-5N HCl. (G.M.3c).
3. 0.88g. lipid mixture ashed at 500° in a furnace for twenty four hours (Exp.12).

The carbohydrate content of the mixture was determined (G.M.11c) only on the water extract from the lipid sample after hydrolysis i.e. No.2. Only after this treatment would all the sugars be expected to be in the free form, saponification would not release them all, and ashing would destroy them.

The results of these analyses are given in Table 19. From this table it appears that the ashing technique results in the loss of some phosphorus from the sample.

200g. of magnesium silicate (Florisil, BDH) were pretreated to remove impurities and fines (G.M. 13) and used to prepare a column with effective dimensions 16.0 x 3.5cm. (G.M. 7).

A lipid mixture of 1.22g. was applied to the column in a solution

TABLE 19

Sample	Inorganic phosphorus (mg/g)	Total phosphorus (mg/g)	Carbohydrate (mg/g)
Alkaline saponified	0	6.2	
Acid hydrolysed	0	6.3	57.3
Ashed	5.1	5.1	

Phosphorus analysis of lipid mixture from freeze-dried rhizomes after alkaline saponification, acid hydrolysis and ashing.
Column 4 gives the carbohydrate analysis after only acid hydrolysis.

TABLE 20

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO & P (%)
1 C-M 9:1	0.84	69.0	Phosphorus	0	0
			Carbohydrate	9.7	14.0
2 C-M 2:1	0.21	17.2	Phosphorus	0	0
			Carbohydrate	28.0	40.0
3 MeOH	0.31	25.5	Phosphorus	2.3	33.0
			Carbohydrate	22.7	33.0

Phosphorus and carbohydrate analysis of Fractions 1, 2 and 3 from Florisil column (Exp. 12).

TABLE 21

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
1220 lipid	1360	110
7 P	2.3	33.0
69 CHO	60.4	87.5

Percentage recovery of lipid, phosphorus and carbohydrate from Florisil column (Exp. 12).

of chloroform-methanol, 9:1 v/v, and eluted using the system of Benson and O'Brien (186) (Exp.12). This employed chloroform-methanol, 9:1 v/v, to remove neutral lipids, followed by chloroform-methanol, 2:1 v/v, to elute glycolipids. A third fraction was eluted off the column with methanol.

All three fractions were hydrolysed with ethanol-chloroform-5N HCl (G.M. 3c) and their aqueous extracts analysed for phosphorus (G.M.12c) and carbohydrate (G.M. 11c) (Table 20).

It is apparent from Table 20, column 6, that a complete separation of the phospholipids in the mixture was achieved; all the phosphorus eluted (33.0%) being confined to Fraction 3 as desired. However it is equally apparent that glycolipids were not confined to Fraction 2 as was hoped, but were spread throughout all three fractions. Fraction 2 did, however, contain more of the carbohydrate elutedfrom the column than Fractions 1 or 3 (40% as compared to 14% and 33%). It was difficult to compare the results with those found by Benson and O'Brien (186), as they do not give any analytical figures for either carbohydrate or phosphorus in their account of the fractionation of a lipid mixture from alfafa leaves on Florisil.

In Table 21 showing the percentage of total lipid, carbohydrate and phosphorus, the 111% recovery, by weight of the total lipid applied to the column is thought to have been caused by contamination of all three fractions by silicone fluid from the rotary evaporators used in reducing their volume. As a result in further experiments requiring the process, a Buchi rotary evaporator, requiring no silicone fluid, was used in place of the Towers apparatus hitherto used.

The majority of the sugar (88%) applied to the column was recovered. No specific reasons can be put forward for the remaining 12% lost, but

TABLE 22

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO a P (%)
1 C-M 19:1	0.63	59.5	Phosphorus	0	0
			Carbohydrate	4.6	7.7
2 C-M 1:1	0.22	20.7	Phosphorus	0	0
			Carbohydrate	37.6	62.6
3 MeOH	0.20	19.1	Phosphorus	3.0	48.5
			Carbohydrate	12.4	20.6

Phosphorus and carbohydrate analysis of Fractions 1, 2 and 3 from Florisil column (Exp. 13).

TABLE 23

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
1060 lipid	1050	99.2
6.2 P	3.0	48.5
60.0 CHO	54.6	91.0

Percentage recovery of lipid, phosphorus and carbohydrate from Florisil column (Exp. 13).

some of this was probably due to experimental error.

It was concluded that although this experiment achieved only a partial separation of the glycolipids from the charged phospholipids, this fractionation could be improved upon by varying the proportions of the eluting agents. The objective, as previously stated, was to increase the percentage sugar in fraction two while retaining all the neutral lipids in fraction one and all the phospholipids in fraction three.

To this end a second Florisil column, similar to the previous one, was prepared (G.M. 7) 1.06g. of lipid mixture was applied and it was this time eluted with chloroform-methanol, 19: 1 v/v, chloroform-methanol, 1: 1 v/v and finally with methanol (Exp.13).

All three fractions were analysed for carbohydrate (G.M. 11c) and phosphorus (G.M. 12c) after hydrolysis with ethanol-chloroform- 5N HCl (G.M. 3c) (Table 22 and 23).

As can be seen from Table 22 an improved separation was achieved; Fraction 2 containing 62% carbohydrate as apposed to 40% previously obtained, while Fraction 3 still contained all the phosphorus eluted from the column (48%). Some carbohydrate was still present in Fractions 1 and 3 but this had been reduced considerably. The two previous experiments confirm that magnesium silicate is a stronger absorbent than silicic acid. The elution of the silicic acid column removed 88.5% of the phosphorus (Table 16), whereas elution of the Florisil column removed only 48.5% of the phosphorus.

According to Rouser (180) free fatty acids which pass straight through a silicic acid column are retained to some degree by magnesium silicate. If this is the case any free fatty acids present in this lipid mixture, after chromatography on Florisil, may be present in Fraction 2 as well as in Fraction 1.

From these two magnesium silicate experiments it was concluded that,

although not quantitative, it could be a very useful technique in helping to fractionate the components present in the lipid mixture from bracken.

3. Successive column chromatography of the lipid mixture from bracken.

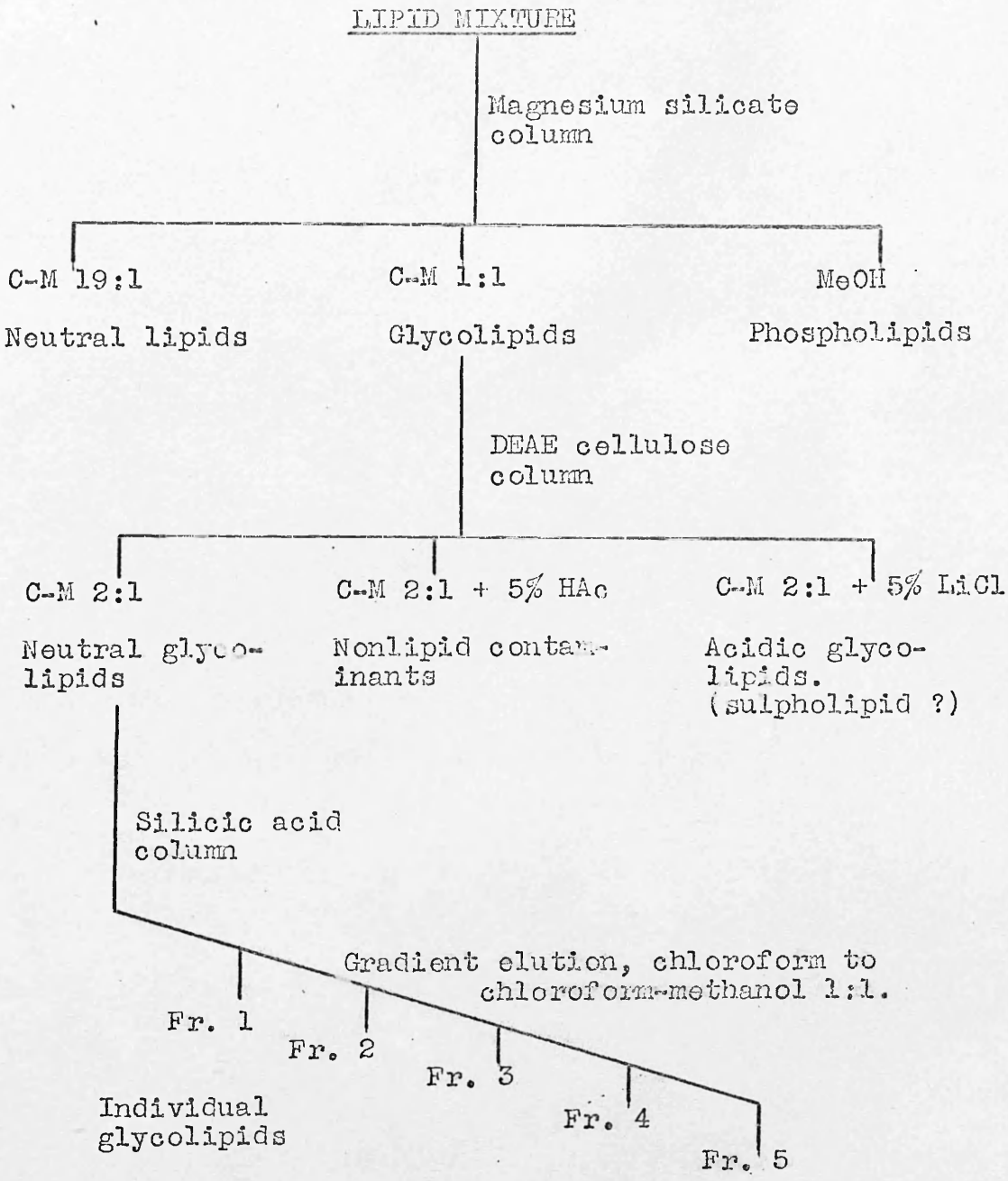
As stated in the introduction to Part 2 of this thesis, and as can be seen from the results of previous experiments (Exps.11,12, & 13) no one column technique is sufficient to separate a lipid mixture to its individual components. Therefore, using the information gathered from these preliminary studies it was decided, at this stage in the investigation, to try and combine these column procedures in such a way that a more complete fractionation of the glycolipids from bracken could be achieved.

A flow-sheet of the scheme used is shown in Fig.21. It was reasoned that if the lipid mixture is initially fractionated on a magnesium silicate column, as previously described (Exp.13), a sample of the glycolipids, free from contamination by neutral lipids and phospholipids, would be obtained. Previous experiments (Exps. 6 and 8) and published evidence (186) have shown that if this glycolipid mixture is further resolved on a DEAE cellulose column, any acidic glycolipids present, such as sulpholipid, would be retained by the column, while neutral glycolipids would pass through. Finally a separation of the neutral glycolipids could be achieved using a silicic acid column and a gradient of methanol in chloroform, as was previously suggested in Exp.11 (page 111).

The initial part of this plan (Exp.14) involved the preparation of a magnesium silicate column with effective dimensions of 18.0 x 3.5cm. (G.M.7). 1.124g. of lipid mixture from freeze-dried rhizomes were applied to the column and three separate fractions eluted using the same system as was employed in Exp. 13 (page117).

All three fractions were analysed for carbohydrate (G.M. 11c) and

Fig. 21



Flow sheet of scheme used in Exp. 15, for the fractionation of the lipid mixture from bracken into individual glycolipids.

phosphorus (G.M. 12c), after hydrolysis with ethanol-chloroform - 5N HCl (G.M. 3c).

The results (Tables 24 and 25) show that the second fraction did, as was expected, contain the majority of the glycolipids (61%). It did however also contain a small amount of phosphorus (4.5%). This slight contamination by phosphorus was, however, considered not to be serious, as expressed in actual weight it only amounted to 0.3mg. P.

The three fractions were also analysed by unidimensional thin-layer chromatography on silicic acid using a solvent mixture of chloroform-methanol-acetic acid (G.M. 4). Iodine vapour was used to detect the spots (G.M. 5) (Fig 22). This, while confirming the carbohydrate and phosphorus analysis in that it showed an almost complete separation between Fraction 2 and 3, also confirmed that the vast majority of the neutral lipids were confined to Fraction 1. Fraction 3 did contain, other than the very slow running spots, one component which was also present in Fraction 2 and ran quite close to the solvent front. No attempt was made to identify this or any other spots at this stage.

Paper chromatography using ethyl-acetate - pyridine - water (G.M. 4) of the aqueous hydrolysate (G.M. 3c) of the three fractions was inconclusive in that all the fractions gave a similar sugar pattern. Fraction 3 did, however, contain a much greater amount of the charged sugar (Rg.38) than did Fraction 2. This fact appears to contradict the findings of O'Brien and Benson to some extent (186) who found that the plant sulpholipid, 6 - sulpho - α - D - quinovo - pyranosyl - (1- 1') - diglyceride, was eluted mainly in the second fraction from Florisil columns.

Fraction 2, 0.26g., from the Florisil column was further resolved on a DEAE cellulose column with effective dimensions 22.0 x 2.2 cm. (G.M. 7). It will be recalled from the introduction (page 60) and from previous

TABLE 24

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO a P (%)
1 C-M 19:1	0.58	51.0	Phosphorus	0	0
			Carbohydrate	1.5	2.3
2 C-M 1:1	0.29	25.0	Phosphorus	0.3	4.5
			Carbohydrate	40.5	61.0
3 MeOH	0.15	13.0	Phosphorus	0.62	9.4
			Carbohydrate	4.5	6.8

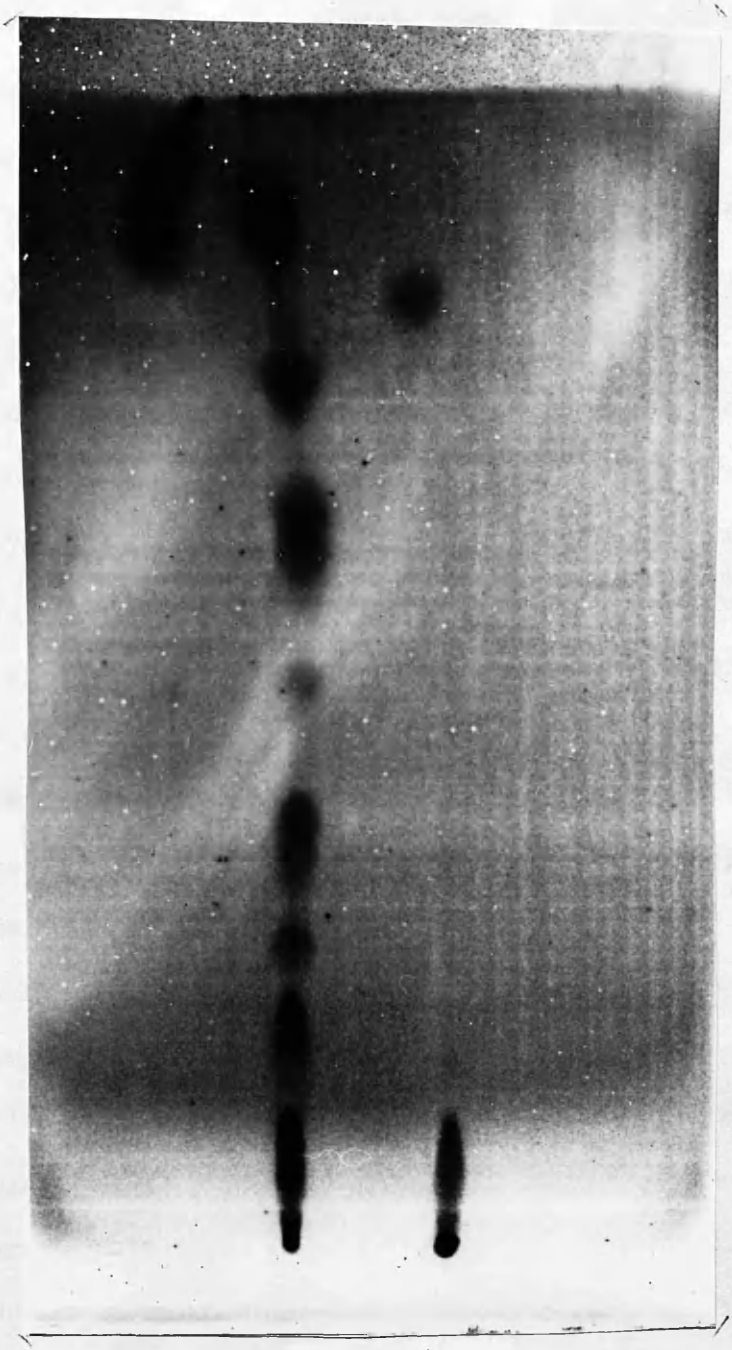
Phosphorus and carbohydrate analysis of Fractions 1, 2 and 3 from Florisil column (Exp. 14).

TABLE 25

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
1124 lipid	1020	90.5
6.6 P	0.9	14.8
64.1 CHO	46.5	72.5

Percentage recovery of lipid, phosphorus and carbohydrate from Florisil column (Exp. 14).

Fig. 22



Unidimensional TLC of Fractions 1, 2 and 3 from magnesium silicate column (Exp. 14).
Solvent: chloroform-methanol-acetic acid (85:25:1 v/v).

experiments (Exps. 6 and 8) that the elution scheme used for this separation was that of Svennerholm (188). This was adhered to here and the three fractions obtained analysed, after hydrolysis (G.M. 3c), for carbohydrate content (G.M. 11c) (Tables 26 and 27).

It can be seen from Table 26 that, as required, the vast majority of the glycolipids had passed straight through the column and were present in Fraction 1.

Unidimensional thin-layer chromatography on silicic acid (G.M. 4) of the three fractions, showed that the first and third were composed of basically the same components, but that Fraction 3 was much less distinct (Fig.23). This would tend to support the idea previously expressed (Exp. 6) that a polar effect as well as an ion-exchange one may have some influence on the elution of lipids from DEAE cellulose columns. Fraction 2 showed up on the thin-layer plates as a streak indicating that, as expected (188), it was mainly composed of nonlipid material or modified lipids.

Paper chromatography (G.M. 4) of the aqueous hydrolysates (G.M. 3c) of the three fractions confirmed previous results from DEAE columns (Exps. 6 & 8) in that it showed the acidic sugar (Rg.38) confined to Fraction 3. Also while Fraction 1 contained the majority of the neutral sugars (Table 26) these were also present to a much lesser extent in the other fractions. To summarise, therefore, the scheme proposed in Fig.21 had, so far, been mainly successful. A sample of glycolipid mixture from bracken, free from neutral lipids and almost free from phospholipids, was obtained using the Florisil column. This mixture was further fractionated on a DEAE cellulose column to give a sample containing neutral glycolipids (Fraction 1) and a sample containing an acidic glycolipid plus a small amount of neutral glycolipids (Fraction 3).

The final stage of Exp.14 was the resolution of the neutral glycolipid

TABLE 26

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO (%)
1 C-M 2:1	0.23	88.5	Carbohydrate	24.0	66.0
2 C-M 2:1 + 5% HAc	0.02	5.4	Carbohydrate	3.0	8.2
3 C-M 2:1 + 5% LiCl	0.01	4.2	Carbohydrate	1.2	3.2

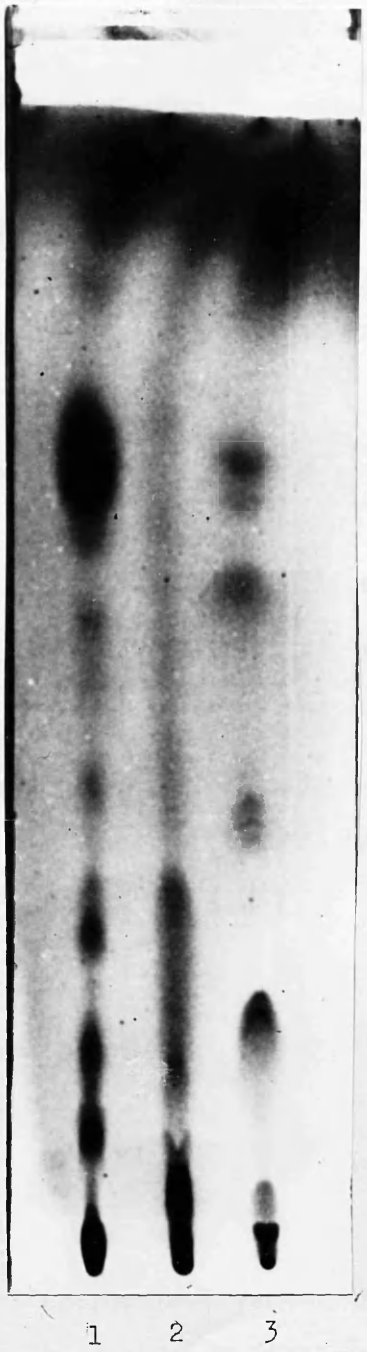
Carbohydrate analysis of Fractions 1, 2 and 3 from DEAE cellulose column (Exp. 14).

TABLE 27

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
260 lipid	255	98.0
36.5 CHO	28.2	78.0

Percentage recovery of lipid and carbohydrate from DEAE cellulose column (Exp. 14).

Fig. 23



Unidimensional TLC of Fractions 1, 2 and 3 from DEAE cellulose column (Exp. 14).
Solvent: chloroform-methanol-acetic acid (85:25:1 v/v).

mixture from the DEAE cellulose column into its individual components using a silicic acid column.

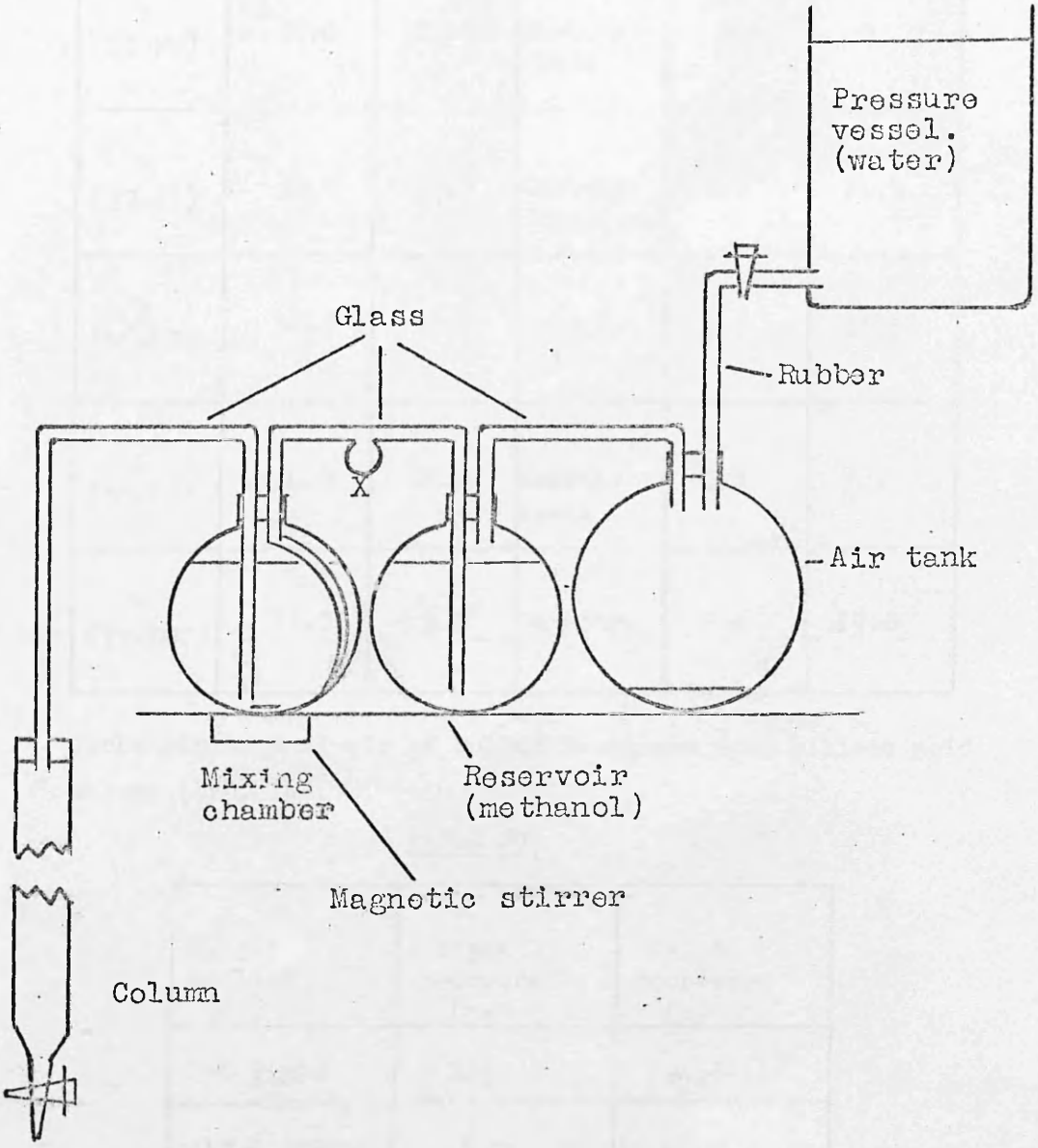
A column, 42.0 x 3.2 cm. in dimension was uniformly packed (G.M.7) with pretreated silicic acid (G.M. 13). The fractionation was performed in a sealed system under pressure as shown in Fig. 24. The water reservoir had a large diameter thus ensuring an almost uniform pressure throughout the separation. Neither rubber nor polythene was used, all connections where necessary being of glass tubing. This was to avoid the possible extraction of contaminants from rubber or polythene by the chloroform-methanol eluting mixture.

