THE COMPLEXING OF CALCIUM AND MAGNESIUM BY ORGANIC PLANT CONSTITUENTS

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ABSTRACT

The definition, occurrence and aetiology of hypomagnesaemic tetany is discussed as an introduction to the practical implications of the present investigation. The current hypotheses, involving an unfavourable pasture chemical composition, accounting for the binding of Ca^{++} and Mg^{++} in the intestinal tract of ruminants are reviewed. The role of undigested or partly-digested plant cell wall materials is advanced as another such hypothesis, and the present investigation of the cell wall polymers of a typical pasture grass is outlined.

The comprehensive analysis of the grass Yorkshire fog (Holcus lanatus) involves, initially, the extraction and purification of pectic substances, lignin, hemicelluloses and cellulose. The non-volatile organic acid content of the grass is also determined.

Analytical methods are developed and evaluated in order to assess the homogeneity of these isolated cell wall fractions, and their chemical constitution investigated to aid in determining any possible relationship between cation complexing and polymer (or monomer) structure.

The pectic fraction isolated from Yorkshire fog is approximately 90% polygalacturonic acid while the hemicelluloses are basically arabinoxylans with varying hexose and uronic acid content. Attempts to fractionate the predominant hemicellulose, hemicellulose B, into homogenous arabinoxylans gives inconclusive results. Most of the chemical evidence, however, indicates the presence of three discreet polysaccharides in this fraction - a simple arabinoxylan, an acidic galactoarabinoxylan and a neutral glucan.

Infrared and ultraviolet spectroscopy is utilised to

determine the purity of the isolated lignin as well as the presence of typical lignin functional groups. Yorkshire fog lignin has a moderate phenolic hydroxyl and -OMe content and, like most other monocotyledonous lignins, gives yields of syringaldehyde, vanillin and p-hydroxybenzaldehyde on alkaline nitrobenzene oxidation. The outstanding feature of the lignin is its appreciable content of etherified hydroxyl groups in the 4-position of the aromatic ring and the low yield of syringaldehyde.

The water-soluble, non-volatile organic acids are quantitatively determined by anion-exchange resin chromatography and their identity confirmed by paper chromatography. The major acid is the tricarboxylic acid, trans-aconitic acid, which is determined spectrophotometrically. The normal plant acids, citric and malic, are present in moderate quantities while the alicyclic acids, quinic anc shikimic, are only present in minor amounts.

A limited amount of data on the seasonal fluctuation of these organic fractions in Yorkshire fog is presented.

Electrolytes and the concept of ionic activity are discussed in the introduction to the study of the ability of these plant fractions to bind Ca^{++} and Mg^{++} in an aqueous salt solution of cationic composition similar to that of the intestine of a ruminant. A cation exchange method is developed whereby changes in the activity of Ca^{++} or Mg^{++} on the introduction of a plant fraction into the salt solution are reflected in the cationic composition of the equilibrium resin. An investigation is undertaken of a large number of calibration solutions varying in $[Ca^{++}]$ and $[Mg^{++}]$, but constant in $[Na^{+}]$, $[K^{+}]$ and $[NH_4^{+}]$, the latter cations being present in excess as 'swamping' cations.

Regression expressions relating solution cation concentration to the equilibrium resin cation concentrations are derived and used as calibration equations to determine the amounts of bound and ionic Ca^{++} and Mg^{++} in solutions in equilibrium with the plant fractions.

The pectic substances, lignin and the organic acids are effective in complexing a large proportion of the solution Ca in a non-ionic form but only lignin and the organic acids display a significant complexing of solution Mg^{++}. Except for hemicellulose B (branched) at a slightly alkaline pH, the hemicelluloses and cellulose have little ability to complex either Ca^{++} or Mg^{++}.

The complexing results are discussed in terms of the relationship of polymer structure to observed cation affinity. Factors involved in cation binding are:

- the charge and degree of hydration of the cation itself;
- distribution and degree of esterification of carboxyl groups in the polymer;
- monomer conformation;
- type of glycosidic linkage in the polymer;
- the possibility of hydrogen-bonding and non-bonded interactions between substituents on the polymers;
- solution pH.

The in vivo implications of the results are finally discussed in the context of general ruminant nutrition and alkaline-earth metal absorption discussed in the introduction.

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PART 1 HYPOMAGNESAEMIC TETANY AND ITS RELATIONSHIP TO THE CHEMISTRY OF DIETARY CALCIUM AND MAGNESIUM

1.1. DEFINITION, OCCURRENCE AND AETIOLOGY OF HYPOMAGNESAEMIC TETANY

Hypomagnesaemic tetany, or grass tetany, (commonly called 'grass staggers' in New Zealand) in ruminants is the clinical manifestation of a metabolic disorder characterised by an abnormally low level of magnesium in the blood serum (hypomagnesaemia). The disease is one of a group of metabolic diseases that includes milk fever and ketosis and is most prevalent in temperate countries having wet cool climates in the range 40^{o} - 60^{o}F (t'Hart, 1960). The ubiquitous factors involved in the grass tetany syndrome have been extensively researched in the past 40 years and while most workers seem to agree that the disorder is primarily of nutritional origin, it also seems that a variety of physiological and environmental factors are implicated (Allcroft and Burns, 1968). Typically, however, outbreaks in Europe are associated with a change from stall to fresh grass feeding while in New Zealand a change in the chemical nature of the feed is also implicated since the severest outbreaks occur when there is an early spring flush of feed, combined with mild weather (Metson, Saunders, Collie and Graham, 1966). Various workers have put the incidence of loss through clinical tetany at 1 - 3% of their national herd population but these figures gloss over the marked localised nature of the outbreaks as well as the unknown economic losses through associated diminished production. Internationally, the increasing preponderance of grass tetany and related metabolic diseases in cattle can be considered a function of more intensive husbandry methods and the necessity to increase agricultural production.