130 mg. of neutral glycolipid mixture from Fraction 1 of the DEAE cellulose column were applied to the column and eluted with a linear gradient of chloroform to chloroform-methanol. The small bulb, x, between the mixing chamber and the methanol reservoir was to ensure that the gradient started at zero, by preventing the methanol in the reservoir from overflowing too soon into the mixing chamber, when the pressure was applied at the water reservoir.

10.0ml. fractions were collected automatically using a Towers fraction collector and every other fraction analysed by unidimensional TLC on silicic acid using a solvent mixture of chloroform-methanol-acetic acid (G.M. 4). The spots were located using iodine vapour (G.M. 5). On the basis of the TLC analysis the appropriate tubes were bulked and the five bulked samples analysed for sugar content (G.M. 11c), after hydrolysis with ethanol-chloroform - 5N HCl (G.M. 3c) (Tables 28 and 29).

However, more information regarding the success of the fractionation was obtained from the TLC analysis (G.M. 4) of the five bulked fractions (Fig.25). This showed that there was considerable cross contamination between the fractions and that only in the first bulked sample was a single

Fig. 24



Pressurised system used for the fractionation of the glycolipid mixture on a silicic acid column using a gradient elution of chloroform to C-M 1:1. (Exp. 14).

TABLE 28

Fraction (tube No)	Weight (mg)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO (%)
1 (13-29)	30.0	23.5	Carbohydrate	0	0
2 (32-45)	31.0	23.8	Carbohydrate	2.9	21.5
3 (46-49)	36.0	27.7	Carbohydrate	3.1	23.5
4 (56-62)	14.0	10.8	Carbohydrate	0.7	7.9
5 (79-107)	12.0	9.2	Carbohydrate	2.3	17.0

Carbohydrate analysis of bulked fractions from silicic acid column (Exp. 14).

TABLE 29

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
130 lipid	123	94.5
13.5 CHO	9.0	66.6

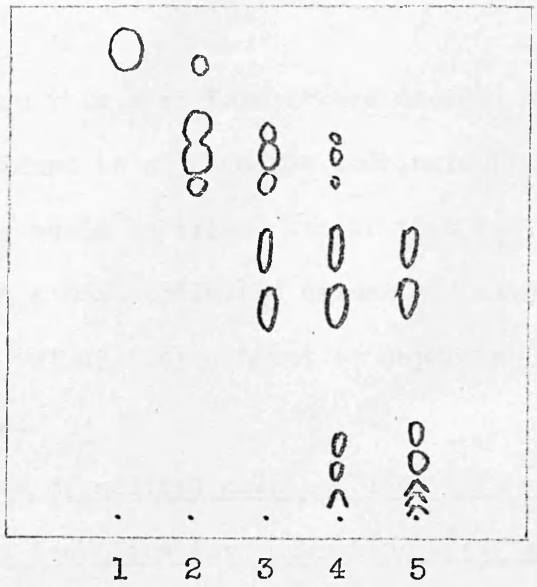
Percentage recovery of lipid and carbohydrate from silicic acid column (Exp. 14).

component detected and as can be seen from Table 28 this sample contained no carbohydrate and, as a result, was suspected of containing, not glycolipid, but free fatty acids. An investigation into what sugars were present in the bulked samples, after hydrolysis (G.M. 3c), was carried out using paper chromatography (G.M. 4). This showed(Fig.26) that, as expected, no sugars were present in the first bulked fraction. It also showed that in the second and third samples glucose was predominant with only a trace of galactose, while in the fourth and fifth samples galactose was dominant with only a trace of glucose. Samples 1 and 2 did not contain glycerol, whereas in samples 3, 4 and 5, the amount of glycerol detected increased successively. A connection between the glycerol and galatose was therefore apparent, possibly in the form of galactoglyceride as was previously suggested. However the glucose containing lipid did not appear to contain glycerol and the fact that it was eluted off the column well ahead of the glycolipid containing glycerol and galactose would suggest that the glucose was associated with a more lyophilic moiety than glycerol. Only one glucose containing plant glycolipid so far known fits this requirement; the esterified steryl glucoside discovered by Lenase (176).

As expected no charged sugar was detected in the bulked samples using paper chromatography, as any present in the lipid extract would be completely retained by the DEAE cellulose column. Only in Fraction 4 and 5 were faint spots detected, running similar to the pentose sugars previously mentioned (page 85). There did not appear to be any significant degree of fractionation between them; three faint spots being located in both fractions.

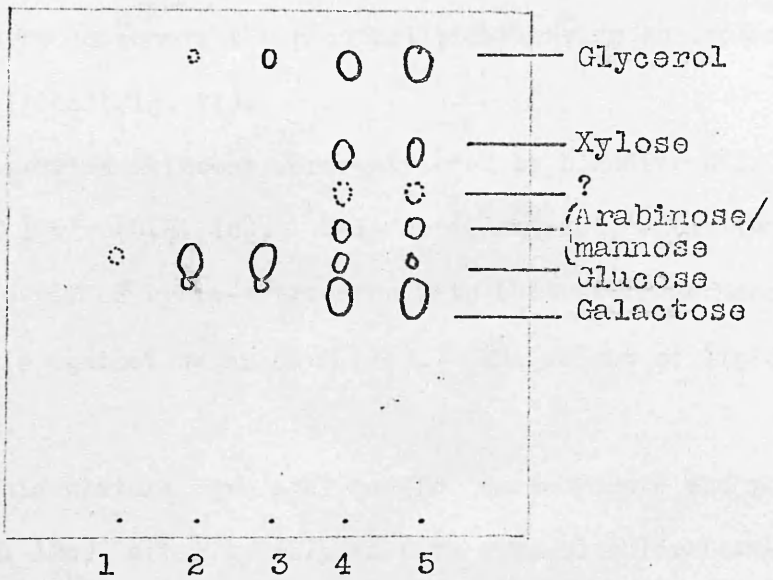
It appeared, therefore, that this particular sequence of columns, and the silicic acid one in particular, was not of any significant value in coping with this exacting fractionation. It was also apparent from this experiment that even the combined powers of resolution of the various

Fig. 25



TLC of the five bulked fractions from silicic acid column (Exp. 14). Solvent: chloroform-methanol-HAc.

Fig. 26



Paper chromatography of sugars present in the five bulked fractions from silicic acid column (Exp. 14).

Solvent: ethyl acetate-pyridine-water (120:50:40 v/v).

column chromatographic techniques were not sufficient to effect a complete fractionation of the glycolipid components of the lipid mixture from bracken.

Following from this therefore it was decided to carry out a further preliminary experiment in which a new combination of silicic acid and magnesium silicate would be tried, and if this was found to be successful, to combine it with a DEAE cellulose column and also preparative thin-layer chromatography in yet another attempt to separate the components in the glycolipid mixture.

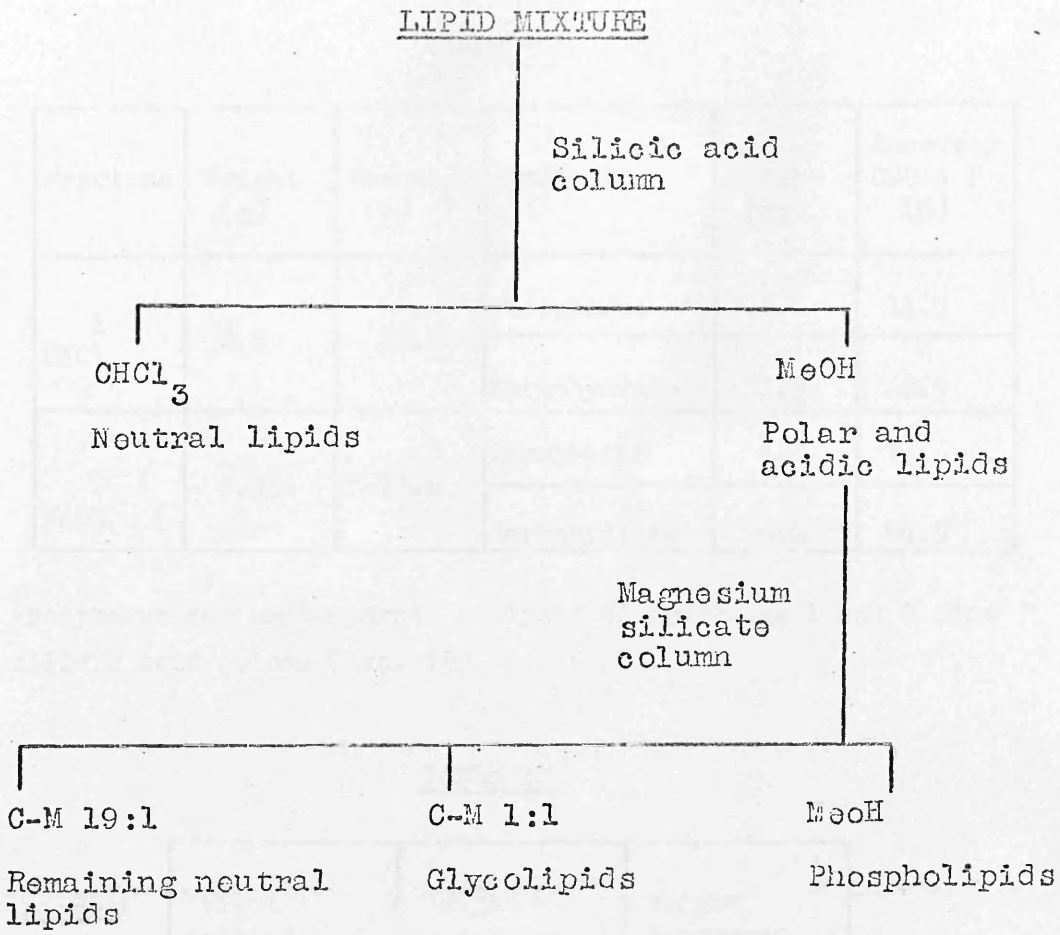
4. The evaluation of silicic acid combined with magnesium silicate as a technique for separating lipid mixtures.

It was decided to carry out an initial fractionation of the lipid mixture using a silicic acid column as in Exp.13. This would remove all the neutral lipids, including fatty acids, leaving a mixture of phospholipids and glycolipids. Further fractionation of this polar mixture on a Florisil column would remove the phospholipids leaving an uncontaminated mixture of glycolipids (Fig. 27).

0.5kg. freeze-dried rhizomes were extracted by blending with chloroform-methanol, 2: 1 v/v (G.M. 1c). This crude extract, after removal of the solvent, was purified by re-extraction with chloroform-methanol, 2:1 v/v, and dialysis against water (G.M. 1c). The weight of lipid mixture obtained was 4.9g.

Samples of this mixture were analysed for carbohydrate and phosphorus content (G.M. 11c. 12c). after hydrolysis with ethanol chloroform-5N HCl (G.M. 3c) (Table 30). The amount of carbohydrate and phosphorus found in this preliminary analysis showed a slight variation compared to that found in previous lipid extracts from freeze-dried rhizomes (Tables 10 and 19). However it was thought that this variation was not sufficient to cause any

Fig. 27



Flow sheet of scheme used in Exp. 15.

TABLE 30

Analysis	Mg./g. mixture
Sugar	46.7
Phosphorus	5.7

Sugar and phosphorus analysis of lipid mixture from freeze-dried bracken rhizomes.

TABLE 31

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO & P (%)
1 $CHCl_3$	0.8	68.0	Phosphorus	1.0	14.9
			Carbohydrate	1.5	2.9
2 MeOH	0.356	29.8	Phosphorus	4.8	71.6
			Carbohydrate	46.4	84.5

Phosphorus and carbohydrate analysis of Fractions 1 and 2 from silicic acid column (Exp. 15).

TABLE 32

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
1175 lipid	1156	98.0
6.7 P	5.9	86.5
54.9 CHO	47.9	87.0

Percentage recovery of lipid and carbohydrate from silicic acid column (Exp. 15).

TABLE 33

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 1																			+	+
	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	

2 dimensional TLC analysis of fractions from silicic acid column. (Exp. 15).

anxiety.

1.175g. of the lipid mixture were applied to a silicic acid column (Exp.15) prepared (G.M. 7) using pretreated material (G.M. 13) and separated into two fractions eluting with chloroform followed by methanol.

Both fractions were analysed for carbohydrate and phosphorus (G.M. 11c, 12c), after hydrolysis (G.M. 3c) (Tables 31 and 32). This showed that a good separation was achieved; Fraction 2 containing the majority of the glycolipids and phospholipids present in the mixture.

Analysis of the two fractions by two dimensional thin-layer chromatography (G.M. 4), using 20% perchloric acid to detect the spots (G.M. 5) (Table 33), confirmed the carbohydrate and phosphorus results. It showed that an almost complete fractionation was achieved, spot No. 19 being the only component visible in both fractions. The spots in Table 33 were numbered according to the 'total lipid map' in Fig.16. Spot No.20 (Table 33). which represents neutral lipid material present in the mixture, was confined entirely to Fraction 1, while the remaining polar lipids were confined to Fraction 2.

340 mgs. of polar lipid mixture from Fraction 2 were further fractionated on a Florisil column (Exp.15) prepared (G.M. 7) with pretreated material (G.M. 13). The mixture was separated into three fractions using the following elution sequence, chloroform-methanol, 19: 1 v/v, chloroform-methanol, 1: 1 v/v and finally methanol. As will be recalled this was the sequence used for this fractionation in Exp.13 (page 117).

All three fractions were analysed for carbohydrate and phosphorus content (G.M. 11c and 12c), after hydrolysis (G.M. 3c). The results (Tables 34 and 35) showed that Fraction 1 did not contain any carbohydrate or phosphorus and was consequently discarded.

The contents of Fractions 2 and 3 were further investigated by the two dimensional TLC technique of Lepage (G.M. 4) as was previously used to

TABLE 34

Fraction	Weight (mg)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO a P (%)
1 C-M 19:1	17.0	4.8	Phosphorus	0	0
			Carbohydrate	0	0
2 C-M 1:1	187.0	55.0	Phosphorus	0.8	17.4
			Carbohydrate	27.3	61.4
3 MeOH	102.0	30.0	Phosphorus	1.7	37.0
			Carbohydrate	11.0	24.8

Phosphorus and carbohydrate analysis of Fractions 1. 2 and 3 from Florisil column (Exp. 15).

TABLE 35

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
340 lipid	306	90.0
4.6 P	2.5	54.4
44.3 CHO	38.3	86.3

Percentage recovery of lipid, phosphorus and carbohydrate from Florisil column (Exp. 15).

identify the components of the total lipid mixture (Tables 11 and 12). 20% perchloric acid was used as a general detecting reagent, molybdenum /H_2SO_4 spray to locate phospholipids and α - napthol reagent to locate glycolipids (Table 36, Nos. 1, 2 and 3).

Table 34 shows that Fraction 2 contained the majority of the carbohydrate (61.5%) applied to the column. Two dimensional TLC analysis (Table 36, No.3) confirmed that this carbohydrate material was in the form of all the glycolipids so far identified to be present in the lipid mixture (Table 11). Apart from a small amount of phosphorus (0.8mgs.) also present in Fraction 2, this separation had succeeded in providing a sample of glycolipid mixture free from phospholipid contamination.

The most significant aspect of Fraction 3 was that the lipid compound represented by spot No.9. was present in this fraction in greater quantities than in Fraction 2 (Table 36, No.3). This spot, being identified as a glycolipid on the basis of its positive reaction with α - naphthol reagent and on the results of previous fractionations using Florisil (Exp.14), was suspected of being an acidic glycolipid, possibly a sulpholipid.

This belief was supported by paper chromatographic analysis of the sugars present in the two fractions, after hydrolysis with 2N H_2SO_4 (G.M. 3) using a solvent mixture of ethyl acetate - pyridine - water (G.M. 4). This showed that Fraction 3 contained a greater amount of slow running acidic sugar (Rg.38) than did Fraction 2. It was, therefore, thought not unreasonable to assume a connection between the acidic sugar predominant in Fraction 3 and the glycolipid, represented by spot No.9 also predominant in Fraction 3.

To summarise, this sequence of columns successfully performed the fractionation required. The silicic acid effectively rid the mixture

TABLE 36

20% PERCHLORIC ACID — No. 1

Spots numbered as in "LIPID MAP" (Fig. 16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 2	w +			m +					w +		w +			w +		s +	s +	s +		
Fraction 3	s +		m +	m +				m +	m +	w +	w +	w +		?	?	w +	vw +	vw +		

MOLYBDENUM/H_2SO_4 — No. 2

Fraction 2								vw +				vw +								
Fraction 3			s +		?	?		s +		w +		m +								

α-NAPHTHOL — No. 3

Fraction 2	m +?			m +					w +		m +			w +		s +	s +	s +		
Fraction 3	m +?			w +					m +		w +			?		w +		vw +		

2 dimensional TLC analysis of fractions from magnesium silicate column. (Exp. 15).

of all the neutral lipids, including free fatty acids, and the second fraction from the Florisil column contained a mixture of glycolipids almost completely free from phospholipids. Following from this it was reasoned that the mixture of glycolipids could be further resolved into neutral and acidic glycolipids with the aid of DEAE cellulose column and finally, a separation of the neutral sugar lipids effected using preparative thin-layer chromatography, as was previously suggested (page 131).

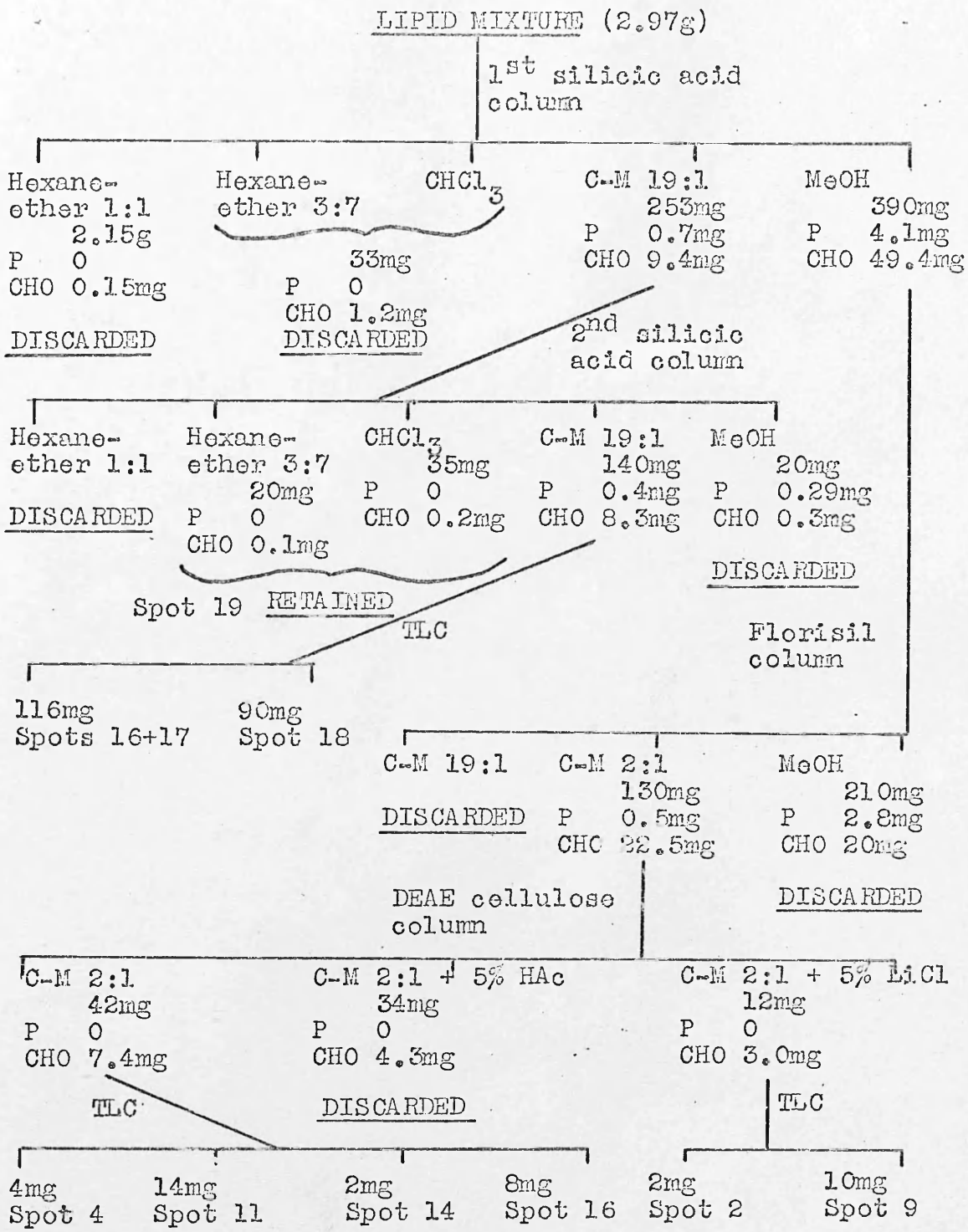
5. Fractionation of the lipid mixture from bracken using both column and thin-layer chromatography.

A flow-sheet of the column sequence used to achieve the complete fractionation of the glycolipids present in bracken is given in Fig.28. From this it can be seen that instead of the normal elution system of chloroform and methanol being used for the initial silicic acid column it was decided to substitute the hexane-ether-chloroform-methanol system used by Lepage to fractionate esterified steryl glucoside from potatoes(167). In doing this it was hoped that if a similar glycolipid were present in bracken it too would be fractionated.

The lipid mixture used for this experiment was extracted from freeze-dried bracken by blending with chloroform-methanol, 2: 1 v/v (G.M. 1c). This crude extract was purified, after solvent removal, by re-extraction with chloroform-methanol and dialysis against water (G.M. 1c). The weight of the lipid mixture obtained was 4.5g.

As was customary with all fresh lipid mixtures a preliminary carbohydrate and phosphorus analysis was performed (G.M. 11c, 12c) (Table 37)

Fig. 28



Flow sheet for the fractionation of the lipid mixture from bracken using column and thin-layer chromatography. (Exp. 16)

TABLE 37

Analysis	Mg/g
Carbohydrate	24.0
Phosphorus	3.0

As can be seen, the values obtained for this analysis were approximately half that normally found for a lipid extract from freeze-dried rhizomes. However two dimensional TLC of the mixture (G.M. 4) showed that all the spots normally present were located (Fig.16). As this last fact was considered to be most important it was decided to continue using this fresh lipid mixture in the present investigation. No explanation can be submitted as to why the carbohydrate and phosphorus values were so low in this case.

An initial fractionation of 2.97g. lipid mixture was carried out using a silicic acid column (G.M. 7) (Exp.16). The column was eluted with hexane-ether, 1: 1 v/v, to remove neutral lipids, followed by hexane-ether, 3: 7 v/v, to remove esterified steryl glucoside if present, next with chloroform to remove any remaining esterified steryl glucoside, followed by chloroform-methanol, 19: 1 v/v, to remove galactolipids and finally the column was eluted with methanol to strip off any remaining lipids still held by it. Fractions 2 and 3 were combined and subsequently referred to as Fraction 2.

Samples of all four fractions were hydrolysed (G.M. 3c) and analysed for carbohydrate and phosphorus content (G.M. 11c, 12c) (Tables 38 and 39).

The components of all four fractions were further investigated by two dimensional TLC on silicic acid (G.M. 4). The detecting reagents were those previously used; 20% perchloric acid, molybdenum/H_2SO_4 and

α-naphthol (G.M. 5) (Table 40, No. 1, 2 and 3).

From the above analyses Fraction 1 and 2 were shown to be composed almost entirely of neutral lipids and consequently were discarded.

Fraction 4 (253 mgs.) contained only a small amount of carbohydrate (13.2%) as well as less than one milligram (7.8%) of phosphorus. TLC analysis of this fraction however did not detect any phospholipids and only spot Nos. 16, 17, 18 and 19, all representing glycolipid components, plus a trace of spot No.20 (neutral lipid) were located. Spot No.19 gave here, for the first time, a positive reaction with α-naphthol and as a result was considered to be a glycolipid. This spot was not previously thought to be a sugar lipid, but perhaps being freed from contamination by neutral lipid had allowed it to be more positively identified with α-naphthol in the present investigation. Figure 29 is a photograph of the separation obtained for Fraction 4 using TLC. The spots were located with 20% perchloric acid. Fraction 4 was retained.

Fraction 5 (390mgs.) as can be seen from the results, was more complex. It contained the remaining glycolipid compounds (spot Nos. 4, 9, 11, 14 and 16) as well as the phospholipids shown (Table 40 No.2). The majority of the carbohydrate (69.5%) was in this fraction but this was contaminated with phosphorus(46.0%). This fraction was also retained for further fractionation.

This column had suceeded in removing all but a trace of neutral lipid from the mixture. A complete fractionation was achieved between the glycolipid compounds in Fraction 4 and those in Fraction 5; Fraction 4 being almost exclusively glycolipid while Fraction 5 also contained a certain amount of phospholipid material.

Previous information (Exp.14 page 129) showed that a glucose containing lipid not attached to glycerol was eluted off a silicic acid column

TABLE 38

Fraction	Weight (g)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO a P (%)
1 Hexane-ether 1:1	2.15	72.2	Phosphorus	0	0
			Carbohydrate	0.15	0.2
2+3 Hexane-ether 3:7 + $CHCl_3$	0.033	1.1	Phosphorus	0	0
			Carbohydrate	1.2	1.7
4 C-M 19:1	0.253	8.5	Phosphorus	0.7	7.8
			Carbohydrate	9.4	13.2
5 MeOH	0.39	13.1	Phosphorus	4.1	46.0
			Carbohydrate	49.4	69.5

Phosphorus and carbohydrate analysis of Fractions 1, 2+3, 4 and 5 from first silicic acid column (Exp. 16).

TABLE 39

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
2970 lipid	2830	95.0
8.7 P	4.8	54.0
71.0 CHO	60.2	84.5

Percentage recovery of lipid, phosphorus and carbohydrate from first silicic acid column (Exp. 16).

TABLE 40

20% PERCHLORIC ACID — No. 1

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 1																				+s
Fraction 2																			+m	+w
Fraction 4																+s	+s	+s	+m	+w
Fraction 5	?	?		+m	+w	+w		+s	+m	+w	+s	+s		+m	?	+w				

MOLYBDENUM/H_2SO_4 — No. 2

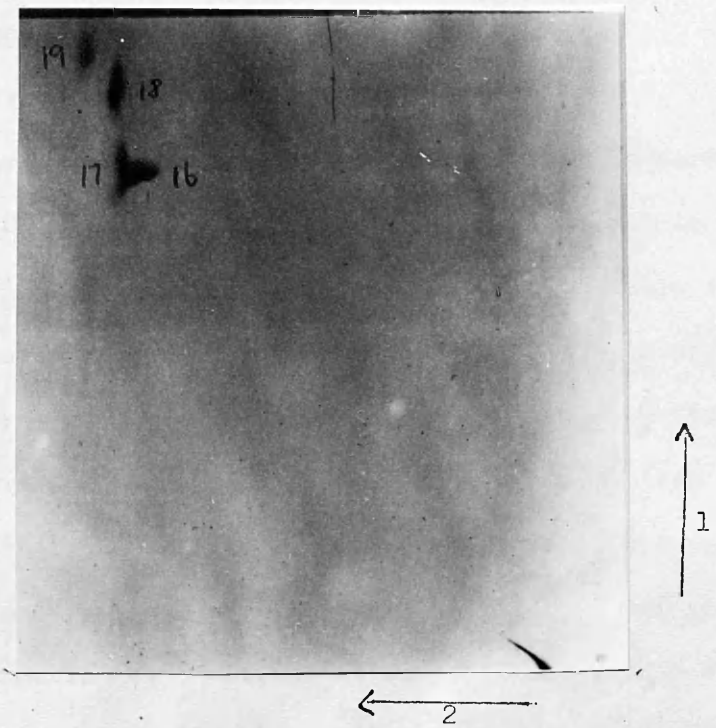
Fraction 1																				
Fraction 2																				
Fraction 4																				
Fraction 5			?		+m	+m		+s		+w?			+s							

α-NAPHTHOL — No. 3

Fraction 1																				
Fraction 2																			+w	
Fraction 4																+s	+ s	+s	+m	
Fraction 5	?	?		+m					+m		+s			+m		+w				

2 dimensional TLC analysis of fractions from first silicic acid column. (Exp. 16).

Fig. 29



Photograph of the two dimensional TLC analysis of Fraction 4 from the first silicic acid column (Exp. 16) showing spot Nos. 16, 17, 18 and 19. The spots were located using 20% perchloric acid.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10 v/v).

near the start. It was decided therefore to rechromatograph Fraction 4 on a similar silicic acid column as the previous one, in an attempt to fractionate further the four glycolipids present.

A fresh silicic acid column, 14. x 0 x 2.5cm. in dimension was prepared, (G.M. 7). 230 mgs. from Fraction 4 were rechromatographed (Exp.16) using the same elution sequence as in the first silicic acid column.

Fraction 1 (11.0 mgs.) was discarded, being presumably all neutral lipid. The four remaining fractions were analysed for carbohydrate and phosphorus content in the usual manner (G.M. 11c, 12c), after hydrolysis (G.M. 3c) (Tables 41 and 42).

The four fractions were further analysed by the normal two dimensional TLC method (G.M. 4). Again three reagents were used to detect the spots; 20% perchloric acid, molybdenum, and α-naphthol (Table 43 No. 1, 2 and 3).

From the results it can be seen that there was a significant degree of success in separating one of the glycolipid components (spot No.19) from the other three (spot Nos. 16, 17 and 18.). Fraction 2 and 3 were composed entirely of this single sugar-lipid, while the remaining three were confined, almost entirely, to Fraction 4. A trace of phosphorus was still present in Fraction 4. However it was so small (0.28% of the total fraction) as to be considered negligible. It was in fact so small that no phospholipid compounds were detected by thin-layer chromatography (Table 43, No.2).

Fractions 2 and 3 were combined, labelled Fraction 2 + 3 and retained. Fraction 4 was also retained.

Fraction 5 contained a very small amount of carbohydrate but this was insignificant (3.5%) and this fraction was discarded.

Figures 30 and 31 are photographs of the separations obtained by TLC analysis of Fraction 2 + 3 and Fraction 4 respectively. The spots were

TABLE 41

Fraction	Weight (mg)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO a P (%)
2 Hexane-ether 1:1	20.0	8.7	Phosphorus	0	0
			Carbohydrate	0.1	1.2
3 $CHCl_3$	35.0	15.5	Phosphorus	0	0
			Carbohydrate	0.2	2.4
4 C-M 19:1	140.0	61.0	Phosphorus	0.4	60.0
			Carbohydrate	8.3	96.5
5 MeOH	20.0	8.7	Phosphorus	0.29	43.0
			Carbohydrate	0.3	3.6

Phosphorus and carbohydrate analysis of Fractions 2, 3, 4 and 5 from second silicic acid column (Exp. 16).

TABLE 42

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
230 lipid	226	98.2
0.67 P	0.69	101.5
8.6	8.9	103.7

Percentage recovery of lipid, phosphorus and carbohydrate from second silicic acid column (Exp. 16).

TABLE 43

20% PERCHLORIC ACID — No. 1

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 2																			+ m	+ vw
Fraction 3																			+ s	
Fraction 4																+ s	+ s	+ s		
Fraction 5																				

MOLYBDENUM/H_2SO_4 — No. 2

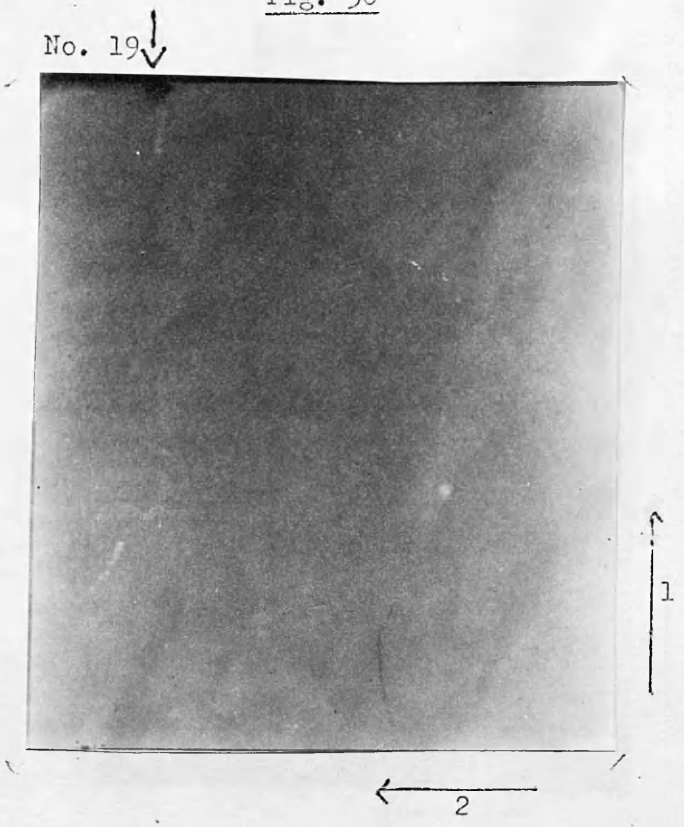
Fraction 2																				
Fraction 3																				
Fraction 4																				
Fraction 5																				

α NAPHTHOL — No. 3

Fraction 2																			+ m	
Fraction 3																			+ s	
Fraction 4																+ s	+ m	+ s		
Fraction 5																				

2 dimensional TLC analysis of fractions from second silicic acid column. (Exp. 16).

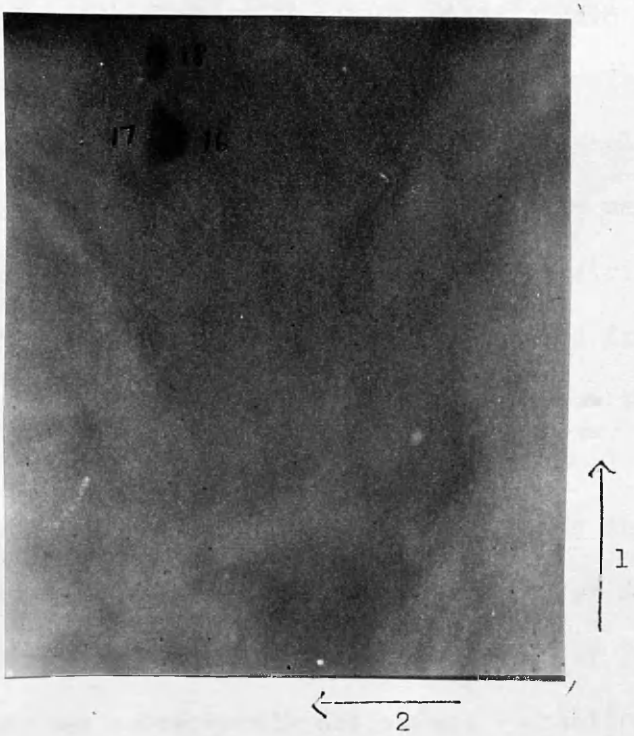
Fig. 30



Photograph of the two dimensional TLC analysis of Fraction 2+3 from the second silicic acid column (Exp. 16) showing spot No. 19. The spots were located using 20% perchloric acid.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10 v/v).

Fig. 31



Photograph of the two dimensional TLC analysis of Fraction 4 from second silicic acid column (Exp. 16) showing spot Nos. 16, 17 and 18. The spots were located using 20% perchloric acid.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10 v/v).

located with 20% perchloric acid.

It will be recalled from the first silicic acid column in this fractionation (Table 38) that Fraction 5, while containing the majority of the carbohydrate (69.5%) in the form of the remaining glycolipids (represented by spot Nos. 4, 9, 11, 14 and 16) also contained a quantity of phosphorus (4.1mg. or 46%). It was decided therefore to attempt the removal of this phosphorus contamination with the aid of a magnesium silicate column.

348mgs. of Fraction 5 from the first silicic acid column (page 142) were fractionated on a column of Florisil (G.M. 7) using the following stepwise elution sequence (Exp.16) chloroform-methanol, 19: 1 v/v, chloroform-methanol, 2: 1 v/v and finally the column was eluted with methanol. Chloroform-methanol, 2: 1 v/v, was substituted for chloroform-methanol, 1: 1 v/v, normally used to elute the second fraction, to ensure that as little phosphorus as possible was washed from the column.

The amount of material in Fraction 1 was so small (less than 2%) that it was discarded. The remaining two were subjected to the usual carbohydrate and phosphorus analysis (G.M. 11c, 12c) (Tables 44 and 45). The results showed that most of the phosphorus (2.8mg. or 78%) was eluted in Fraction 3, which was subsequently discarded. Fraction 2 was also subjected to analysis by two dimensional TLC (G.M. 4) (Table 46).

The results indicated that this column was largely successful in removing the phosphorus from the lipid sample applied to it. Fraction 2 while containing only half (51.0%) of the carbohydrate applied to the column, contained next to no phosphorus (0.5mg. or 13.9%). TLC analysis also showed that only two phospholipid spots were weakly located, while all the glycolipid spots (Nos.4, 9, 11, 14 and 16) were easily detected.

TABLE 44

Fraction	Weight (mg)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO & P (%)
2 C-M 2:1	130	37.5	Phosphorus	0.5	13.9
			Carbohydrate	22.5	51.0
3 MeOH	210	60.0	Phosphorus	2.8	78.0
			Carbohydrate	20.0	45.0

Phosphorus and carbohydrate analysis of Fractions 2 and 3 from magnesium silicate column (Exp. 16).

TABLE 45

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
348 lipid	340	97.5
3.6 P	3.3	91.9
44.1 CHO	42.5	96.0

Percentage recovery of lipid, phosphorus and carbohydrate from magnesium silicate column (Exp. 16).

TABLE 46

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20% perchloric acid	?	?		+ W					+ S	+ VW	+ S	+ W		+ S	+ VW	+ W				
Molybdenum/H_2SO_4										+ VW		+ W								
α-naphthol	?	?		+ W					+ S		+ S			+ S		+ m				

2 dimensional TLC analysis of fraction two from magnesium silicate column. (Exp. 16).

Also present in Fraction 2 were several spots near the origin of the TLC plate which gave a positive reaction with α-naphthol. These spots were, however, only very faintly detectable.

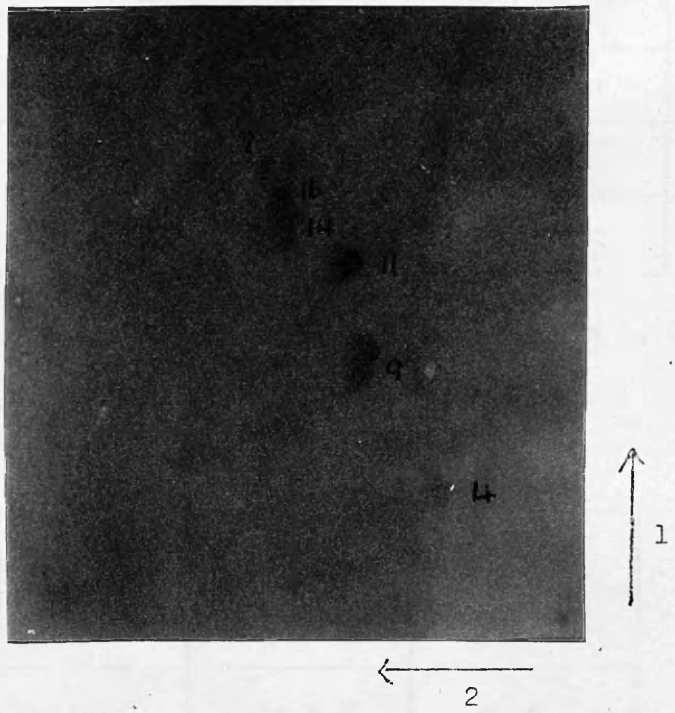
It was decided therefore that as Fraction 2 was composed almost entirely of the glycolipid compounds under investigation and as only a very small amount of phosphorus contamination was present, no further attempts at removing the 0.5mgs. phosphorus left would be carried out. This fraction was retained. Figure 32 is a photograph of the separation obtained of Fraction 2 using two dimensional TLC and 20% perchloric acid to detect the spots.

Fraction 2 from the Florisil column as previously stated contained five glycolipid compounds (represented by Nos. 4, 9, 11, 14 and 16). From the results of previous experiments (Exp.15) the glycolipid represented by the spot No.9 was believed to be acidic, and possibly a sulpholipid. It was decided therefore to try and separate it from the others with the aid of a DEAE cellulose column. 93.0mgs. of Fraction 2 from the Florisil column (page 151) were fractionated on a DEAE cellulose column, 20.0 x 2.5cm. in dimension (G.M. 7) using the stepwise elution sequence of Svennerholm as described in the introduction (page 60).

The three fractions obtained were analysed in the usual manner for carbohydrate and phosphorus (G.M. 11c, 12c) (Tables 47 and 48) and also their composition was investigated by two dimensional TLC (G.M. 4) (Table 49, Nos. 1, 2 and 3).

The results indicated an almost complete separation of the charged glycolipid from the rest, the former being the only compound definitely located in Fraction 3, while the latter were wholly confined to Fraction 1. A very faint trace of spot No.9 was located in Fraction 1 by the TLC

Fig 32



Photograph of the two dimensional TLC analysis of Fraction 2 from the Florisil column (Exp. 16) showing the glycolipid spots, Nos. 4, 9, 11, 14 and 16. All the spots were located using 20% perchloric acid.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10 v/v).

TABLE 47

Fraction	Weight (mg)	Recovery (%)	Analysis	Weight (mg)	Recovery CHO & P (%)
1 C-M 2:1	42.0	45.0	Phosphorus	0	0
			Carbohydrate	7.4	46.0
2 C-M 2:1 + 5% HAc	34.0	36.5	Phosphorus	0	0
			Carbohydrate	4.3	26.0
3 C-M 2:1 +5% LiCl	12.0	12.9	Phosphorus	0	0
			Carbohydrate	3.0	18.0

Phosphorus and carbohydrate analysis of Fractions 1, 2 and 3 from DEAE cellulose column (Exp. 16).

TABLE 48

Weight applied (mg)	Weight recovered (mg)	Weight recovered (%)
93.0 lipid	88.0	94.5
0.34 P	0	0
16.1 CHO	14.7	91.2

Percentage recovery of lipid, phosphorus and carbohydrate from DEAE cellulose column (Exp. 16).

TABLE 49

20% PERCHLORIC ACID — No. 1

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fraction 1				+w					+w		+m			+s		+w				
Fraction 2																				
Fraction 3		?							+s											

MOLYBDENUM/H_2SO_4 — No. 2

Fraction 1																				
Fraction 2																				
Fraction 3																				

α-NAPHTHOL — No. 3

Fraction 1				+m					+w		+s			+s		+m				
Fraction 2																				
Fraction 3		?		+w					+s											

2 dimensional TLC analysis of fractions from DEAE cellulose column. (Exp. 16).

analysis (Table 49). Fractions 1 and 3 were retained.

Fraction 2, while containing a considerable quantity of sugar (over 25%) did not reveal any worthwhile information as to its composition from the TLC analysis and was, as a result, discarded.

No phosphorus was detected in Fraction 1 or 3 by either Kirg's method or by TLC analysis using the molybdenum/ H_2SO_4 reagent specific for phospholipids.

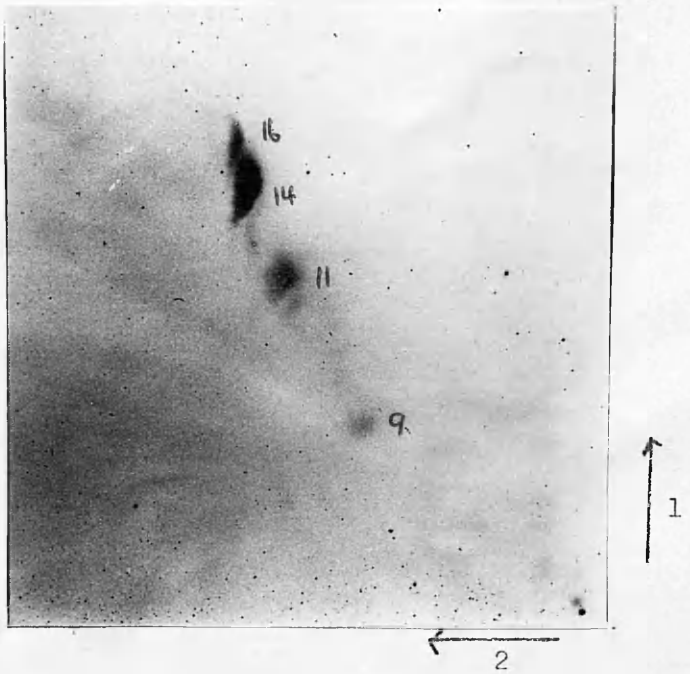
Figures 33 and 34 are photographs of the separations obtained by TLC of Fractions 1 and 3 from the DEAE column. It can be seen from these photographs that the charged glycolipid(spot No.9) was present almost entirely in Fraction 3.

The fact that this DEAE cellulose column fractionation was successful, while previous ones (Exp. 6 and 8) resulted in a degree of cross-contamination of the fractions, may be due to the very small load (93.0mgs. to 20g. DEAE cellulose) used, and also to the fact that it had been extensively purified beforehand by previous column techniques. This therefore supports Rouser's view that this medium has a very low capacity (180, 181).

The purpose of the successive column chromatography performed was twofold; firstly to rid the total lipid mixture of as much non-glycolipid material as possible (phospholipids and neutral lipids) while still retaining all the sugar-lipids intact, and secondly to attempt a fractionation of the glycolipids into individual compounds or groups of compounds as free from cross-contamination as possible.

In summarising, therefore, as far as the first goal was concerned the two silicic acid columns removed all the neutral lipids, while the magnesium silicate column removed the majority of the phospholipid material, the amount left falling continually with subsequent chromatographic experimen-

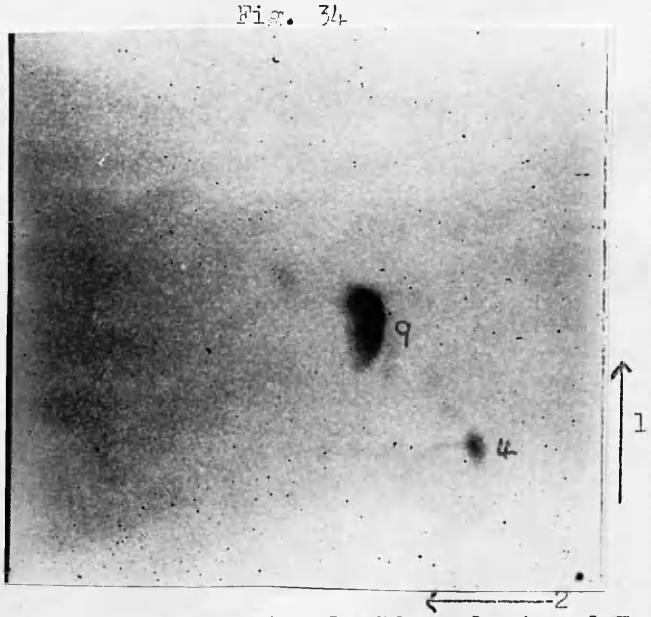
Fig. 33



Photograph of the two dimensional TLC analysis of Fraction 1 from the DEAE cellulose column (Exp. 16) showing spot Nos. 9 (faint trace), 11, 14 and 16. The spots were located using 20% perchloric acid.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10).

Fig. 34



Photograph of the two dimensional TLC analysis of Fraction 3 from the DEAE cellulose column (Exp. 16) showing spot Nos. 4 (trace), and 9.

Solvents: 1 chloroform-methanol-water (65:25:4 v/v),
2 diisobutyl ketone-acetic acid-water (80:50:10).

TABLE 50

Spots numbered as in "LIPID MAP" (Fig.16 No.2)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fr.2+3, 2nd silicic acid column																			s +	
Fr.4, 2nd silicic acid column																s +	s +	s +		
Fr.1, DEAE cellulose column				m +					w +		s +			s +		m +				
Fr.3, DEAE cellulose column		?		+ w					+ s											

Summary of the fractionation obtained of the glycolipid compounds present in the lipid extract from bracken, using acombination of column and preparative thin-layer chromatography. (Exp. 16).

TABLE 51

Fraction	Weight (mg.)
2+3, 2nd silicic acid column	30
4, 2nd silicic acid column	130
1, DEAE cellulose column	40
3, DEAE cellulose column	10

Summary of the weights of the fractionated glycolipid samples. (Exp. 16).

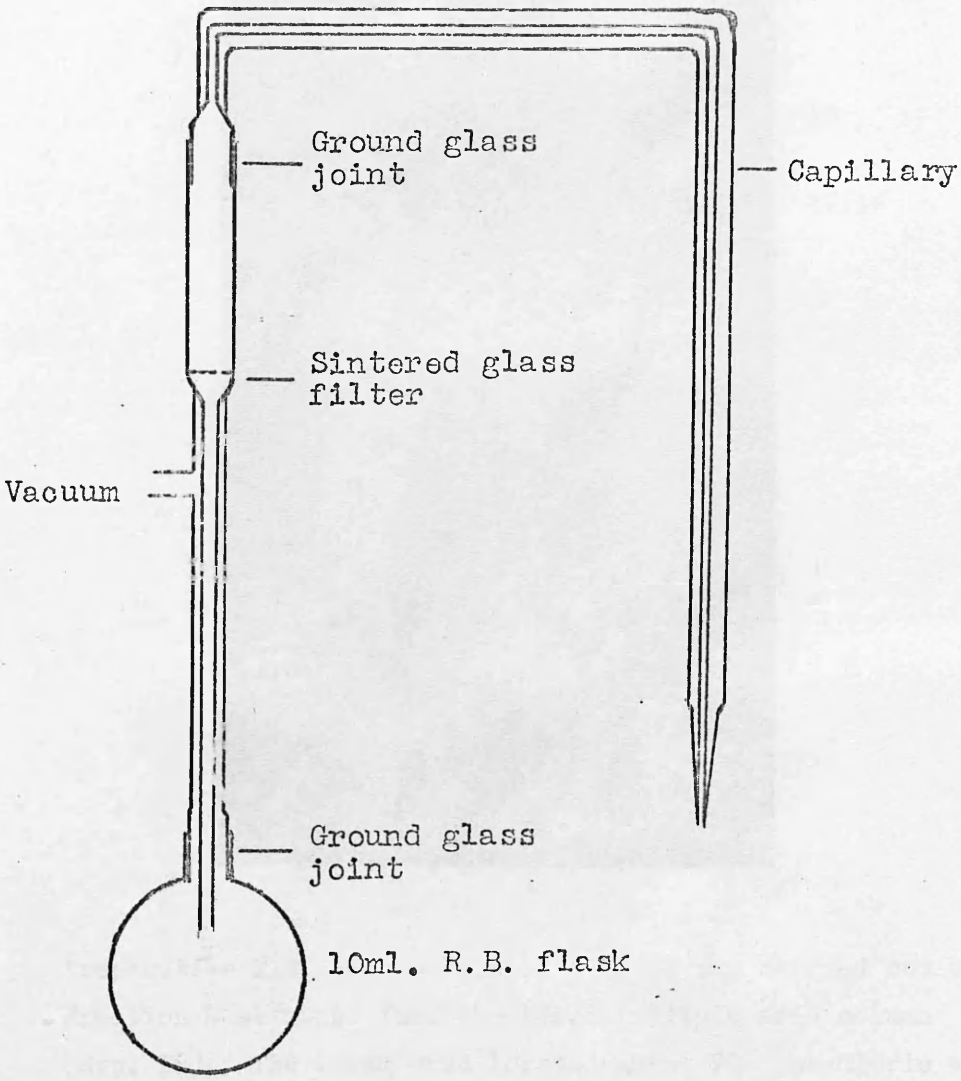
tation until no noticeable amount was detected.

A summary as to how far the second goal was achieved is given in Tables 50 and 51. It can be seen (Table 50) that the suspected glycolipids were resolved either completely or into smaller groups, thus facilitating attempts to obtain pure samples of each.

The three fractions retained from the column fractionation which contained more than one glycolipid compound, were further resolved by preparative thin-layer chromatography (G.M. 4). The bands of separated compounds were located using a Rhodamine 6G spray and observation in U.V. light (Hanovia Chromatolite) (G.M. 5). Each band was collected and eluted off the silicic acid using the apparatus shown in Fig.35 which, operating under suction, removed the coating of silicic acid from the glass plate. In each case a preliminary test was carried out using a 10 x 20 cm. plate in which the bands were located visibly with 20% perchloric acid (G.M. 5). This was to determine the correct quantities to apply to the plates in each case for optimum resolution of the mixture. Figure 36 is a photograph of such a test performed on a sample of Fraction 4 from the second silicic acid column. This sample contained three glycolipid compounds, represented by Nos.16, 17 and 18. The top band in Figure 36 is No.18 and the lower, larger band is Nos. 16 and 17 combined.

This preparative TLC separation was performed on the following samples; Fraction 4 from the second silicic acid column, Fraction 1 from the DEAE cellulose column and Fraction 3 also from the DEAE cellulose column. The separation of Fraction 4 from the second silicic acid column (Fig. 37, No.1) produced two separate bands as previously mentioned; the topmost contained the glycolipid represented as No.18 and the lower was composed of a mixture of the other two glycolipids, Nos. 16 and 17. Fraction 3 from the DEAE

Fig. 35

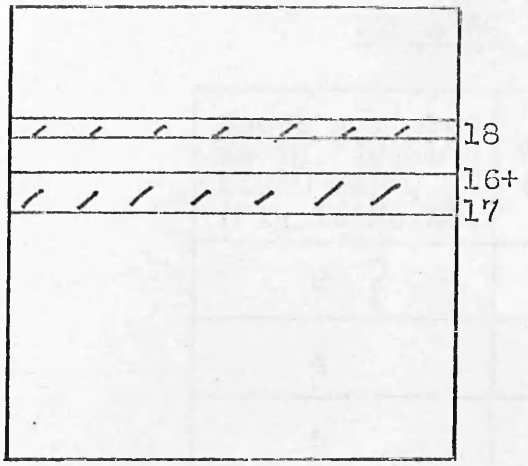


Apparatus used for removing spots from preparative thin- layer plates.

Fig. 36

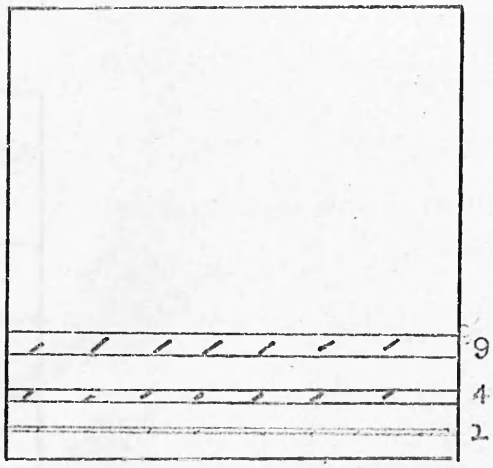
Preparative TLC. Photograph of a trial run carried out using Fraction 4 obtained from the second silicic acid column (Exp. 16). The bands were located using 20% perchloric acid.

Fig. 37



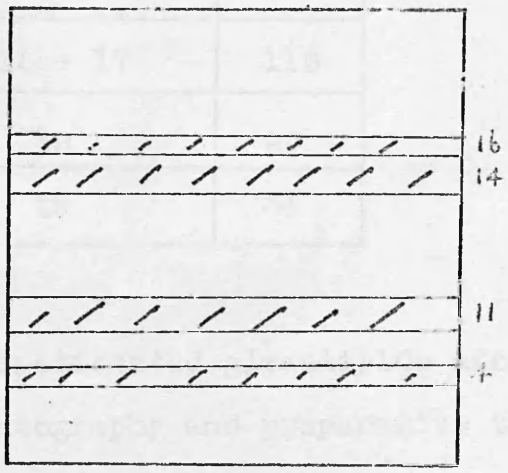
No. 1

Fraction 4 from second silicic acid column.



No. 2

Fraction 3 from DEAE cellulose column.



No. 3

Fraction 1 from DEAE cellulose column.

Preparative TLC of partially fractionated glycolipids after successive column chromatography. The glycolipid bands are numbered as in "TOTAL LIPID MAP" (Fig.16, No. 2).

TABLE 52

Spots numbered as in "TOTAL LIPID MAP", (Fig.16 No.2).	Weight (mg.)
2 ?	2
4	11
9	10
11	14
14	2
16	8
16 + 17	116
18	90
19	34

Weights of fractionated glycolipids after successive column chromatography and preparative thin-layer chromatography. (Exp. 16).

cellulose column was completely resolved (Fig. 37, No.2) and a pure sample of the charged glycolipid (No.9) was obtained. Fraction 1 from the DEAE cellulose column was not so simple. Four separate bands were obtained (Fig. 37, No.3) but the top two, Nos. 16 and 14 were not completely separated from each other. However, No.11 and No.4 were, and pure samples of each were collected. Bands 16 and 14 were collected as one and rechromatographed to obtain a better separation. They were then collected separately.

Table 52 summarises the glycolipid compounds so far obtained in a pure form from this experiment.

6. GLC Analysis of the sugar content of the glycolipid compounds obtained from the previous column and TLC fractionation.

As was previously stated at the beginning of Section B, the intention of this set of experiments was to obtain a pure sample of each suspected glycolipid compound, followed by the confirmation of the presence of carbohydrate in each sample. Since some of the samples obtained were very small it was decided to use gas-liquid chromatography as the preferred method for sugar analysis because of its greater sensitivity.

Each glycolipid sample was dissolved in chloroform-methanol, 2: 1 v/v. Half of each was taken and the residues, after solvent removal, were hydrolysed with ethanol-chloroform - 5N HCl (G.M. 3c). The remaining halves were retained for fatty acid analysis. The hydrolysates were extracted with n - hexane and water successively. The aqueous phases, containing any water soluble compounds such as sugars, were reduced to dryness under vacuum, and the residues thoroughly dried by washing with dry methanol (G.M. 6). The sugar residues were then converted, in the usual manner, into their trimethylsilyl derivatives with a 2: 1 v/v mixture of hexamethyldisilazane and trimethylchlorosilane (G.M. 15).

Standard sugars were also converted into their TMS derivatives in a

TABLE 53

Sugar	Anomers	Rt. rel. to α glucose (std)
Glycerol		0.069
Arabinose	β	0.42
"	α	0.47
Fucose	α	0.52
Xylose	α	0.59
"	β	0.70
Mannose	α	0.83
"	β	1.06
Galactose	α	0.94
"	β	/
Glucose	α	1.0
"	β	1.33

Rt. values for standard sugars obtained by GLC analysis on an SE-30 column.

TABLE 54

Sample, as in "LIPID MAP"	Peak	Rt. relative to α glucose (std., TABLE 53)	Main peaks	Possible identificatn.
	1	0.07		Glycerol
No. 4	2	0.255	+	?
	3	0.60	+	α xylose
	1	0.069	+	Glycerol
	2	0.256		?
	3	0.43		β arabinose
No. 9	4	0.60		α xylose
	5	0.82		α mannose
	6	0.95	+	α galactose
	7	1.40		?
	1	0.071	+	Glycerol
	2	0.26		?
	3	0.43		β arabinose
No. 11	4	0.59	+	α xylose
	5	0.82	+	α mannose
	6	0.89		?

GLC analysis of sugars obtained from fractionated glycolipids in Exp. 16. The Rt. values obtained were compared with standard sugars (TABLE 53). PTO

TABLE 54 (contd.)

No. 11	7	0.93	+	α galactose
	8	1.40		?
No. 14	1	0.26		?
	2	0.60		α xylose
	1	0.255	+	?
	2	0.59	+	α xylose
	3	0.93	+	α galactose
No. 16	4	1.0	+	α glucose
	5	1.21		?
	6	1.33		β glucose
	7	1.60		?
	1	0.257	+	?
No. 16 +17	2	0.59	+	α xylose
	3	0.93	+	α galactose
	4	1.0	+	α glucose
	1	0.257	+	?
No. 18	2	0.59	+	α xylose
	3	0.93		α galactose
	4	1.32		β glucose

PTO

TABLE 54 (contd.)

	1	0.072	+	Glycerol
	2	0.26		?
	3	0.60	+	α xylose
No. 19	4	0.93	+	α galactose
	5	1.0		α glucose
	6	1.32		β glucose

similar manner (G.M. 15).

A commercial Pye 104 dual flame ionisation chromatograph was used for the analysis (G.M. 16). The separations were performed using glass columns, 152.0 x 0.4cm. in dimension, containing 2.5% S.E. 30 (Applied Science Lab, Penn., U.S.A.). The columns were packed using freshly prepared material (G.M. 13).

1 to 10 γ l. samples of the TMS derivatives of the unknown mixtures were injected into the top of the column. The separations were carried out using temperature programmed conditions of 130-160° with a linear rise in temperature of 2°/min. The separated compounds were recorded automatically as peaks using a Smiths Servoscribe recorder.

Samples of the standard derivatives were also run under exactly similar conditions as the unknowns.

The retention times of the unknown peaks and the known peaks were calculated relative to α-glucose (Tables 53 and 54).

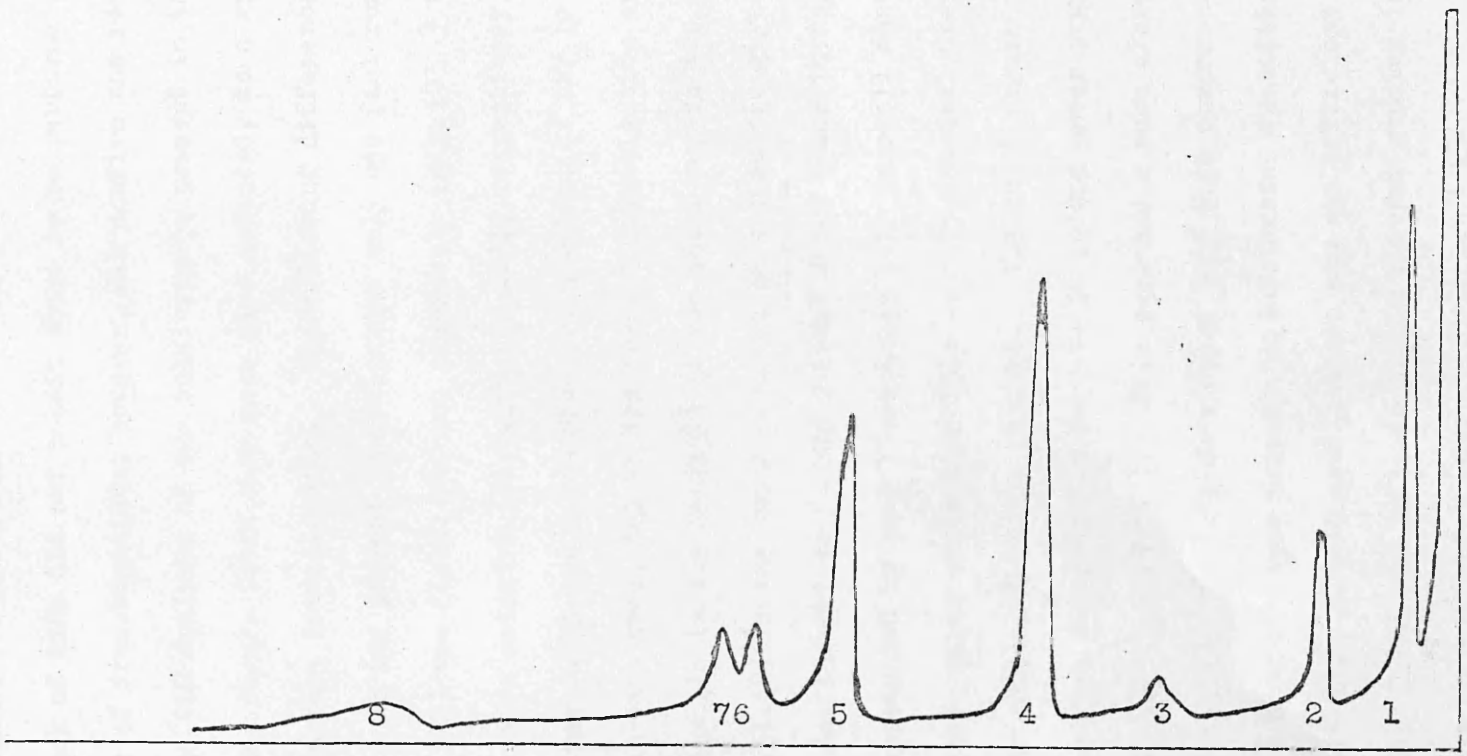
The chromatographic separation of the unknown sugar mixtures was repeated but included this time in each case was an internal standard of α - glucose.

Figure 38 shows the separation obtained for the sugars present in the glycolipid sample from spot No.11.

The results (Table 54) indicated that no single sugar could be assigned to each glycolipid sample. Several sugars were identified for each sample analysed, indicating even yet, a degree of crosscontamination between the samples.

As many of the sugars were detected only in trace amounts, column three in Table 54 was compiled to show the main peaks obtained from each sample analysed. Although this helped to clarify the rather complex results obtained, before any definite conclusion as to the identity of the

Fig. 38



GLC analysis of the sugar content of fractionated glycolipid spot No. 11. The separation was performed on an SE-30 column using temperature programmed conditions of 130-160° with a linear rise of 2°/min. (TABLE 54).

glycolipid samples could be made and also before any of the sugars found could be said definitely to be part of the structure of the various glycolipid compounds, further confirmatory studies would be necessary. Unfortunately lack of time did not permit these to be performed.

This series of investigations, however, did confirm the results obtained from the GLC analysis of the total sugars present in the lipid mixture. All the sugars found then were also detected, to a greater or lesser extent, in this investigation. The following differences were however noted. In the present investigation only one fast running component (Rt.0.255) was found, whereas previously (page 103) two fast running unknowns were detected (Rt.0.155 and 0.245 respectively). Since the retention times 0.255 and 0.245 are very similar, it may be that it is the same sugar in each case, but in the present investigation only one anomer was found while in the analysis of the total sugars two were found.

Peak 7 (Rt. 1.40) in the sugar analysis of the acidic glycolipid (spot No.9) was not thought to be the charged sugar under investigation. The component represented by peak 7 was found only in small amounts whereas previously conducted paper chromatographic studies has indicated that this sugar was a major component in the mixture. The total sugars present in the lipid mixture were separated on an S.E. 30 column under programmed conditions of 160-120° (page 100). This produced a much slower (Rt. 2.85) and larger peak (No.11). It was considered that this component fitted the requirements of a charged sugar present in substantial quantities, more than peak No.7 in Table 54. Unfortunately there was not sufficient quantities of this particular glycolipid left, to enable further studies into this aspect of the work to be carried out.

The fact that the unknown fast running peak (Rt. 0.255) and also α -xylose (Rt.0.56) were nearly always present, in every sample

cannot be explained.

In conclusion, although the column and TLC studies previously conducted indicated that each sample was relatively pure, the GLC analysis appeared to indicate otherwise and that there was still some degree of cross-contamination. As a consequence the results obtained were complex. This fact and some unexplained observations imply that much more work is required before definite conclusions can be made concerning the sugar content of each glycolipid compound.

7. Investigation into the fatty acid content of the total lipid mixture and also the individual glycolipid mixtures.

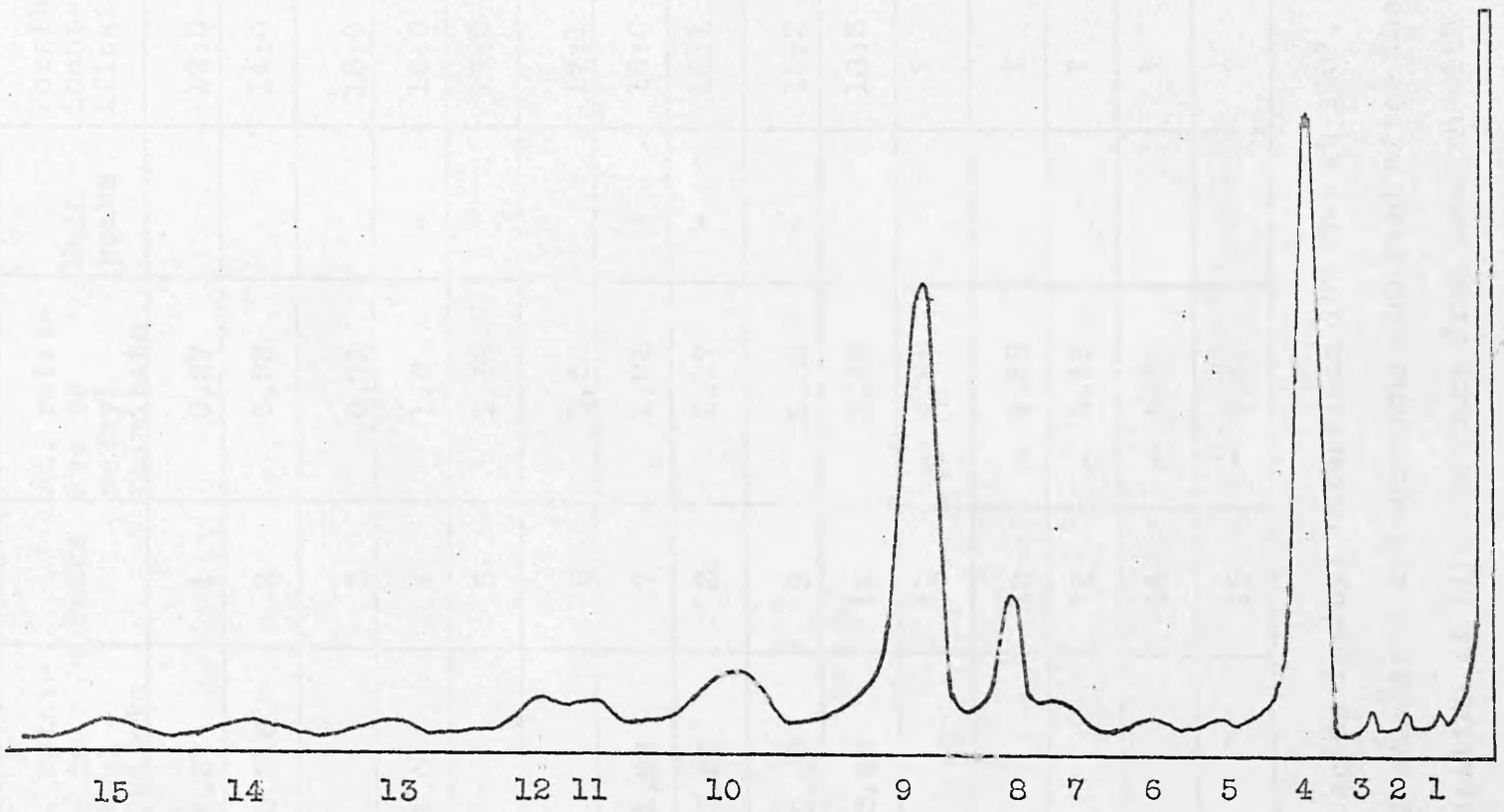
A preliminary qualitative analysis into the fatty acid content of the total lipid extract was carried out. This was followed by an examination of the fatty acids present in the glycolipid samples obtained from the column and preparative thin-layer chromatography experiment. (Exp.16).

A lipid mixture of 500 mgs. from freeze-dried rhizomes were methanolysed with dry methanolic HCl. (201) (G.M. 15b). The methyl esters of the fatty acids formed were extracted from the mixture with n - hexane, which was reduced in volume on a steam bath and samples used for the analysis.

A commercial Pye 104 dual flame ionisation detector chromatograph was used for the analysis (G.M. 16). The separation was performed using glass columns, 152.0 x 0.4cm. in dimension, packed with a polar phase of 10% ethylene glycol adipate, EGA (202). The columns were packed using freshly prepared material (G.M. 13).

.1 to 10μl samples of the unknown fatty acid mixture were injected onto the column and the separation carried out at an isothermal temperature of 180° (202). The separated components were recorded automatically as peaks on a Smiths Servoscribe recorder.

Fig. 39



GLC of total fatty acids (methyl esters) obtained from lipid mixture from bracken rhizomes, after transesterification with 0.5N dry methanolic HCl. The separation was performed on a column of 10% EGA at a temperature of 180°. (TABLE 55).

TABLE 55

Standards	Rt. relative to methyl palmitate	Peaks	Rt. relative to methyl palmitate	Main peaks	Possible identification.
12:0	0.27	1	0.27		12:0
14:0	0.516	2	0.52		14:0
		3	0.71		15:0
16:0	1.0	4	1.0	+	16:0
		5	1.38		17:0
		6	1.58		17:1
18:0	1.92	7	1.93	+	18:0
18:1	2.19	8	2.17	+	18:1
18:2	2.69	9	2.66	+	18:2
18:3	3.48	10	3.45	+	18:3
		11	~ 4.28		?
		12	~ 4.58		?
		13	~ 5.49		?
		14	~ 6.36		?
		15	~ 7.6		?

GLC of fatty acids (methyl esters) on 10% EGA at 180°. Rt. values of standards and unknowns obtained after the transesterification of lipid mixture from bracken with 0.5N dry methanolic HCl.

The methyl esters of standard fatty acids were prepared using ethereal solution of freshly prepared diazomethane at 0°C. (202) (G.M.15b), and run under exactly similar conditions as the unknown mixture.

Figure 39 is the separation obtained of the total fatty acid content of the lipid mixture.

The retention times of the unknown peaks and the standard peaks were calculated relative to methyl palmitate, and compared (Table 55).

As the results indicate, a whole series of acids were detected but the major ones were easily identified as palmitic, linoleic, oleic, linolenic and stearic acid. Any others detected were present only in trace amounts.

The remaining half of the individual glycolipid samples obtained from the column and preparative TLC (page167) were hydrolysed, after solvent removal, with dry methanolic HCl (G.M. 4c), and the methyl esters of the fatty acids extracted with n - hexane as before, and used for the analysis (G.M. 16).

1 to 10γl. samples were used for the separation using again a column of 10% EGA and an isothermal temperature of 180° (202).

A comparison of the retention times of the unknown components with those for standard fatty acids (Table 56) showed that several fatty acids were detected for each glycolipid sample analysed. However, a great many of the acids detected were present only in trace amounts. When only the major fatty acid components were taken into account a much clearer picture was obtained (Table 56, Col 3). In doing this it was possible to assign to each glycolipid sample definite major fatty acid components.

With one exception no fatty acid was detected in the analysis of the individual glycolipid samples (Table 56) which was not also detected in the

TABLE 56

Samples as in "LIPID MAP"	Peak	Rt. relative to methyl palmitate (TABLE 55)	Main peaks	Possible identificat^{n}.
	1	0.265		12:0
No. 4	2	0.515		14:0
	3	0.713		15:0
	4	1.0	+	16:0
	1	1.0	+	16:0
No. 9	2	2.17	+	18:1
	3	2.66		18:2
	1	1.0	+	16:0
No. 11	2	1.37		17:0
	3	2.14	+	18:1
	4	2.62		18:2
	1	0.71		15:0
	2	1.0	+	16:0
No. 14	3	1.35		17:0
	4	1.89		18:0

GLC analysis of fatty acids (methyl esters) obtained after the transesterification of the glycolipid samples obtained from Exp. 16, with 10% dry methanolic HCl. Rt. values obtained were compared with standards.

P'TO

TABLE 56 (contd.)

No. 14	5	2.59		18:2
	1	0.265		12:0
	2	0.51		14:0
	3	0.71		15:0
	4	1.0	+	16:0
No. 16	5	1.37		17:0
	6	1.9		18:0
	7	2.14		18:1
	8	2.65		18:2
	9	3.86	+	20:0 ?
	1	0.51		14:0
	2	0.71		15:0
	3	1.0	+	16:0
	4	1.38		17:0
No. 16 + 17	5	1.94	+	18:0
	6	2.16	+	18:1
	7	2.68	+	18:2
	8	4.3	+	?
	9	4.58	+	?
No. 18	1	0.507		14:0

PTO

TABLE 56 (contd.)

	2	0.705		14:0
	3	1.0	+	16:0
	4	1.37		17:0
	5	1.92		18:0
No. 18	6	2.15	+	18:1
	7	2.65	+	18:2
	8	3.45	+	18:3
	9	?		?
	10	?		?
	11	?		?
	1	0.26		12:0
	2	0.385		?
	3	0.508		14:0
	4	0.715		15:0
No. 19	5	1.0	+	16:0
	6	1.38		17:0
	7	1.92	+	18:0
	8	2.15	+	18:1
	9	2.64	+	18:2
	10	3.46		18:3

fatty acid analysis of the whole glycolipid extract (Table 55). The one exception to this being a twenty carbon fatty acid which appeared to be present in sample No.16 (identified by comparison of the results with published data (202)). This acid was not detected in the analysis of the whole glycolipid extract.

As can be seen from Table 56 the number and proportion of the major fatty acids in each sample varied considerably. However, because of the purely preliminary nature of this experiment no attempt was made to ascertain any structural characteristics of the glycolipids involved.

As well as the twenty carbon acid observed in No.16, several other long chain acids were detected in other glycolipid samples and in the whole lipid sample. Unfortunately time did not permit any further work to identify these acids.

8. A comparison of the sugar content of the nucleotide, polysaccharide, and glycolipid fractions extracted from bracken rhizomes.

At this terminal stage therefore a comparison was made between sugar content of the above three biochemical fractions from bracken as was suggested in the introduction to this thesis. This is done in Table 57. Column one gives the sugar content of the nucleotide fraction (3), column two the sugar content of the polysaccharide fraction as reported in Part I and column three the sugar content of the glycolipid fraction as reported in Part II of this thesis.

All three fractions did not contain the same sugars. However in attempting to demonstrate a connection between the nucleotide, polysaccharide and glycolipid fractions it is not essential that all three have a completely similar sugar content. Hence in the present context the nucleotides containing N - acetyl glucosamine and rhamnose and the glycolipid containing 6 - .

TABLE 57

Sugars found in nucleotide fraction	Sugars found in polysaccharide fraction	Sugars found in glycolipid fraction
D-galactose	D-galactose	D-galactose
L-arabinose	L-arabinose	L-arabinose
D-xylose	D-xylose	D-xylose
L-fucose ?	L-fucose	
	D-mannose	D-mannose
D-glucose	D-glucose *	D-glucose
	Uronic acid	
		6-sulpho-α-D-quinovo-pyranose
N-acetyl-glucosamine		
L-rhamnose		
		Unknown

A comparison of the sugars found in the nucleotide, polysaccharide and glycolipid fractions from bracken rhizomes.
* trace.

- sulpho - D - quinovo pyranose are irrelevant, as these sugars do not appear as structural units of the mucilagenous polysaccharide.

If however, certain glycolipids, along with nucleoside diphosphate sugars do play a role in the biosynthesis of polysaccharides, then it would be important to show that the sugars found in the polysaccharide were also present in the other two fractions. This is the case with D - galactose D - xylose and L - arabinose. The uronic acid component of the polysaccaride was not present in either of the two other fractions. However its presence in the polysaccaride could result from enzymic oxidation of either glucose or galactose, depending on whether it was a glucuronic or galacturonic acid. Mannose was present in the polysaccharide and glycolipid fractions but not in the nucleotide fraction, while fucose was found in the polysaccharide and nucleotide fractions but not in the glycolipid. As stated in the introduction to this thesis it was thought that if L - fucose could be shown to be present in all three fractions this would be strong evidence of a link between them. That it was not found in the glycolipid extract was therefore disappointing, but this, on the other hand, was not considered proof that there is no connection between the three fractions. It may well be present as part of a glycolipid but was not extracted or was extracted in such small amounts as to remain undetected.

A certain similarity therefore does exist between the sugar content of the polysaccharide and that of the nucleotide and glycolipid fractions. However it is certainly not close enough to enable a definite statement to be made concerning a possible connection between them. Further research into this particular aspect of polysaccharide synthesis would be worthwhile. This work could involve the isolation of glycolipid fractionsand their use in enzymic experiments in an attempt to synthesis polysaccharides in vitro.

Because of the objectives set out in the introduction of this thesis, the work performed was of necessity general in nature. Consequently more specific topics of interest which arose during the work were unable to be pursued fully, because time did not permit. Such topics included; in Part 1, the more detailed structure of the mucilagenous polysaccaride; in Part II, the problems associated with the extraction of the lipid material from bracken and also a study in depth of the individual glycolipids in turn as opposed to en masse, in particular the more unusual sugar-lipids suspected of being present such as sulpholipid and steryl glycoside. However, interesting as they were, it was decided that the pursuit of such studies could only be minimal if the main aims stated were to be realised.

EXPERIMENTAL SECTION.

INDEX.

GENERAL METHODS

EXPERIMENTS PART I

GENERAL METHODS

G.M. 1. EXTRACTION METHODS (LIPIDS)

a) 3.0kg. washed bracken rhizomes were chopped into lengths of one inch. These were blended in four separate lots with a total of 12.0 litres methanol using a high speed Waring blender (Waring Products Corporation, Winstead, Conn., U.S.A.). The resultant mixture was filtered under suction and the filtrate reduced to 2.0 litres using a large scale vacuum distillation apparatus and finally this was reduced to dryness by rotary vacuum distillation at a temperature not more than 45°. The syrupy residue was then extracted by refluxing with 250 ml. chloroform-methanol, 2: 1 v/v. This was filtered under suction and the filtrate placed in a 400 ml. beaker which was subsequently placed inside a 15.0 litre container. The outside container was slowly filled with deionised water, and the system left for twenty-four hours. The small beaker was removed and the chloroform layer collected after filtration, with the aid of a separating funnel. The lipid mixture was recovered by rotary vacuum distillation, redissolved in 100 ml. water, filtered and the purification step repeated with a further 10 litres water. The weight of the lipid mixture obtained was 4.7g.

b) The powdered bracken preparation (freeze-dried or heated at 110°) was packed into a glass column, 16.0 x 60.0 cm. in dimension, with a fat free cotton wool plug at the bottom. The powdered bracken was eluted with a total of 40 litres hot chloroform-methanol. 2: 1 v/v. The eluant was reduced to dryness by large scale vacuum distillation followed by rotary vacuum distillation at 45°. The crude lipid residue was extracted with chloroform-methanol, 2: 1 v/v (200 ml.) by shaking in

a flask. This was filtered, reduced to dryness by rotary vacuum distillation, and the residue re-extracted by shaking it with 100ml chloroform-methanol, 2:1 v/v, in a flask. This was filtered, the filtrate placed in a dialysis sac and immersed in 10 litres deionised water with continuous stirring by a magnetic stirrer. The water was changed four times over a period of sixty hours. After this time the contents of the sac were filtered under suction and the filterate made homogeneous by the addition of methanol, with stirring. This was reduced to dryness by rotary vacuum distillation at 45° to yield the purified lipid mixture.

c) The chopped bracken rhizome preparation (fresh or freeze-dried) was blended with chloroform-methanol, 2:1 v/v (2 - 10 litres), in a high speed Waring blender. The mixture was filtered and the filtrate reduced to dryness by large scale vacuum distillation followed by rotary vacuum distillation (occasionally the residue from the filtration was re-extracted by blending with chloroform-methanol, 2:1 v/v (5 l.), filtered again and the filtrates combined.) The crude residue was dissolved in 200ml. chloroform-methanol, 2:1 v/v, by shaking in a flask, filtered, and the filtrates taken to dryness by rotary distillation at 45°. The residue was again dissolved in chloroform-methanol 2:1 v/v (100ml.), filtered and the filtrate dialysed against 10.0 litres deionised water, with constant stirring. The water was changed four times over a period of approximately 48-60 hours. The contents of the dialysis sac were filtered and the filtrate made homogeneous by the addition of methanol with stirring. This solution was reduced to dryness by rotary vacuum distillation at 45°, to yield the purified lipid mixture.

G.M. 2. RECYCLISING OF ION-EXCHANGE RESINS.

a) Amberlite IR. 120. H^+. 200g. of this resin were recycled to give the hydrogen form by washing successively with 3.0 litres N NaOH, distilled water to neutrality, N HCl and finally with distilled water again until the supernatant had a pH of 7.0.

b) Dowex 1, 1-4%, 200 - 400 mesh, CH_3COO^-, 200g. of the resin were prepared by washing successively with 3.0 litres N NaOH, distilled water to neutrality, N CH_3COOH and distilled water to neutrality.

c) Biodeminrolite Mixed Bed Resin. This resin was used as obtained from the bottle, after it was thoroughly washed with distilled water to ensure the removal of all water soluble impurities.

G.M. 3 METHODS OF HYDROLYSIS

a) 1.5-2N sulphuric acid. Up to 2g. of the material was refluxed for two hours with 50ml. 1.5-2N H_2SO_4. The cooled hydrolysate was neutralised by the addition of fine mesh $BaCO_3$. Filtration and centrifugation, to remove the barium sulphate and excess barium carbonate, followed.

b) 1.5 - 2N sulphuric acid. 2g. of the material were refluxed for two hours with 50ml. 1.5 - 2N H_2SO_4. The cooled hydrolysate was then neutralised by the addition of a slight excess of barium hydroxide. The pH was then brought back to neutrality by bubbling gaseus CO_2 through the solution. This was then filtered and centrifuged.

c) A sample of the material, up to 2g., was refluxed with 50ml. ethanol-chloform - 5N HCl, 7.4: 6: 3, for two hours. The hydrolysate was reduced to dryness by rotary vacuum distillation at a temperature not more than 50°. The residue was then extracted with water and/or chloroform and filtered. For the quantitative determination of

carbohydrate and phosphorus in lipid samples a known weight of the sample was hydrolysed with ethanol-chloroform - 5N HCl, 7.4: 6 :3 v/v, (5-50ml.) for 2.0 hours. The hydrolysate was reduced to dryness by rotary vacuum distillation at a temperature not more than 50°. The residue was extracted with a known volume of deionised water (10-30ml.), and chloroform (30ml.). The aqueous phase was collected after standing for several hours, with the aid of separating funnel. A known volume (1-10mls.) of the aqueous extract was diluted to a standard volume in a volumetric flask (100 -500 ml.) and 1.0ml. samples pipetted from this for the carbohydrate or phosphorus analysis (G.M. 11c and 12c). Occasionally where the phosphorus concentration was low, samples were taken directly from the aqueous extract for the determination.

G.M. 4 PAPER AND THIN-LAYER CHROMATOGRAPHY

a) One dimensional paper chromatography. Paper chromatography was carried out using Whatman No.1 paper. The papers, 56 x 25.5cm. were marked off 9cm. from one end. Using a capillary, spots of the unknown compounds and the standards were placed along this line 3 cm. apart. The opposite end of the papers were serrated to prevent glutting with solvent as it dripped off the end. The papers were then developed in a descending manner in glass tanks 53 x 30 x 19cm. which had been pre-equilibriated with the solvent for twelve hours. The tanks were kept in a constant temperature free from draughts. After development the papers were dried thoroughly at room-temperature before the spots were located.

The following solvents were used:-

1. Butanol-pyridine-water (3: 1: 1.5 by vol., top layer plus 1 vol pyridine.

2. Phenol-water (160 : 40 w/v).

3. Ethyl acetate-pyridine-water (120: 50: 40 by vol.).

4. Formic acid-methylethyl ketone - butanol - water (15: 30: 40: 15 by vol).

b) Two dimensional paper chromatography. This was also done on Whatman No.1 paper. The papers, 54 x 46cm. were spotted approximately 10cm. from one corner. The end of the papers were serrated and then developed in a tank 58 x 53 x 22 cm. in dimension, which was pre-equilibriated with the solvent and kept at a constant temperature. After the first development, the papers were thoroughly dried and developed in the second solvent at right angles to the first, again in a descending fashion. The papers were then finally dried once again and the spots located.

The following solvents were used:-

1. Butanol-pyridine-water (3: 1: 1.5 by vol., top layer plus 1 vol. pyridine).

2. Phenol-water (160: 40 w/v.)

c) Unidimensional thin-layer chromatography. Glass plates of the following sizes were used for this purpose, 5 x 20 cm., 10 x 20 cm. and 20 x 20 cm. All traces of grease and dirt were removed from the plates by soaking them in chromic acid for several hours and then washing them with deionised water.

Sugars were resolved on powdered cellulose plates (Chromedia CC 41, Whatman). The cellulose was spread to a thickness of 0.3mm. using a slurry of 30g. Chromedia in 63ml. distilled water. A Shandon thin-layer spreader was employed for this purpose. The plates were allowed to dry at room-temperature, before being used.

Lipids were resolved on silicic acid plates (kieselgel, G. Nach Stahl, E. Merch, Darmstadt). The silicic acid was coated onto the plates to a depth of 0.25mm. using a suspension of 25g. silicic acid in 55ml. distilled water. The plates were activated by a final heating at 100° for thirty minutes.

Using a template and a micro capillary, spots were placed 1 cm. from the bottom of the stationary phase and no less them 1cm. apart. The plates were developed in pre-equilibrated TLC tanks (Shandon Lab., Equipment) at a constant temperature. To ensure that the atmosphere inside the tanks was totally saturated, the tanks were lined with filter paper soaked in solvent. The plates were allowed to develop until the solvent was 2-3cm. from the top. They were then removed dried at room temperature and the spots detected.

The following solvents were used for the resolution of lipids:-

1. Chloroform-methanol-acetic acid (85: 25: 1 by vol.)
2. Chloroform-methanol-water (65: 25: 4 by vol.)

d) Two dimensional thin-layer chromatography. This was used only for the resolution of lipids. The silicic acid plates were 20 x 20cm. in dimension. They were prepared in a similar manner to the plates used for one dimensional TLC. A single spot of lipid material was applied 2cm. from one corner of the plate. The plate was then developed in the first solvent, removed, allowed to dry and developed in the second solvent at right angles to the first. The plate was finally removed, dried and the spots located.

The following solvents were used:-

1. Chloroform-methanol-water (65: 25: 4 by vol.).
2. Disobutyl ketone-acetic acid-water (80: 5: 10 by vol.).

e) Preparative thin-layer chromatography. This was performed only on lipid mixtures. The silicic acid plates were spread to a thickness of 0.4mm., in a similar fashion as before. A narrow band of the lipid mixture to be separated was applied along the bottom of the plates about 1cm. from the edge of the stationary phase. This was done using a micro capillary. The plates were developed unidimensionally, as before, removed, dried and the separated bands located.

The following solvent was used:-

1. Chloroform-methanol-water (65: 25: 4 by vol.).

G.M. 5 THE DETECTION OF COMPOUNDS ON PAPER AND THIN LAYER PLATES.

a) The following reagents were used to detect sugar spots on paper chromatograms:-

1. ALKALINE SILVER NITRATE

Reagents

a) 1 ml. of a saturated aqueous solution of silver nitrate made up to 100 ml. with acetone and water added dropwise, with stirring, until the precipitate just redissolved.

b) 4g. NaOH dissolved in 10ml. water and made up to 200 ml. with alcohol to give a 0.5N solution.

c) 200 ml. 5N aqueous ammonia.

Procedure:-

The paper was first dipped in the silver nitrate, allowed to dry, then dipped in the alcoholic NaOH and left until the spots had developed sufficiently. The excess reagent was then removed by dipping the paper in the aqueous ammonia and washing it in running water for several hours.

2. BENZIDINE

Reagent:-

0.5g benzidine was dissolved in 10ml. glacial acetic acid and this made up to 100ml. with absolute alcohol.

Procedure:-

The paper could be dipped through the reagent or the reagent sprayed onto the paper. Both procedures were performed in a fume cupboard. The spots were then developed by heating the paper at 100° for five minutes.

3. p-ANISIDINE-HCl

Reagent:-

3g. P-anisidine-HCl dissolved in n -butanol and the solution diluted to 100ml.

Procedure

The reagent was sprayed onto the paper which was then heated at 100° for three minutes.

4. ANILINE OXALATE

Reagent:-

100ml. 0.1M oxalic acid plus 0.9ml. redistilled aniline were shaken until the precipitate redissolved.

Procedure:-

The paper was sprayed with the reagent then heated at 100° for 5-10 minutes.

5. α -NAPHTHOL PHOSPHORIC ACID

Reagent:-

An 0.2% solution of α -naphthol in ethanol was mixed with 0.1 vol. orthophosphoric acid.

Procedure:-

The paper was sprayed with the reagent and heated at 90° for ten minutes.

b) Thin-Layer Plates. The following reagents were used to detect sugars on cellulose thin-layer plates:-

1. MODIFIED ALKALINE SILVER NITRATE

Reagents:-

a) 1ml. of a saturated water solution of silver nitrate was made up to 100ml. with acetone and water added, dropwise with stirring, until the precipitate just redissolved.

b) 4g. NaOH dissolved in 10ml. water followed by the addition of ethanol and acetone alternatively, keeping the precipitate in solution, until the volume was 200ml.

c) 100ml. concentrated ammonia dissolved in 300ml. acetone.

Procedure:-

The plate was immersed gently into the silver nitrate making sure that the cellulose surface did not come away from the plate. It was removed, allowed to dry and again immersed in the NaOH solution until the spots were developed sufficiently and then removed, and finally dipped in the acetone-ammonia solution to remove excess reagent. The plate was not washed with water.

2. BENZIDINE

Reagent:-

0.5g. benzidine dissolved in 10ml. glacial acetic acid and made up to 100ml. with absolute alcohol.

Procedure:-

The plate was lightly sprayed with reagent and heated at 100° for two to three minutes.

c) Thin-layer Plates. The following reagents were used to detect lipid spots on silicic acid coated thin-layer plates.

1. IODINE VAPOUR - General.

Reagent:-

Iodine crystals enclosed in a suitable vessel.

Procedure:-

The plate was placed in the glass vessel containing the crystals until the lipid spots developed sufficiently.

2. PERCHLORIC ACID General.

Reagent:-

A 20% aqueous solution of perchloric acid.

Procedure:-

The reagent was sprayed very finely onto the plate which was then heated at 100° until the spots had charred sufficiently (5-10 minutes) to be visible.

3. RHODAMINE 6G General.

Reagent:-

A 0.001% solution of Rhodamine 6G.

Procedure:-

The plate was finely sprayed with the rhodamine until damp, then viewed under a suitable source of ultra violet light.

4. NINHYDRIN Aminolipids.

Reagent:-

0.2g. ninhydrin dissolved in 99ml. n - butanol and made up to 100ml. with pyridine.

Procedure:-

The reagent was sprayed onto the plate which was heated for

five minutes at 100°.

5. MODIFIED DRAGENDORFF. Choline lipids/glycolipids.

Reagents:-

a) Stock solution. 8.0g. bismuth subnitrate dissolved in 25ml. 30% nitric acid. This was then added, slowly with stirring, to a solution containing 28g. KI, 1ml. 5N HCl. and 5ml. water. The solution was cooled, filtered and made up to 100ml. with water. Stored in a dark bottle in 'fridge.

b) Developing solution. 20ml. water, 5ml. 5N HCl , 20ml. stock solution and 5ml. 5N NaOH in that order. A few drops of 5N HCl can be added if precipitate was not wholly redissolved. Stored in 'fridge.

Procedure:-

The plate was sprayed with the developing solution upon which choline containing lipids appear as orange/yellow spots, followed by glycolipids and possibly aminolipids. No heating required.

6. SCHIFF'S REAGENT Phospholipids/glycolipids.

Reagents:-

a) 100ml. 1% aqueous sodium periodate

b) Gaseous SO_2.

c) 100ml. 0.5% p-rosaniline in water which was freshly decolourised by bubbling SO_2 gas through it.

Procedure:-

The plate was sprayed with 1% sodium periodate until damp. The excess periodate on the plate was then destroyed by exposure to SO_2 gas. The plate was then sprayed with 0.5% p-rosaniline. The spots appear gradually as a blue colour without heating.

7. MOLYBDENUM Phospholipids

Reagents:-

a) 40.11g. Mo O_3 plus 1.01 25N H_2SO_4 boiled till all the MoO_3 was dissolved.

b) 500ml. of solution a) plus 1.78g. powdered molybdenum metal gently boiled for fifteen minutes. The solution then cooled and decanted.

c) Equal volumes of a) and b) mixed and diluted with 2 volumes of water.

Procedure:-

The plate was sprayed with reagent c) upon which phospholipids showed up as blue spots. No heating required.

8. α -NAPHTHOL Glycolipids.

Reagents:-

a) 0.5% solution of α -naphthol in methanol water, 1:1 v/v.

b) 95% H_2 SO_4.

Procedure:-

The plate was sprayed with 0.5% α -naphthol, air dried, then finely sprayed with 95% H_2 SO_4 and heated at 120° for a few minutes. Glycolipids appear as blue/purple spots. Do not overheat.

G.M. 6 DRYING OF SOLVENTS

A) Pyridine

Apparatus:-

Five litre flask, fitted with a two-necked adaptor. To one neck was attached a reflux condenser and in the other was inserted a thermometer.

Procedure:-

250g. of barium oxide and 2.5 litres pyridine were placed in the flask

and refluxed for eight hours. If, at the end of this time, the refluxing temperature was not in the range 113-115° then a further 250g. barium oxide were added and refluxing continued until the temperature was within the above limits. The pyridine was then distilled off, collecting the fraction boiling at 113-115° in a flask fitted with a drying tube. It was stored over a suitable drying agent such as molecular sieve or Na_2SO_4.

B) METHANOL

Apparatus:-

A three-necked five litre flask fitted with an efficient stirrer, reflux condenser and dropping funnel. All the apparatus was thoroughly dry. The magnesium turnings used in this reaction were worked with dry ether in a current of air. It is also advisable to suspend the apparatus over a bath of ice-water, so that it may be cooled if the reaction becomes too vigorous.

Procedure:-

Approximately 15g. of dry magnesium turnings were placed in the flask plus a crystal of resublimed iodine. 100ml. of dry methanol were run in slowly, through the dropping funnel. Once the reaction was going the mixture was stirred and 900ml. methanol added slowly.

After completion of the reaction the mixture was refluxed for two hours with stirring then the methanol was distilled off and collected, discarding the first 25ml., in a flask.

C) CHLOROFORM

Apparatus:-

A five litre flask and fractionating column , condenser and three litre receiving flask.

Procedure:-

Three litres of chloroform were redistilled by collecting the fraction boiling at 61°. The first 100-200 mls. were discarded. The redistilled chloroform was stored over molecular sieve or Na_2SO_4.

D) ACETONE

Apparatus:-

Shaking apparatus and a Winchester.

Procedure:-

Two-three litres acetone were shaken over anhydrous sodium sulphate for eighteen hours and stored over this dehydrating agent until required.

E) DI-ISO-BUTYL KETONE

Apparatus:-

Three litre flask, fractionating column, condenser, and a two litre receiving flask.

Procedure:-

The di-iso-butyl ketone was redistilled using a fractionating column, discarding the first 50-100mls. It was stored over molecular sieve until required.

F) ETHER

British Drug Houses' anhydrous ether was used as supplied.

G) HEXANE

Apparatus:-

Two-litre flask, fractionating column, condenser, and a two-litre receiving flask.

Procedure:-

The hexane was redistilled, collecting the fraction boiling at 69°. It was stored over molecular sieve.

G.M. 7 COLUMN PREPARATION

a) Ion-Exchange Columns

Ion-exchange columns were prepared by pouring a slurry of the ion-exchange material in deionised water into the column which was previously half-filled with water. The particles were allowed to settle to the desired height by draining the water from the bottom of the column.

b) Cellulose Columns

1. In the preparation of powdered cellulose columns for accurate and quantitative fractionations of sugars the stationary phase was packed into the column in a more uniform manner. This was done by firstly filling the column, the top of which was connected to a large filter funnel, with solvent. Into the funnel was poured a slurry of the stationary phase in the same solvent as was in the column (water saturated n-butanol). This slurry was stirred continually in the funnel with an electric motor and the cellulose was allowed to sediment and pack into the column uniformly by slowly draining the solvent from the bottom of the column. This was continued until the desired height was obtained.

2. Powdered cellulose columns for the fractionation of lipid mixtures were prepared by making a slurry of cellulose (not previously treated) in methanol-water, 9: 1 v/v, and pouring this slurry into the column, which was half filled with the same solvent mixture, and allowing the cellulose to settle by draining the column slowly. After the cellulose had reached the desired height it was washed in the following order with 5 bed volumes each of, methanol-water, 1:1 v/v, methanol-water, 9:1 v/v, chloroform-methanol, 1: 1 v/v and finally chloroform-methanol, 9:1 v/v saturated with water was passed through the columns until only one phase came out the bottom. This was to prevent the formation of channels

caused by a sudden change in polarity of the solvent.

c) SILICIC ACID COLUMNS

For fractionations involving the use of a gradient elution of chloroform to methanol, the silicic acid (Mallinckrodt) was packed into the column in a similar manner as the cellulose in b). However the pretreated silicic acid was packed in a slurry of dry methanol (G.M. 5) and once the column was at the desired height, it was replaced with chloroform by washing through one litre redistilled chloroform.

Otherwise the pretreated silicic acid (G.M.12) was poured in a slurry with dry methanol(G.M. 5) into the column which was half filled with methanol and the silicic acid allowed to settle by draining the methanol from the bottom of the column. The methanol in the column was then replaced with chloroform or hexane.

d) MAGNESIUM SILICATE COLUMNS

A slurry of pre-treated magnesium silicate (Florisil, BDH) (G.M.12) in dry methanol (G.M. 5) was poured into the column, half filled with methanol and with a glass wool plug. The Florisil was packed by slowly running the methanol out the bottom of the column. The column was then washed with 300ml. methanol and finally with one litre dry chloroform (G.M. 5) to replace the methanol.

e) DEAE CELLULOSE COLUMNS

Pretreated DEAE cellulose (Whatman) (G.M. 12) was poured as a slurry in chloroform-methanol, 2:1, into the column, again half filled with the same solvent mixture and with a glass wool plug. The cellulose particles were allowed to settle by draining the solvent mixture from the bottom. Before being used the column was washed with one litre chloroform-methanol, 2:1.

f) GAS-LIQUID CHROMATOGRAPHY COLUMNS

The circular glass columns, 152.5 x 0.4cm. in dimension, were filled with stationary phase in the following manner. The prepared stationary phase (G.M.12) was placed on a watch glass, five or six inches in diameter. The outlet of the column was plugged with glass wool and connected to a water pump, applying gentle pressure. The inlet to the column was then used to scoop up about 1g. of the stationary phase at a time. This was sucked to the bottom of the column where even packing was ensured by holding the column at different angles and tapping the sides with a spatula. This was repeated until the column was completely filled. Before the columns were used for the separation of sugar or fatty acid mixtures they were pre-conditioned at a temperature 20-30° higher than that which they were likely to be used. This was done with a stream of nitrogen flowing through the column to wash out, into the atmosphere, any volatile compounds present in the stationary phase. The S.E.30 columns were heated at 230-240° and the EGA columns 200° for twentyfour hours before being used.

G.M. 8. DILUTION TECHNIQUE FOR STANDARDS

The method used for the preparation of standard solutions of known concentration involved the initial preparation of a 'stock solution'. From this stock solution was taken the required volume which was subsequently diluted appropriately to give the desired volume of known concentration. This was repeated for the other concentrations within the range. These final standards were then used for the preparation of standard graphs.

G.M.9 ELECTROPHORESIS OF SUGARS

Electrophoresis of monosaccharides was carried out on strips of Whatman No.1 paper, 10 x 45cm. in dimension. A line 10 cm. from one end was

drawn, along which the sugar band was placed. The ends of the paper were then dipped in buffer solution which was allowed to run up to the line, by capillary action, taking care to avoid diffusion of the spots. The paper was blotted and the centre portion immersed in dry carbon tetrachloride while the end of the strip were placed in separate buffer solution on either side of the CCl_4.

The two electrodes were placed each in the correct buffer solution and a constant voltage run across the paper for a set period of time. The paper was removed, dried and the spots located.

Buffer solutions used were:-

0.025M. citrate pH5.0
0.025M. citrate pH 2.5
} 800 volts for $\frac{1}{2}$ to $\frac{3}{4}$ hour.

G.M. 10 SAPONIFICATION OF LIPID EXTRACTS

1-3g. lipid extract were saponified by refluxing with 50ml. 10% methanolic KOH for two hours. The reaction mixture was then taken to pH 4-5 by carefully adding diluted (5N) sulphuric acid with constant stirring. The mixture was filtered and the filtrate extracted with either chloroform or ether to remove non saponifiable lipids and free fatty acids, while saponifiable material remained in the aqueous phase.

For the quantitative determination of phosphorus in lipid mixtures after saponification the following procedure was used. A known weight of the lipid mixture was saponified with 10% methanolic KOH (25mls.) for 2.0 hours. The saponified mixture was made slightly acidic (pH 4) with the addition of 2N H_2SO_4. This was then extracted with 40-60° petroleum ether (30ml. x 2). The aqueous phase was removed with the aid of a separating funnel, pipetted into a volumetric flask (250ml.) and made up to the mark with deionised water. 1.0ml. samples from this were used

for the phosphorus determination (G.M.12c).

G.M.11 SUGAR ANALYSIS

a) THE DETERMINATION OF SUGARS IN AN ALCOHOLIC SOLUTION USING THE METHOD OF GARDELL (129)

Reagent:-

50ml. cold ethanol added to 64ml. 8.5N TCA in a flask immersed in an ice bath. To this was added 4ml. redistilled aniline and the solution diluted to 220ml. with cold ethanol. The reagent was stored in the refrigerator.

Procedure:-

0.5ml. sugar samples were placed in test tubes immersed in an ice bath. 1ml. of the reagent was added to each sample which was shaken for three minutes and then placed in a boiling water bath for fifteen minutes, the tubes being loosely stoppered with marbles. The tubes were then removed, cooled for five minutes and to each tube was added 2ml. 95% ethanol. The colour intensity of each tube was then read at 370mμ using an SP.500 spectrophotometer (UNICAM). The values found for unknown samples were then referred to a standard graph.

b) REDUCING POWER BY THE SOMOGYI (1952) NELSON METHOD (133)

Reagent:-

1. Copper solution. 12g. rochelle salt and 24g. anhydrous sodium carbonate were dissolved in 250 ml water. A solution of 4g. copper sulphate in water was added, followed by 16g. sodium carbonate. A solution of 180g. anhydrous sodium sulphate in 500ml. water was boiled to expel air. The two solutions were combined and diluted to 1 litre. After standing for one week the clear supernatant was used. The solution was stored at 37°.

2. ARSENOMOLYBDATE SOLUTION. 25g. ammonium molybdate were dissolved in 450ml. distilled water and 21ml. concentrated $H_2 SO_4$ added. 3.0g. diisodium hydrogen arsenate in 25ml. water were added and the solution kept at 37° for forty-eight hours. The solution was stored in a brown bottle.

Procedure:-

1ml. samples of the unknown sugar solutions plus 1ml. of the copper reagent were mixed and heated in a boiling water bath for twenty minutes, the tubes being loosely stoppered with marbles. After cooling for five minutes in tap-water, 1ml. arsenomolybdate was added with shaking. The solution was then made up to 10ml. with water and after ten minutes the colour was measured with an EEL colorimeter at 660 mμ the results determined after adjustment for the blank reading.

c) PHENOL SULPHURIC ACID METHOD (192)

Reagents:-

1. 5% aqueous phenol

2. Concentrated $H_2 SO_4$ (98% Analar).

Procedure:-

1ml. of the sugar solution (10-100γ) was mixed with 1ml. 5% aqueous phenol and 5ml. concentrated $H_2 SO_4$ added quickly with continual shaking. After ten minutes the tube was cooled in tap water for five minutes and then the colour intensity measured with EEL colorimeter at 490 mμ.

d) HEXOSES IN THE PRESENCE OF OTHER SUGARS - PRIMARY REACTION WITH L-CYSTEINE SULPHURIC ACID (142)

Reagents:-

1. 86% sulphuric acid (Analar)

2. 3% aqueous L - cysteine HCl monohydrate

Procedure:-

To 1ml. of the hexose sample in a test tube (10-100γ) was added 5ml. 86% sulphuric acid, with cooling in an ice bath. After two minutes the mixture was gently shaken in the ice bath, placed in the tap water for one minute and vigorously boiled for three minutes, the tubes being loosely stoppered with marbles. After cooling in tap water 0.1ml. 3% L-cysteine-HCl monohydrate was added and the sample vigorously shaken. After a few minutes the yellow colour produced was read using an SP.500 spectrophotometer (UNICAM) at 414 mμ and 380 mμ, the difference taken and used for the determination.

e) 6-DEOXYHEXOSES IN THE PRESENCE OF OTHER SUGARS - PRIMARY L-CYSTEINE SULPHURIC ACID REACTION (143)

Reagents:-

1. 86% sulphuric acid (Analar)
2. 3% aqueous L-cysteine - HCl monohydrate.

Procedure:-

As for hexoses, except that the colour intensity was read after standing for two hours and at dichromate readings, 396 mμ and 426-428 mμ the difference being used to determine the amount of 6-deoxyhexose.

f) PENTOSES IN THE PRESENCE OF OTHER SUGARS (144, 145)

Reagent:-

Freshly prepared reagent containing 110ml. glacial acetic acid, 2ml. concentrated HCl, 4.5ml. of a freshly prepared 5% solution of phloroglucinol in ethanol and 1ml. of an 0.8% solution of D-glucose.

Procedure:-

To 0.4 ml. of sugar sample (10-50γ) was added 5ml. reagent. The reaction mixture was heated in a boiling water bath for fifteen minutes

with the test tube loosely stoppered with a marble. The intensity of the purple colour produced was immediately read, after cooling, at 522 mμ and 510 mμ using an SP.500 spectrophotometer (UNICAM). The difference in optical density was then used to determine the amount of pentose present.

G.M. 12 PHOSPHORUS ANALYSIS - KING (123)

Reagents:-

1. 72% perchloric acid

2. 5% ammonium molybdate

3. Amidol. 0.5g. 1 - amino - 2 - naphtho-sulphonic acid, 3.0g. sodium metabisulphite and 6.0g. sodium sulphite were dissolved in 250ml. water and the solution filtered and stored in a brown bottle. This solution was stable for about two weeks.

Procedure:-

a) Inorganic Phosphate

To 1ml. of the sample was added 1 ml. perchloric acid, 1 ml. ammonium molybdate and 0.5ml. amidol. The solution was then made up to 10ml. with water. The contents were mixed and stood at room temperature for fifteen minutes upon which the colour intensity was read using an EEL colorimeter at 660 mμ. The phosphorus content was then calculated by reference to a standard graph.

b) Acid Labile Phosphate.

Acid labile phosphate was determined as inorganic phosphate after heating the sample with N HCl. in a boiling water bath for seven minutes.

c) Organic Phosphate

Organic phosphate was determined as inorganic phosphate after digestion

with 72% perchloric acid. The digestion was performed by heating 1 ml. of the sample with 1.2ml. perchloric acid until the charred material had vanished to leave a clear solution. The solution was then cooled and 1 ml. ammonium molybdate added, plus 0.5ml. amidol and the solution diluted to 10ml. and the colour intensity read as in the inorganic phosphate.

G.M. 13 PRETREATMENT OF CHROMATOGRAPHIC MATERIAL

a) COLUMN MATERIAL

1. Ion-Exchange Resins.

All ion-exchange resins were recyclised before being used (G.M. 1). Mixed bed resins which were not recyclised were thoroughly washed with distilled water several times before being used as column material.

2. Powdered Cellulose (Whatman)

Cellulose for the column fractionation of monosaccharides was pre-washed with water saturated n-butanol several times, decanting the fines with the supernatant each time. Finally it was filtered and then made into a slurry with water saturated n-butanol in readiness for column preparation.

3. Silicic Acid (Mallinckrodt Chemical Works, U.S.A.)

200g. silicic acid, 100 mesh, were washed with 3 litres N HCl, with stirring, filtered and the silicic acid repeatedly washed with distilled water, decanting any fines formed after about 10-15 minutes, until the pH of the supernatant was neutral. The washed material was spread on aluminium foil and dried overnight at 120°, cooled and stored in a well stoppered bottle.

4. Magnesium Silicate (Florisil B.D.H.)

200g. Florisil, 60-100 mesh, were repeatedly washed with 7 litres deionised water, allowing it to stand for 10-15 minutes then decanting the supernatant. Finally the water was removed by filtration, the Florisil spread on aluminium foil and dried at 120° for twenty-four hours, cooled in a stoppered bottle and stored under dry methanol until required.

5. DEAE CELLULOSE (WHATMAN)

This was washed several times with chloroform-methanol, 2:1. Finally it was filtered before being made into a slurry with chloroform-methanol. 2:1, in readiness for column preparation.

b) THIN-LAYER CHROMATOGRAPHY MATERIAL

1. Powdered cellulose (Chromedia, Whatman)

Powdered cellulose used for the preparation of thin-layer plates was washed twice with deionised water, filtered and dried in a desiccator under vacuum and at room temperature.

2. Silicic Acid (Merck, Darmstadt, Germany)

300g. of silicic acid were initially washed several times with 3-4 litres chloroform-methanol 2:1. Finally it was filtered, and the silicic acid spread on aluminium foil and dried at 100° for twenty four hours. It was cooled and stored in a well stoppered bottle.

c) GAS-LIQUID CHROMATOGRAPHY MATERIAL

1. Gas-Chrom 'S' (Applied Sciences Lab., Penn, U.S.A.)

This supporting material was used in the preparation of both S.E.30 columns and EGA columns. 200-300g. of Gas-chrom 'S' were washed with N HCl, filtered and washed with deionised water until the supernatant was neutral. This washed material was then dried in an oven at 60-80° for twenty-four hours. After drying it was passed through an 85-100 mesh sieve to grade the particles.

2. 2.5% S.E. 30 (Applied Sciences Lab., Penn., U.S.A.)

This column packing was used in the GLC of TMS derivatives of monosaccharides. It was prepared as follows: 0.375g. S.E. 30 were dissolved in 50ml. methylene chloride. To this was added 14.625g. pretreated Gas-chrom 'S' with continual GENTLE shaking. This mixture was placed on a st eam bath and GENTLY shaken (not stirred) until almost all of the solvent has been removed. The packing was left on the steam bath for forty-five minutes, stirring occasionally, to drive off the remainder of the methylene chloride. This 2.5% S.E. 30 preparation was then removed and stored until required, in an air tight flask.

3. 10% EGA (Applied Science Lab., Penn., U.S.A.)

This was prepared by dissolving 1.5g. ethylene glycol adipate in 50ml. methylene chloride. To this was added 13.5g. pretreated Gas-Chrom 'S'. The flask was placed on a steam bath and GENTLY shaken until almost all the methylene chloride was removed. The flask was then left on the steam bath for forty-five minutes to drive off the remainder of the solvent, occasionally shaking or stirring the packing. The 10% EGA preparation was stored in a well stoppered flask, before being used to prepare a column.

G.M. 14 PREPARATION OF DRY METHANOLIC-HCl.

Reagents:-

1. Concentrated sulphuric acid
2. Solid sodium chloride
3. Dry methanol.

Procedure:-

0.5N dry methanolic - HCl was prepared by dripping concentrated H_2SO_4 onto solid NaCl. The HCl gas given off was bubbled through concentrated H_2SO_4 to remove any traces of moisture. It was then passed

into 200ml. dry methanol (G.M. 5) 10ml. samples of methanol were removed every so often and titrated with N NaOH until the normality of the HCl was 0.5. It was stored in a well stoppered bottle.

G.M. 15 PREPARATION OF DERIVATIVES FOR GLC ANALYSIS

a) Sugars (Trimethyl silyl derivatives (118, 119)

Reagents:-

1. Dry pyridine
2. Hexamethyldisilazane
3. Trimethylchlorosilane

Procedure:-

Approximately 10mgs. carbohydrate were dissolved in dry pyridine (G.M. 5). Dissolution of the sugar samples in the pyridine was aided by shaking or gently heating in a warm water bath. To this was added 0.2ml. hexamethyldisilazane and 0.1ml. trimethychlorosilane. This mixture was shaken vigorously for thirty seconds and allowed to stand at room temperature for ten minutes before samples were removed for chromatography.

b) Fatty Acids (Methyl ester derivatives)

Reagent:-

An ethereal solution of diazomethane. This was prepared by setting up a distillation apparatus. In the distillation flask was put 350ml. ether, 55ml. diethylene glycol mono-methyl ether and 70ml 40% NaOH. 22.5g. Nitrosan were added to the mixture. The flask was well shaken and warmed gently in a warm water bath to distil off the ether diazomethane mixture which was collected in the receiving flask which also contained ether. The receiving flask was cooled in an ice-salt bath. The ether layer in the distillation flask was taken off until only 0.5 inches remained when the distillation was stopped. The ether/diazomethane

mixture was dried by the addition of KOH pellets to the flask which was left for ten minutes and then the supernatant transferred to another flask or container. It was stored in the deep-freeze. This whole preparation was performed in a fume cupboard taking all essential precautions against accidental leakage of this poisonous gas and also against any possible explosion of the gas. The residue in the distillation flask was destroyed by the addition of acetic acid.

Procedure:-

1-3ml. of an ether solution containing 1-5mgs. fatty acid mixture were placed in a conical centrifuge tube immersed in an ice bath. To this was added a three-fold excess of a freshly distilled ethereal solution of diazomethane at 0^{o}. The reaction mixture was allowed to stand for thirty minutes at room temperature and then the excess diazomethane was removed by warming on a water bath until the yellow colour had vanished. The fatty acid methyl esters were not stored in ether but in a solution of light petroleum and kept in the deep-freeze over anhydrous sodium sulphate.

2. Reagent:-

0.5N dry methanolic $\overset{HCl}{\wedge}$ (G.M. 14)

Procedure: (20)

The lipid mixture, free from solvent, was hydrolysed with the reagent (20 fold excess) by refluxing for two hours. After this time the methanol was removed by heating the mixture over a steam bath. The residue was then extracted by the addition of n -hexane ($\sim$ 5ml). This was filtered and the $\sim$ -hexane containing the fatty acid methyl esters was thoroughly dried by storage over anhydrous sodium sulphate.

G.M.16 GAS-LIQUID CHROMATOGRAPHY

a) Sugars

Procedure:-

1. A commercial Pye series 104 dual flame ionisation chromatograph was used for the analysis (W.G. Pye & Co.Ltd., Cambridge, England). 5 ft. blank and sample glass columns containing 2.5% S.E. 30 were employed (G.M. 7). 1-10γl. samples (containing 5-100γ of the TMS derivatives) were injected onto the column. The mixtures were separated using a linear temperature programme. The column has an initial temperature of 130°. After injection of the mixture this was held for two minutes then the temperature rose linearly at a rate of 2°/min., holding under isothermal conditions when 160° was attained. The rate of flow of the carrier gas (oxygen free nitrogen) was 75ml./min. The amplifier attenuation setting varied from 1×10^3 to 5×10^3. The separated components in the mixture were detected as they left the column by the flame ionisation detector, the current amplified and the sugars recorded automatically as peaks using a Smiths Servoscribe potentiometric recorder (Kelvin Electronics Company, Wembley, Middlesex.) The sensitivity of the recorder was 2 mV. and the chart speed 600mm/hour.

2. For slower running sugars (charged sugars and disaccharides) the conditions and settings were the same as above except that a different temperature programme was employed. That was, an initial temperature of 160° holding for two minutes after injection of the sample, then rising linearly at a rate of 2°/min., holding isothermally when 200° was attained.

b) Fatty Acids

Procedure:-

Fatty acid methyl esters (G.M.15) were separated using a single 5 ft.

glass column packed with 10% EGA (G.M.7). 1-10 γ-l of the ethereal solutions (containing up to 100 γ fatty acid methyl esters) were injected onto the column. The separation was effected at an isothermal temperature of 180°. The flow rate of the nitrogen carrier gas was 45ml./min. The amplified attenuation settings varied from 1 x 10^3 to 5 x 10^3. The separated fatty acids were detected as they left the column and recorded as peaks. The sensitivity of the recorder was set at 2mV. and the chart speed was 600m.m./hour.

EXPERIMENTS (Exp.)

PART I

Exp.1 THE EXTRACTION OF A MUCILAGENOUS POLYSACCHARIDE FROM BRACKEN

1kg. freshly dug bracken rhizomes were washed and chopped into one inch lengths. They were extracted for one minute with a total of 1.5l. chilled TCA (5%), in four separate batches, using a high speed Waring Blender (Waring Products, Winsted, Conn., U.S.A.). The mixture was pressed through muslin and the residue re-extracted with 2.0l.water, filtered through muslin and the two extracts combined. The residue was discarded. The brown viscous extract was centrifuged at 1,000g. for twenty mins. at 2°. The supernatant in each centrifuge tube (250ml.) was extracted in a separating funnel twice with two volumes of ether, to remove the TCA. The combined supernatants were poured, with stirring, into three volumes chilled absolute alcohol, and left for twenty-four hours to allow the precipitate to settle. The supernatant was then poured off and the polysaccaride collected by centrifugation at 500g. for ten minutes. The polysaccharide in each centrifuge tube was washed with 100ml.

alcohol, followed by 100ml. ether. The combined polysaccharide was then allowed to dry in vacuo over paraffin wax. The polysaccharide was redissolved by firstly moistening it with benzene then adding small amounts od water and blending the mixture in a small Waring blender until it was all in solution. The alcoholic purification step was then repeated to yield 7.5g. of dry polysaccharide.

Exp.2 PURIFICATION BY CETYLTRIMETHYL AMMONIUM BROMIDE

2.5g. polysaccharide were dissolved in 1 litre deionised water. This was centrifuged and passed through a column (20 x 3.0cm.) containing Amberlite IR 120H$^+$ (G.M. 2). The effluent at pH 3.5 was titrated with an excess of a 10% solution of CETAB. The precipitate formed was centrifuged off and the pH of the supernatart raised to 7.5 by slowly adding N NaOH with continual stirring. A second precipitate formed at pH 7.5 was also collected by centrifugation. Both fractions were dissolved in 500ml. 10% LiCl. These two solutions were then poured into two separate lots of 3 volumes chilled absolute alcohol, with continual stirring. The two polysaccharide fractions were collected by decantation and centrifugation and then dried. Samples of both fractions were hydrolysed with 2N H_2SO_4 (G.M. 3) and analysed by paper chromatography for their sugar content (G.M. 4 and 5).

This purification step was repeated but this time with a 500ml. solution containing 0.1% polysaccharide. The pH was lowered to 2 by the addition of N H_2SO_4 with stirring. It was then titrated with an excess of 10% CETAB. No precipitate appeared initially. The pH of the solution was then raised slowly by the addition, with stirring, of drops of 0.5N NaOH. Two precipitates were then obtained, one at pH 3.5 and another at pH 7-7.5. Both were collected and dissolved in 500ml. 10% LiCl., and the

two polysaccharide fractions obtained by alcoholic precipitation. The two fractions were collected and dried. Samples of both fractions were hydrolysed with H_2SO_4 (G.M. 3) and examined by paper chromatography for their constituent monosaccharides (G.M. 4 and 5).

Exp.3 CELLULOSE COLUMN CHROMATOGRAPHY OF POLYSACCHARIDE CONSTITUENTS

Pretreated powdered cellulose (G.M. 3) was packed into a column 105.0 x 3.5cm. in dimension, (G.M. 7) to a height of 100 cm. The column was then washed with 2 litres water saturated n-butanol.

2g. of polysaccharide were hydrolysed with H_2SO_4 (G.M. 3) and the hydrolysate placed on top of the column in 4ml. water. The top of the column was attached to a reservoir containing 5 litres water saturated n-butanol. The flow rate of the column was 1ml./minute. 10ml. fractions were collected automatically. Alternate fractions were analysed for sugar content by the method of Gardell (G.M. 11a) using 1ml. samples. The appropriate peaks were bulked, reduced to dryness *in vacuo* and examined by paper chromatography (G.M. 4 and 5).

Polarimetry. Peak one, shown to be L-fucose by paper chromatography, was reduced to dryness and dissolved in 2ml. water to give a concentration of 5.1%. Its optical rotation was then measured using one decimeter cells with an internal diameter of 3mm. against a blank of water and using a Hilger and Watts standard polarimeter. From the reading, the specific rotation of peak one was calculated.

Osazone Derivative. The 2 mls. water containing approximately 100mgs. of peak one were placed in a conical centrifuge tube. To this was added 0.4ml. phenylhydrazine and 0.4ml. glacial acetic acid. This mixture was heated in a water bath for 1.5 hours, removed and allowed to cool in a refrigerator for thirty minutes. The yellow crystals formed were collected

by filtration and washed with water then ether. The crystals were recrystallised from absolute alcohol until a constant melting point was obtained (174-176°).

Exp.4 ION-EXCHANGE FRACTIONATION OF POLYSACCHARIDE SUGAR COMPONENTS

Dowex 1, 200-400 mesh, in the acetate form (G.M. 2) was used to prepare a column with effective dimensions of 15 x 1.5cm. (G.M. 7). 1g. polysaccharide was hydrolysed with H_2SO_4 (G.M. 3) and the syrup dissolved in 4ml. 0.02 N NaCH to prevent lactone formation.

This was applied to the top of the column which was eluted with a linear gradient of 1 litre water to 1 litre N. acetic acid. 25ml. fractions were collected automatically and each was analysed for sugar content, using 1 ml. samples by the Somogyi reaction (G.M. 11b). The appropriate tubes were bulked to give there separate peaks (Fig. 7) which were then examined by paper chromatography for their sugar content (G.M. 4 and 5).

Exp. 5 QUANTITATIVE ANALYSIS OF POLYSACCHARIDE

Moisture. 0.267g. polysaccharide were placed in a drying pistol under P_2O_5, the system was evacuated and the temperature raised to 85° for twenty-eight hours. The sample was cooled in the pistol, removed and weighed immediately. This was repeated until a constant weight was obtained.

ASH. 0.35g. polysaccharide, obtained after alcoholic precipitation, were heated in a silica crucible over a bunsen until completely charred. It was then transferred to a muffle furnace at 500° for twenty-four hours. The sample was removed, cooled in a desiccator over pellets of KOH and weighed. This analysis was repeated using 0.475g. of polysaccharide, obtained after cetyl trimethyl ammonium bromide precipitation.

Sulphate. The method used here was that of Treon and Crutchfield (141). 2.14g. of polysaccharide, after alcoholic precipitation, were charred over a bunsen then placed in a muffle furnace at 500° for twelve hours. The ash was cooled in a desiccator and digested on a sand bath for ten minutes with 5ml. N HCl. This was filtered into a 500ml. volumetric flask, washing thoroughly both the beaker and filter paper with water (deionised). The volume was made up to the mark with deionised water. 1g. fine mesh $BaCO_3$ was then added, the mixture well shaken for five minutes and the turbidity read immediately at 370 mμ using an SP.500 spectrophotometer (UNICAM) against a blank of water. The reading obtained was referred to a standard graph of 0-120mgs. $SO_4^{=}$/ml. which was prepared using 2.0N H_2SO_4 as the source of sulphate ions. From this graph the mgs. $SO_4^{=}$/g. polysaccharide was calculated. This experiment was repeated using 1.05g. of polysaccharide, obtained after precipitation with CETAB.

Uronic acid. 0.25g. polysaccharide were dissolved in 400ml. water. This was performed by moistening the sample with benzene then adding small amounts of water at a time, and blending the mixture in a small Waring blender. This was then poured through an Amberlite IR 120H$^+$ column (G.M. 2 and 7) 15 x 3.0cm. in dimension. The effluent was freeze-dried and the freeze-dried sample left for forty-eight hours to equilibrate with atmospheric moisture. The weight of this sample was 1.6g. This was dissolved in 400ml. water and the solution made up to 500ml. 100ml. portions of this were titrated with 0.01 N NaOH using phenylphthalein as an indicator. The acidity found was presumed to be due solely to uronic acid groups and from the value found the percentage uronic acid present was calculated taking the molecular weight of a hexuronic acid to be 176.

Hexose and Methyl Hexose. 0.265g. polysaccharide was dissolved in 500ml.

water, firstly by damping it with benzene then adding small amounts of water at a time with blending in a small Waring blender (Solution Y). 10, 20 and 30ml. samples were taken from Solution Y and made up to 100ml. in volumetric flasks with water. 1ml. samples from each were then used for the determination using L-cysteine and sulphuric acid. The hexose readings were taken after ten minutes and the methyl hexose after two hours (G.M. 11d and e). A blank of water was also run with the samples. The readings obtained were referred to a standard graph made up of 0-100γ/ml. of D-galactose and L-fucose (G.M. 8).

Pentose. 10, 20 and 30ml. samples were taken from Solution Y and made up to 100ml. with water in volumetric flasks. 0.4ml. samples were used for the determination using the method of Dische and Borenfreund (G.M. 11f). A blank of water was also run at the same time. The readings were referred to a standard graph made up using samples of 0-50γ/ml. of D-xylose (G.M.8).

PART II SECTION A

Exp. 6 PRELIMINARY SILICIC ACID AND DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF FIRST LIPID MIXTURE

3.5g. lipid extract were dissolved in 10ml. chloroform (G.M. 6) and applied to a silicic acid column, 13.0 x 4.0 cm. in dimension (G.M. 7). The column was eluted with 1.0l. chloroform followed by 1.0l. methanol. The first fraction was discarded while the second was reduced to dryness using a rotary evaporator at a temperature of 45°. The residue was dissolved in 20ml. chloroform-methanol, 2: 1 v/v, and applied to a DEAE cellulose column, 20.0 x 3.2cm. in dimension (G.M. 7). The column was eluted with 1.0l. chloroform-methanol, 2: 1 v/v, 1.5l. chloroform-methanol, 2: 1 v/v plus 5% glacial acetic acid and finally with chloroform-methanol, 2: 1 v/v, plus 5% 0.5M aqueous LiCl (2.0l.). Fractions one and two were

reduced to dryness using a rotary evaporator and each redissolved in 10ml. chloroform-methanol, 2: 1 v/v. Fraction three was also reduced to dryness and dissolved in 100ml. chloroform-methanol, 2: 1 v/v. To this, in a separating funnel, was added 50ml. water with shaking. After standing overnight the lower chloroform layer was run off, taken to dryness and redissolved in 10ml. chloroform-methanol, 2: 1 v/v.

Exp. 7. PRELIMINARY SILICIC ACID AND DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF FIRST LIPID MIXTURE

The remaining halves of the three fractions from the DEAE cellulose column in Exp.6 were taken and fractions one and two combined. The combined fractions and fraction three were reduced to dryness and saponified (G.M.10). The two ether extracts were taken to dryness using a rotary evaporator and the residues redissolved in 8.0ml. chloroform. Each chloroform solution was applied to a separate silicic acid column, 5.0 x 1.5cm. in dimension (G.M. 7) and eluted with 200ml. chloroform, followed by 200ml. methanol. The chloroform fractions were discarded while the methanol fractions were hydrolysed (G.M. 3) and examined for sugar content (G.M. 4 and 5).

Exp.8 PRELIMINARY SILICIC ACID AND DEAE CELLULOSE COLUMN CHROMATOGRAPHY OF SECOND LIPID EXTRACT

2g. lipid mixture were dissolved in 10ml. chloroform (G.M. 6) and applied to a silicic acid column, 20.0 x 2.5cm. in dimension (G.M. 7). The column was eluted with 1.0l. chloroform followed by 1.0l. chloroform-methanol, 4: 1 v/v. The first fraction was discarded while the second was reduced to dryness and the residue (0.7g) redissolved in 4ml. chloroform-methanol, 2: 1 v/v. This was put onto a DEAE cellulose column, 22.0 x 3.5cm. in dimension (G.M. 7) and eluted with 1.5l. chloroform-methanol, 2: 1 v/v, 1.5l. chloroform-methanol, 2: 1 v/v plus 5% glacial

acetic acid and finally with 1.5l. chloroform-methanol, 2: 1 v/v plus 5% 0.5N aqueous LiCl. Fractions one and two were reduced to dryness. Fraction three was also reduced and the residue dissolved in 100ml. chloroform-methanol, 2: 1 v/v. This was shaken in a separating funnel with 50 ml. water, left overnight and the chloroform layer collected and reduced to dryness using a rotary evaporator.

Exp. 9 COMPARISON OF SUGARS OBTAINED FROM LIPID MIXTURES FROM FREEZE-DRIED BRACKEN

a) Soaked in water

b) " " 0.5% $HgCl_2$

c) " " 1.0% $HgCl_2$

0.5Kg. freeze-dried rhizomes were divided into three equal amounts. The first was soaked in 4 litres water, the second in 4 litres 0.5% $HgCl_2$ and the third in 4 litres 1.0% $HgCl_2$. After twelve hours each was pressed through muslin and the residues dried in an oven at 110° for twenty-four hours. Each sample was then extracted by blending with chloroform-methanol, 2: 1 v/v (G.M. 1c).

Exp.10 REMOVAL OF NONLIPIDS USING POWDERED CELLULOSE COLUMN

2.5g. partially purified lipid mixture (G.M. 1c) were dissolved in 10ml. water saturated chloroform-methanol, 9: 1 v/v, and applied to a powdered cellulose column, 27.0 x 3.2cm. in dimension (G.M. 7). The column was eluted with seven bed volumes (~ 600ml.) water saturated chloroform-methanol, 9: 1 v/v, followed by seven bed volumes methanol-water, 9: 1 v/v. The two fractions were reduced to dryness and the residues redissolved in 10ml. chloroform-methanol, 2: 1 v/v.

SECTION B

Exp.11 EVALUATION OF SILICIC ACID CHROMATOGRAPHY OF LIPID MIXTURES

1.75g. lipid mixture were dissolved in 10ml. chloroform (G.M. 6) and applied to a silicic acid column, 18.0 x 2.5cm. in dimension (G.M. 7). The column was eluted with 500ml. chloroform followed by 500ml. methanol, the rate of flow being 3-5ml./min. Both fractions were reduced to dryness using a rotary evaporator, at a temperature of not more than 50°. The residues were each redissolved in 15ml. chloroform-methanol, 2: 1 v/v.

Exp.12 EVALUATION OF MAGNESIUM SILICATE CHROMATOGRAPHY OF LIPID MIXTURES

Phosphorus analysis of the lipid mixture (G.M. 12c) was carried out after saponification (G.M. 10), after hydrolysis with ethanol-chloroform-5N HCl. (G.M. 3c) and after charring. The latter method was carried out by heating 0.875g. of the lipid mixture in a silica crucible over a bunsen until completely charred. This was then transferred to a muffle furnace at 500° and left for twenty-four hours. The sample was then cooled, and the ash digested with 5ml. N HCl on a sand bath for fifteen minutes. This was filtered into a 250ml. volumetric flask, washing the crucible and filter paper thoroughly with deionised water. The flask was made up to the mark with deionised water and 1.0ml. samples used for the phosphorus analysis (G.M. 12c). 1.22g. of the lipid mixture was then dissolved in 10ml. chloroform-methanol, 9: 1 v/v, and put onto a magnesium silicate (Florisil, BDH) column, 16.0 x 3.5cm. in dimension (G.M. 7). The column was eluted with 800ml. chloroform-methanol, 9: 1 v/v, 1.0l. chloroform-

methanol, 2: 1 v/v and finally with 1.0l. methanol. All three fractions were reduced to dryness using a rotary evaporator at a temperature not more than 50° and the residues hydrolysed (G.M. 3c).

Exp.13 REPEAT OF MAGNESIUM SILICATE COLUMN CHROMATOGRAPHY

1.06g. of lipid mixture were dissolved in 5ml. chloroform-methanol, 19: 1 v/v, and applied to a magnesium silicate column, 16.0 x 35cm. in dimension (G.M. 7). The column was eluted with 1.0l. chloroform-methanol, 19: 1 v/v, 1.5l. chloroform-methanol, 1: 1 v/v, and finally with 1.5l. methanol. All three fractions were reduced to dryness as before and the residues hydrolysed (G.M. 3c).

Exp.14 FRACTIONATION OF LIPID MIXTURE BY SUCCESSIVE COLUMN CHROMATOGRAPHY

Magnesium Silicate Column. 1.124g. of lipid mixture were dissolved in 5ml. chloroform-methanol, 19: 1 v/v, and applied to a magnesium silicate column, 18.0 x 3.5 cm. in dimension (G.M. 7). The column was eluted with 800ml. chloroform-methanol, 19: 1 v/v, 2.0l. chloroform-methanol, 1: 1 v/v, and finally with 1.0l. methanol. The flow rate of the column was 5-7ml./min. The three fractions were reduced to dryness using a rotary evaporator at a temperature of 45-50°. The residues were redissolved in 10ml. chloroform-methanol, 2: 1 v/v, and examined by TLC (G.M.4). The remainder of each fraction was made up to 250ml. in a volumetric flask with chloroform-methanol, 2: 1 v/v. 10ml. from each flask were reduced to dryness and the residues hydrolysed with ethanol-chloroform- 5N HCl (G.M. 3c) and analysed for carbohydrate and phosphorus content (G.M. 11c and 12c).

DEAE CELLULOSE COLUMN. The remaining 240ml. chloroform-methanol, 2: 1 v/v, solution containing fraction two from the magnesium silicate column

was reduced to dryness by rotary vacuum distillation to give a residue 0.26g. in weight. This was redissolved in 5ml. chloroform-methanol, 2: 1 v/v, and applied to the top of a DEAE cellulose column, 22.0 x 2.2cm. in dimension (G.M. 7). The column was eluted with 1.0l. chloroform-methanol, 2: 1 v/v, 700ml. chloroform-methanol, 2: 1 v/v plus 5% glacial acetic acid and finally with 600ml. chloroform-methanol, 2: 1 v/v plus 5% 0.5N aqueous LiCl. The flow rate of the column was 5-7ml/min. All three fractions were reduced to dryness using a rotary evaporator. The residues from fractions one and two were dissolved in 10ml. chloroform-methanol, 2: 1 v/v. The residue from fraction three was redissolved in 100ml. chloroform-methanol, 2: 1 v/v and shaken with 50ml. water in a separating funnel. After standing overnight, the chloroform layer was collected, reduced to dryness and the residue redissolved in 10ml. chloroform-methanol, 2: 1 v/v. All three fractions were then examined by TLC (G.M. 4) The remainder of each fraction was then made up to 250ml. in a volumetric flask with chloroform-methanol, 2: 1 v/v. 25ml. samples were removed from each, reduced to dryness using a rotary evaporator and the residues hydrolysed with ethanol-chloroform - 5N HCl (G.M. 3c) and examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

SILICIC ACID COLUMN. 225ml. chloroform-methanol, 2: 1 v/v, containing fraction one from the DEAE cellulose column was reduced to dryness to give a residue weighing 130mgs. This was dissolved in 3.0ml. chloroform and applied to a silicic acid column, 42.0 x 3.2cm. in dimension, which was packed uniformly (G.M. 7). The column was eluted with a linear gradient of chloroform to chloroform-methanol, 1:1 v/v. (2l. chloroform in mixing chamber and 2l. methanol in reservoir). The flow rate of the column was approximately 4ml/min. 10m. fractions were collected automatically using a Towers fraction collector, and every alternate fraction was analysed by

TLC (G.M. 4). The appropriate fractions were bulked (Table 28), reduced to dryness and each residue redissolved in 10ml. chloroform-methanol, 2:1 v/v. Each bulked fraction was analysed by TLC (G.M. 4) (Fig.24). 1.0ml. from each bulked solution was also reduced to dryness, hydrolysed with ethanol-chloroform-5N HCl (G.M. 3c) and analysed for carbohydrate and phosphorus content (G.M. 11c and 12c).

Exp.15 EVALUATION OF SILICIC ACID COMBINED WITH MAGNESIUM SILICATE AS A COLUMN TECHNIQUE IN LIPID FRACTIONATION

Silicic Acid. 1.175g. of lipid mixture were dissolved in 7-8ml. chloroform-methanol, 9:1 v/v and applied to a silicic acid column, 22.0 x 2.5cm. in dimension (G.M. 7). The column was eluted 600ml. chloroform, followed by 500ml. methanol at a rate of 4ml/min. Both fractions were reduced to dryness by rotary distillation and the residues redissolved in 10ml. chloroform-methanol, 2:1 v/v and examined by two dimensional TLC (G.M. 4). The remainder of fraction one was reduced to dryness and hydrolysed with ethanol-chloroform-5N HCl (G.M. 3c). The remainder of fraction two was made up to 50ml. with chloroform-methanol, 2: 1 v/v , in a volumetric flask, and 2ml. of this taken to dryness and hydrolysed (G.M. 3c). Both were examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

Magnesium Silicate. The 48ml. solution of chloroform-methanol, 2:1 v/v containing the remainder of fraction two from the silicic acid column was reduced to dryness to give a residue 340mg. in weight. This was dissolved in 4-5ml. chloroform-methanol, 19: 1 v/v, and applied to a magnesium column 20.0 x 3.5cm. in dimension (G.M. 7). The column was eluted with 500ml. chloroform-methanol, 19: 1 v/v, 1.0l. chloroform-methanol, 1: 1 v/v, and finally 1.0l. methanol. All three fractions were reduced to dryness,

redissolved in 5-10ml. chloroform-methanol, 2: 1 v/v and examined by two dimensional TLC (G.M. 4). The remainder of fraction one was reduced to dryness and the residue hydrolysed (G.M. 3c). Fractions two and three were diluted to 50ml. in a volumetric flask with chloroform-methanol, 2: 1 v/v. 2.0ml. from each were then reduced to dryness and the residues hydrolysed with ethanol-chloroform- 5N HCl (G.M. 3c). All three hydrolysate were examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

Exp. 16 COMBINED USE OF SILICIC ACID, MAGNESIUM SILICATE AND DEAE CELLULOSE CHROMATOGRAPHY FOR THE FRACTIONATION OF LIPID MIXTURES

1st Silicic acid Column. 2.97g. lipid mixture were dissolved in 5ml. hexane-ether, 1:1 v/v. This was applied to a silicic acid column 25.0 x 2.5cm. in dimension and eluted as follows: with 1.0l. hexane-ether, 1:1 v/v, 500ml. hexane-ether 3: 7v/v, 500ml. chloroform, 700ml. chloroform-methanol 19: 1 v/v and finally with 500ml. methanol. Fractions two and three were combined and called fraction two. All four fractions were taken down to dryness and the residues redissolved in 5-10ml. chloroform-methanol, 2: 1 v/v, and analysed by two dimensional TLC (G.M. 4). The remainder of fraction one was reduced to dryness and the residue hydrolysed with ethanol-chloroform-5N HCl (G.M. 3c). All four hydrolysate were analysed for carbohydrate and phosphorus content (G.M. 11c and 12c).

2nd Silicic Acid Column. The remainder of fraction four from the above column (230mg) was dissolved in 3.0ml. chloroform and applied to a silicic acid column, 14.0 x 2.5cm. in dimension(G.M. 7). The column was eluted as in the first silicic acid column. Fraction one was discarded and the remaining four reduced to dryness and the residues redissolved in 5ml. chloroform-methanol, 2: 1 v/v. All four were examined by two dimensional

TLC (G.M. 4). All four fractions were then diluted to 50ml. in a volumetric flask, with chloroform-methanol, 2: 1 v/v. 4.0ml. were removed from each, reduced to dryness and the residues hydrolysed (G.M.3c). The hydrolysates were then examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

Magnesium Silicate Column. Fraction five (348mgs) from the first silicic acid column was dissolved in 3.0ml. chloroform-methanol, 19: 1 v/v, and applied to a magnesium silicate column, 20.0 x 3.5cm. in dimension (G.M. 7). The column was eluted with 500ml. chloroform-methanol, 19: 1 v/v, followed by 800ml. chloroform-methanol, 2: 1 v/v, and finally with 500ml. methanol. Fraction one was discarded. Fractions two and three were reduced to dryness using a rotary evaporator at a temperature of 50°. The residues were redissolved in 5.0ml. chloroform-methanol, 2: 1 v/v, and fraction two only, analysed by two dimensional TLC (G.M. 4). The remainder of fractions two and three were diluted to 50ml. in a volumetric flask with chloroform-methanol, 2: 1 v/v. 4.0ml. were taken from each, reduced to dryness and hydrolysed with ethanol-chloroform-5N HCl (G.M. 3c). Both hydrolysates were examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

DEAE CELLULOSE COLUMN. Fraction two (93.0mg.) from the magnesium silicate column was dissolved in 3.0ml. chloroform-methanol, 2: 1 v/v, and put on a DEAE cellulose column, 20.0 x 2.5cm. in dimension. The column was eluted with 600ml. chloroform-methanol, 2: 1 v/v, followed by 700ml. chloroform-methanol, 2: 1 v/v plus 5% glacial acetic acid, and finally with 700ml. chloroform-methanol, 2: 1 v/v, plus 5% 0.5M aqueous LiCl. Fractions one and two were reduced to dryness and the residues redissolved in 4-5ml. chloroform-methanol, 2: 1 v/v. Fraction three was also reduced to dryness and the residue redissolved in 100ml. chloroform-methanol, 2: 1 v/v.

This was shaken with 50ml. water in a separating funnel, and after standing overnight, the chloroform layer was collected. This was reduced to dryness and the residue redissolved in 5ml. chloroform-methanol, 2: 1v/v. All three fractions were then analysed by two dimensional TLC (G.M. 4). The three fractions were then made up to 25ml. in a volumetric flask with chloroform-methanol, 2: 1 v/v, and 1.0ml. from each taken and hydrolysed (G.M. 3c). All three hydrolysates were examined for carbohydrate and phosphorus content (G.M. 11c and 12c).

BIBLIOGRAPHY.

1. Rees, W.R., Duncan, H.J., Biochem. J., 94, No. 1, 19p, (1965).
2. Rees, W.R., Duncan, H.J., Biochem. J., 94, No. 1, 19p, (1965).
3. Wadsworth, J.R., Vet. Med., 47, 412, (1952).
4. Cullity, M., J. Dept. Agr. West. Australia, 15, 414, (1938).
5. Green, K.R., Agr. Gaz. N. S. Wales, 61, 341, (1950).
6. Forrest, J.D., Proc. British Weed Control Conf., 4th, Brighton, 1958, 184, (1960).
7. Fletcher, W.W., Kirkwood, R.C., Agriculture, 68, 426, (1961).
8. Cori, C.F., Colowick, S.P., Cori, G.T., J. Biol. Chem., 121, 465, (1961).
9. Hanes, C.S., Proc. Roy. Soc., B129, 174, (1940).
10. Cardini, C.E., Paladini, A.C., Caputto, A., Leloir, L.F., Nature, 165, 191, (1950).
11. Strominger, J.L., Physiol. Revs., 40, 55, (1960).
12. Neufeld, E.F., Hassid, W.Z., Adv. in Carbohydrate Chem., 18, 309, (1963).
13. Cabib, E., Annual Rev. Biochem., 32, 321, (1963).
14. Michelson, A.M., "The Chemistry of Nucleosides and Nucleotides", Academic Press, (1963).
15. Leloir, L.F., Cabib, E., J.Amer. Chem. Soc., 75, 5445, (1953).
16. Cardini, C.E., Leloir L.F., Chiviboga, J., J. Biol. Chem., 214, 149, (1955).
17. Leloir, L.F., Cardini, C.E., J. Biol. Chem., 214, 157, (1955).

18. Yamaha, T., Cardini, C.E., Arch. Biochem. Biophys., 86, 127, (1960).

19. Cabib, E., Leloir, L.F., Cardini, C.E., J. Biol. Chem., 205, 1055, (1953).

20. Cabib, E., Leloir, L.F., J. Biol. Chem., 206, 779, (1954).

21. Ginsburg, V., Stumpf, P.K., Hassid, W.Z., J. Biol. Chem., 223, 977, (1956).

22. Sukuzi, S., Biochim. Biophys. Acta., 50, 395, (1961).

23. Baddiley, J., Blumson, N.L., Biochim. Biophys. Acta, 39, 376, (1960).

24. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

25. Glaser, L., J. Biol. Chem., 232, 627, (1958).

26, Glaser, L., Brown, D.H., J. Biol. Chem., 228, 729, (1957).

27. Elbein, A.D., Barber, G.A., Hassid, W.Z., J. Amer. Chem. Soc., 86, 309, (1964).

28. Feingold, D.S., Neufeld, E.F., Hassid, W.Z., J. Biol. Chem., 233, 783, (1958).

29. Goldenberg, S.H., Marechal, L.R., Biochim. Biophys. Acta, 71, 743, (1963).

30. Feingold, D.S., Neufeld, E.F., Hassid, W.Z., J. Biol. Chem., 234, 488, (1959).

31. Glaser, L., Brown, D.H., Proc. Nat. Acad. Sci. (Wash.), 41, 253, (1955).

32. Markovitz, A., Cifonelli, J.A., Dorfman, A., J. Biol. Chem., 234, 2343, (1959).

33. Smith, E.E.B., Mills, G.T., Biochem. J., 82, 42p, (1962).

34. Smith, E.E.B., Mills, G.T., Bernheimer, H.P., J. Biol. Chem., 236, 2179, (1961).

35. Duncan, H.J., " Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

36. Leloir, L.F., Cardini, C.E., L. Amer. Chem. Soc., 79, 6340, (1957).

37. Leloir, L.F., de Fekete, M.A.R., Cardini, C.E., J. Biol. Chem., 236, 636, (1960).

38. Recondo, E., Leloir, L.F., Biochem. Biophys. Res. Commun., 6, 85, (1961).

39. Pottinger, P.K., Oliver, I.T., Biochim. Biophys. Acta, 58, 303, (1962).

40. Frydman, R.B., Cardini, C.E., Biochem. Biophys. Res. Commun., 17, 407, (1964).

41. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

42. Badenhuizen, N.P., Nature, 197, 464, (1963).

43. Buttrose, M.S., Staerke, 15, 85, (1963).

44. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

45. Carter, H.E., McCluer, R.H., Slifer, E.D., J. Amer. Chem.Soc., 78, 3735, (1956).

46. Northcote, D.H., Biochem. J., 83, 20P, (1962).

47. Northcote, D.H., Goulding, K.J., Horne, R.W., Biochem. J., 70, 391, (1958).

48. Northcote, D.H., Goulding, K.J., Horne, R.W., Biochem. J., 77, 503, (1960).

49. Thornber, J.P., Northcote, D.H., Biochem. J., 81, 455, (1961).

50. Hassid, W.Z., Neufeld, E.F., Feingold, D.S., Proc. Nat. Acad. Sci., (Wash.), 45, 905, (1959).

51. Benson, A.A., Daniel, H., Wiser, R., Proc. Nat. Acad. Sci., (Wash.), 45, 1582. (1959).

52. Benson, A.A., Ann. Rev. Plant Physiol., 15, 1, (1964).

53. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

54. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

55. Colvin, J.R., Nature, 183, 1135, (1959).

56. Colvin, J.R., Can. J. Biochem. Physiol., 39, 1921, (1961).

57. Lennarz, W.J., J. Biol. Chem., 239, PC3110, (1964).

58. Neufeld, E.F., Hall, C.W., Biochem. Biophys. Res. Commun., 14, 503, (1964).

59. Duncan, H.J., "Nucleotides and Related Compounds in Plants", Ph.D. Thesis, Glasgow, (1965).

60. Ginsburg, V., O'Brien, P.J., Hall, C.W., Biochem. Biophys. Res. Commun., 7, 1, (1962).

61. Gordon-Young, E., "Carbos. Accumulation of Younger Plants", (Encyclopaedia of Plant Physiology, Vl).

62. Hansen, R.G., Freedland, R.A., Scott, K.M., J. Biol. Chem., 219, 391, (1956).

63. Pigman, W., Platts, D., "Polysaccs., Part 2". (The Carbohydrates, edited by Pigman, Academic Press).

64. Strominger, J.L., J. Biol. Chem., 237, 1388, (1964).

65. Su, J.C., Hassid, W.Z., Biochemistry, 1, 474, (1962).

66. Foster, A.B., Stacey, M., "The Polysaccs. from Lower Plants". (Encyclopaedia of Plant Physiology, V1).

67. Smith, E.E.B., Mills, G.T., Harper, E.M., Biochem. Biophys. Acta, 23, 662, (1959).

68. Stacey, M., Barker, S.A., "Polysaccs. of Micro-organisms", (Oxford Press).

69. Smith, E.E.B., Mills, G.T., Harper, E.M., J. Gen. Microbiol., 16, 426, (1959).

70. Denamur, R., Fauconeau, G., Jarrige-Guntz, G., Compt. Rend., 246, 492, (1958).

71. Stacey, M., Barker, S.A., "Carbohydrates of Living Tissue", (D. Van Nostrand Co., Ltd.).

72. Ballio, A., Casinova, G., Serluppi-Crescenzi, G., Biochem. Biophys. Acta, 20, 414, (1956).

73. Mikaido, A., Jokura, K., Biochem. Biophys. Res. Commun., 6, 304, (1961).

74. Whistler, R.L., Corbett, W.M., "Polysaccs., Part 1. (The Carbohydrates, edited by Pigman, Academic Press).

75. Park, J.T., J. Biol. Chem., 194, 877, (1952).

76. Weir, J., personal communication.

77. Ferguson, W.S., Armitage, E.R., Agr. Sci., 34, 165, (1944).

78. Hunter, J.G., Nature, 153, 656, (1944).

79. Smith, A.M., Wyllie-Fenton, E., J. Soc. Chem. Ind., 63, 218, (1944).

80. Moon, F.E., Pal, A.K., J. Agr. Sci., 32, 296, (1949).

81. Hunter, J.G., J. Sci. Food Agr., 4, 10, (1953).

82. Moon, F.E., Raafat, M.A., J. Sci. Agr., 2, 228, (1951).

83. Muller-Stoll, W.R., Michael, K., Planta, 36, 507, (1949).

84. Hamilton, S., Conny, M.J., Australian J. Biol. Sci., 13, 479, (1960).

85. Bremner, I., Wilkie, K.C.B., Carbohydrate Res., 2, 24, (1966).

86. Paterson, T.M., Proc. New Zealand Weed Pest Control Conf., 18, 24, (1965).

87. Sevag, M.G., Biochem. Z., 273, 419, (1934).

88. Morner, K.A.H., Scand. Arch. Physiol., 6, 332, (1895).

89. Schiller, S., Matthews, M.B., Goldfaber, L., Ludeweig, J., Dorfman, A., J. Biol. Chem., 212, 531. (1955).

90. Jorpes, J.E., Biochem. Z., 204, 354, (1929).

91. Meyer, K., Linker, A., Davidson, E.A., Weismann, B., J. Biol. Chem., 205, 611, (1955).

92. Schoch, T.J., Adv. in Carbohydrate Chem., 1, 247, (1945).

93. Caldwell, G.C., Hixon, R.M., J. Amer. Chem. Soc., 63, 2876, (1941).

94. Samec, Nucic, M.C., Pirkmaier, V., Kolloidzschr. 94, 350, (1941).

95. Gardell, S., Gordon, A.H., Aquist, S., Acta Chem. Scand., 4, 907, (1950).

96. Fuller, K.W., Northcote, D.H., Biochem. J., 64, 657, (1956).

97. Blix, G., Snellman, O.,Ark. Kemi. Min. Geol., 194, 32, (1945).

98. Ringertz, N., Reichard, P., Acta Chem. Scand., 13, 1467, (1959).

99. Ringertz, N., Reichard, P., Acta Chem. Scand., 14, 303, (1960).

100. Peterson, E.A., Sober, H.A., J. Amer. Chem. Soc., 78, 751, (1956).

101. Schmidt, M., Biochim. Biophys. Acta, 63, 346, (1962).

102. Ringertz, N., Acta Chem. Scand., 14, 312. (1960).

103. Porath, J., Flodin, P., Nature, 183, 1657. (1959).

104. Ostling, G., Acta Soc. Med. Upsaliensis, 64, 222, (1960).

105. Porath, J., Biochim. Biophys. Acta, 39, 193, (1960).

106. Scott, J.E., "The Assay of Acidic Polysaccs. from Tissues". Ph.D. Thesis, Manchester, (1956).

107. Scott, J.E., Methods of Biochemical Analysis, 8, 145, (1960). (Interscience Inc.).

108. Jeanes, A., Wise, C.S., Dimler, R.J., Anal. Chem., 23, 415, (1951).

109. Smith, I., Chromatographic Techniques, 166, (1958). (William Heinemann).

110. Fink, K., Cline, R.E., Fink, R.M., Anal. Chem., 35, 389, (1963).

111. Trevelyan, W.E., Proctor, D.P., Harrison, J.S., Nature, 166, 444, (1950).

112. Bacon, J.S.D., Edelman, J., Biochem. J., 48, 114, (1951).

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

114. Bryson, J.L., Mitchell, T.J., Nature, 167, 864, (1951).

115. Horrocks, R.H., Manning, G.B., Lancet, 1042, (1949).

116. Bishop, C.T., Glick, D., Methods of Biochemical Analysis, 10, 1, (1962). (Interscience Inc.).

117. Vanden Heuvel, W.J.A., Horning, E.C., Biochem. Biophys. Res. Commun., 4, 399, (1961).

118. Bentley, R., Sweeley, C.C., Makita, M., Wells, W.W., Biochem. Biophys. Res. Commun., 11, 14, (1963).

119. Sweeley, C.C., Bentley, R., Makita, M., Wells, W.W., J. Amer. Chem. Soc., 85, 2497, (1963).

120. Wells, W.W., Sweeley, C.C., Bentley, R., "Gas Chromatography of Carbohydrates", p 169. (Biomedical Applications of Gas Chromatography, edited by Herman A. Szymansky. Plenum Press, 1964).

121. Oates, M.D.G., Schrager, J., Biochem. J., 97, 697, (1965).

122. Sweeley, C.C., Bull. Soc. Chim. Biol., 47, 1477, (1965).

123. Kylin, H., Z. Physiol. Chem., 83, 171, (1913).

124. Kylin, H., Z. Physiol. Chem., 94, 357, (1915).

125. Percival, E.G.V., Ross, A.G., J. Chem. Soc., 717, (1950).

126. Conchie, J., Percival, E.G.V., J. Chem. Soc., 827, (1950).

127. Aspinall, G.O., Baillie, J., J. Chem. Soc., 1702, (1963).

128. Hough, L., Jones, J.K.N., Wadman, W.H., J. Chem. Soc., 2511, (1949).

129. Gardell, S., Acta Chem. Scand., 5, 1011, (1951).

130. "Data for Biochemical Research", edited by Dawson, Elliot, Elliot and Jones, p 86. (Oxford Press).

131. Heilbron and Bunbury, "Dictionary of Organic Coumpounds", 2, 567. (Eyre and Spottiswoode, 1953).

132. Khym, J.X., Doherty, D.G., J. Amer. Chem. Soc., 74, 3199, (1952).

133. Somogyi, M., J. Biol. Chem., 195, 19, (1952).

134. Hulyalkar, R.H., Ingle, T.R., Bhide, B.V., J. Indian Chem. Soc., 33, 861, (1956).

135. Falconer, E.L., Adams, G.A., Can. J. Chem., 34, 338, (1958).

136. Aspinall, G.O., Das Gupta, P.C., J. Chem. Soc., 3627, (1958).

137. Timmel, T.E., Can. J. Chem., 37, 893, (1959).

138. Timmel, T.E., Methods in Carbohydrate Chem., 1, 301, (1962). (Academic Press).

139. Jones, J.K.N., J. Chem. Soc., 534, (1950-.

140. Aspinall, G.O., Hirst, E.L., Nicholson, A., J. Chem. Soc., 1697, (1958).

141. Treon, J.F., Crutchfield, W.E., Anal. Chem. 14, 119, (1942).

142. Dische, Z., Methods in Carbohydrate Chem., 1, 488, (1962).

143. Dische, Z., Methods in Carbohydrate Chem., 1, 501, (1962).

144. Dische, Z., Methods in Carbohydrate Chem., 1, 487, (1962).

145. Dische, Z., Borenfreund, E., Biochim. Biophys. Acta, 23, 639, (1957).

146. Law, J.E., Ann. Rev. Biochem., 29, 131, (1960).

147. Carter, H.E., Johnson, P., Weber, E.J., Ann. Rev. Biochem., 34, 109, (1965).

148. Allen, C.F., Good, P., J. Amer. Oil Chem. Soc., 42,610, (1965).

149. Allen, C.F., Good, P., Davis, H.F., Fowler, S.C., Biochem. Biophys. Res. Commun., 15, 424, (1964).

150. Wintermans, J.F.G.M., Biochim. Biophys. Acta, 44, 49, (1960).

151. Ferrari, R.A., Benson, A.A., Arch. Biochem. Biophys., 93, 185, (1961).

152. O'Brien, J.S., Benson, A.A., J. Lipid Res., 5, 432, (1964).

153. Carter, H.E., Ohno, K., Nojima, S., Tipton, C.L., Stanacev, N.Z., J. Lipid Res., 2, 215, (1961).

154. Kates, M., Can. J. Botany, 35, 895, (1957).

155. Sastry, P.S., Kates, M., Biochim. Biophys. Acta, 70, 214, (1963).

156. Daniel, H., Lepage, M., Shibuya, I., Benson, A.A., Miyano, M., Mumma, R.O., Yagi, T., J. Amer. Chem. Soc., 83, 57, (1962).

157. Miyano, M., Benson, A.A., J. Amer. Chem. Soc., 84, 57, (1962).

158. Yagi, T., Benson, A.A., Biochim. Biophys. Acta, 57, 601, (1962).

159. Carter, H.E., Galanos, D.S., Hendrickson, H.S., Jann, B., Nakayama, T., Yakazawa, Y., Nichols, B., J. Amer. Oil Chem. Soc., 39, 107, (1962).

160. Carter, H.E., Hendry, R.A., Nojima, S., Stanacev, N.Z., Biochim. Biophys. Acta, 45, 402, (1960).

161. Carter, H.E., Hendry, R.A., Nojima, S., Stanacev, N.Z., Ohno, K., J. Biol. Chem., 236, 1912, (1961).

162. Sastry, P.S., Kates, M., Biochim. Biophys. Acta, 84, 231, (1964).

163. Matlock, M.B., J. Amer. Pharm. Assoc., 18, 24, (1929).

164. Wright, H.E., Burton, W.W., Berry, R.C., J. Org. Chem., 27, 918, (1962).

165. Swift, L.J., J. Amer. Chem. Soc., 74, 1099, (1952).

166. Aylward, F., Nichols, B.W.,Nature, 181, 1064, (1958).

167. Lepage, M., J. Lipid Res., 5, 587, (1964).

168. McKillican, M.E., J. Amer. Oil Chem. Soc., 41, 554, (1964).

169. Duncan, H.J., "Nucleotides and Related Compounds inPlants", Ph.D. Thesis, Glasgow, (1965).

170. Folch, J., Ascoli, I., Lees, M., Meath, J.A., LeBaron, F.N., J. Biol. Chem., 191, 833, (1951).

171. Hanahan, D.J., Lipid Chemistry, (J. Wiley & Sons, 1959).

172. Folch, J., Lees, M., Sloane-Stanley, G.H., Fed. Proc.,13, 209, (1954).

173. Folch, J., Lees, M., Sloane-Stanley, G.H., J. Biol. Chem., 226, 497, (1957).

174. Svennerholm, L., J. Neurochem., 1, 42, (1956).

175. Folch, J., Arsove, S., Meath, J.A., J. Biol. Chem., 191, 819, (1951).

176. Rouser, G., Bauman, A.J., Kritchevsky,G., Heller, D., O'Brien, J.S., J. Amer. Oil Chem. Soc., 38, 544, (1961).

177. Svennerholm, L., Nature, 177, 524, (1956).

178. Wells, M.A., Dittmer, J.C., Biochemistry, 2, 1259, (1963).

179. Siakotos, A.N., Rouser, G., J. Amer. Oil Chem. Soc., 42, 913, (1965).

180. Rouser, G., Kritchevsky, G., Galli, C., Heller, D., J. Amer. Oil Chem. Soc., 42, 215, (1965).

181. Rouser, G., Kritchevsky, G., Heller, D., Leiber, E., J. Amer. Oil Chem. Soc., 40, 425, (1963).

182. Rouser, G., Bauman, A.J., Nicolaides, N., Heller, D., J. Amer. Oil Chem. Soc., 38, 565, (1961).

183. Wren, J.J., J. Chromat., 4, 173, (1960).

184. Radin, N.S., Lavin, F.B., Brown, J.R., J. Biol. Chem., 217, 789, (1955).

185. Carroll, K.K., J. Lipid Res., 2, 135, (1961).

186. O'Brien, J.S., Benson, A.A., J. Lipid Res., 5, 432, (1964).

187. Rouser, G., Bauman, A.J., Kritchevsky, G., Amer. J. Clin. Nutr., 9, 112, (1961).

188. Svennerholm, L., Thorin, H., J. Lipid Res., 3, 483, (1962).

189. Lepage, M., J. Chromat., 13, 99, (1964).

190. Benson, A.A., Advances in Lipid Res., 1, 387, (1963).

191. Radin, N.S., J. Biol. Chem., 219, 977, (1956).

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

193. King, E.J., Biochem. J., 26, 292, (1932).

194. Sims, R.P.A., Larose, J.A.G., J.Amer. Oil Chem. Soc., 39, 232, (1962).

195. Feldman, G.L., Rouser, G., J. Amer. Oil Chem. Soc., 42, 290, (1965).

196. Bregoff, H.M., Roberts, E., Delwiche, C.C., J. Biol. Chem., 205, 565, (1953).

197. Baddiley, J., Buchanan, J., Handschumacher, R.E., Prescott, J.F., J. Chem. Soc., 2818, (1956).

198. Dittmer, J.C., Lester, R.L., J. Lipid Res., 5, 126, (1964).

199. Siakotos, A.N., Rouser, G., J. Amer. Oil Chem.Soc., 42, 913, (1965).

200. Pomeranz, Y., Okkyung, Chung, Robinson, R.J., J. Amer. Oil Chem. Soc., 43, 511, (1966).

201. Sweeley, C.C., Walker, B., Anal. Chem., 36, 1461, (1964).

202. James, A.T., Methods of Biochemical Analysis, VIII, 1, (1960).

203. Hirst, E.L., Endeavour, 10, 106, (1951).

204. Neukom, H., Kuendig, W., Methods in Carbohydrate Chem., V, 14, (1965).

205. Heri, W., Neukom, H., Deuel, H., Helv. Chem. Acta, 44, 1939, (1961).

206. Heri, W., Neukom, H., Deuel, H., Helv. Chem. Acta, 44, 1945, (1961).

207. Rees, W.R., personal communication.

208. Duncan, H.J., "Nucleotides and Related Compounds inPlants", Ph.D. Thesis, Glasgow, (1965).