Hypomagnesaemia Ever since Sjollema (1930) showed that cows suffering from grass tetany were in a hypomagnesaemic state most research into the problem has centred on the cause(s) of this hypomagnesaemia. It is not the function of this introduction to review the vast amount of often conflicting literature on the aetiology of this hypomagnesaemia but the salient points deserve discussion since they are pertinent to the study of the binding of calcium and magnesium carried out in this present investigation.

Early investigators apparently held two dissimilar views (Allcroft and Burns, 1968):

(1) that the disease was the result of a physiological dysfunction in which endochrine and environmental factors were paramount; magnesium content of the herbage was of little importance.

(2) that the disease was related to dietary factors associated with changes in the chemical composition of pastures.

Most research into the hypomagnesaemia problem has been concerned with possible limitations to the flow of magnesium and calcium from the soil through the plant to the animal; consequently, the concept of the 'availability' of Mg and Ca in the feed has arisen (section 1.3.). Today it is generally agreed that a 'low' (if indeed such an arbitrary term has meaning without reference to a standard value) intake of magnesium is not the only factor contributing to a hypomagnesaemic state in the animal. A variety of other factors must all be considered as part of the syndrome:

- low availability or reduced absorptive efficiency of dietary magnesium
- the chemical composition of the herbage
- the requirements for and metabolism of magnesium in the particular animal
- the age and genetic makeup of the animal

- a variety of hypothetical 'stress' factors whether they be endochrine (pregnancy and lactation) or environmental (weather, management patterns).

In particular, Swan and Jamieson (1956) have shown that hypomagnesaemia is a necessary, although not a sufficient pre-requisite for grass tetany and there is a considerable body of evidence indicating that clinical outbreaks may depend on a number of 'triggering' stress factors. Allcroft and Burns (1968) recognise a variety of hypomagnesaemic states which may give rise to varying degrees of tetany, the most common being hypomagnesaemia accompanied by hypocalcaemia. Furthermore, the speed with which blood magnesium falls (Allcroft and Green, 1938; Rook, 1963) appears to be an important factor in determining the onset of tetanic convulsions.

Magnesium is lost from the body of a ruminant by three main routes - in the milk, urine and faeces - and there is considerable empirical evidence indicating that with increasing age, the animal is less able to mobilise magnesium reserves to allay physiological stresses or dietary scarcity. Consequently, an adequate daily magnesium intake is necessary, especially during lactation, to maintain the animal in a positive magnesium balance. Hypomagnesaemia and clinical cases of grass tetany have been produced by simply underfeeding cows (Swan and Jamieson, 1956) or by feeding a magnesium deficient diet (Blaxter, Rook and MacDonald, 1954; Dishington and Tollersrud, 1967). However, most pastures contain an '<u>absolute</u>' level of Mg which is more than adequate for the animals' needs and the problem has devolved into elucidating the factors which are impairing the absorption of these elements from the digestive tract of the animal.

1.2. ABSORPTION OF CALCIUM AND MAGNESIUM FROM THE DIGESTIVE TRACT OF RUMINANTS

Before discussing nutritional factors which may be involved in reducing the availability of calcium and magnesium in the feed, it is pertinent to briefly review the literature on the site and mode of absorption of these elements in the ruminant. The selective absorption of particular ions from the digesta would require cell membranes endowed with properties of specific ionic permeabilities. This permeability may depend upon the relative sizes of the ions and the pores in the membrane; such a theory, assuming the pore wall to form part of the ion hydration sphere during passage through the membrane, has been advanced by Mullins (1961). On the other hand, however, a reasonable amount of evidence suggests that some ionic transport mechanisms may require a specific chemical interaction of the ion with specialised components of the membrane.

Two general types of transport mechanisms exhibiting such specificity are 'active' and 'passive' (facilitated diffusion) transport. Although these have certain common characteristics, they can be distinguished on the grounds that active transport involves a net transfer against an electrochemical gradient (and is therefore an energy-requiring process linked with metabolism) while passive transport depends upon such a gradient and is not an energy-requiring process. Such an increase in electrochemical gradient across a membrane can be considered as an increase in the entrophy of the system.

One concept which is particularly applicable to the transportation of alkaline earth metals is that of chelation (Rubin, 1963). The reaction of such hydrated metal ions with hydrated ligands involves the displacement of water molecules from the

co-ordination spheres of both ions and a simultaneous neutralisation of charge. The favourable decrease in free energy of this reaction accounts for the stability of these ring-structures which tend to increase their chelation strength with an increase in ionic charge on the metal and ligand and a decrease in the crystallographic ionic radius (r_{cryst}) of the metal ions (Martell and Calvin, 1952). Hence, alkaline-earth metal chelation strength would be expected to decrease in the order Mg> Ca> Sr> Ba. However, this order is also that of decreasing ionic hydration and, consequently, chelation and hydration can be considered as competing processes since they both increase with an increase in the ratio e^2/r_{cryst} (see Table 13) since the chelating ligand displaces water molecules from the ion hydration sphere according to the following general equation:

$$M(H_2O)_x + \frac{x}{n}L \quad — \quad M(H_2O)_x - \frac{x}{n}(L)\frac{x}{n} + (H_2O)\frac{x}{n}$$

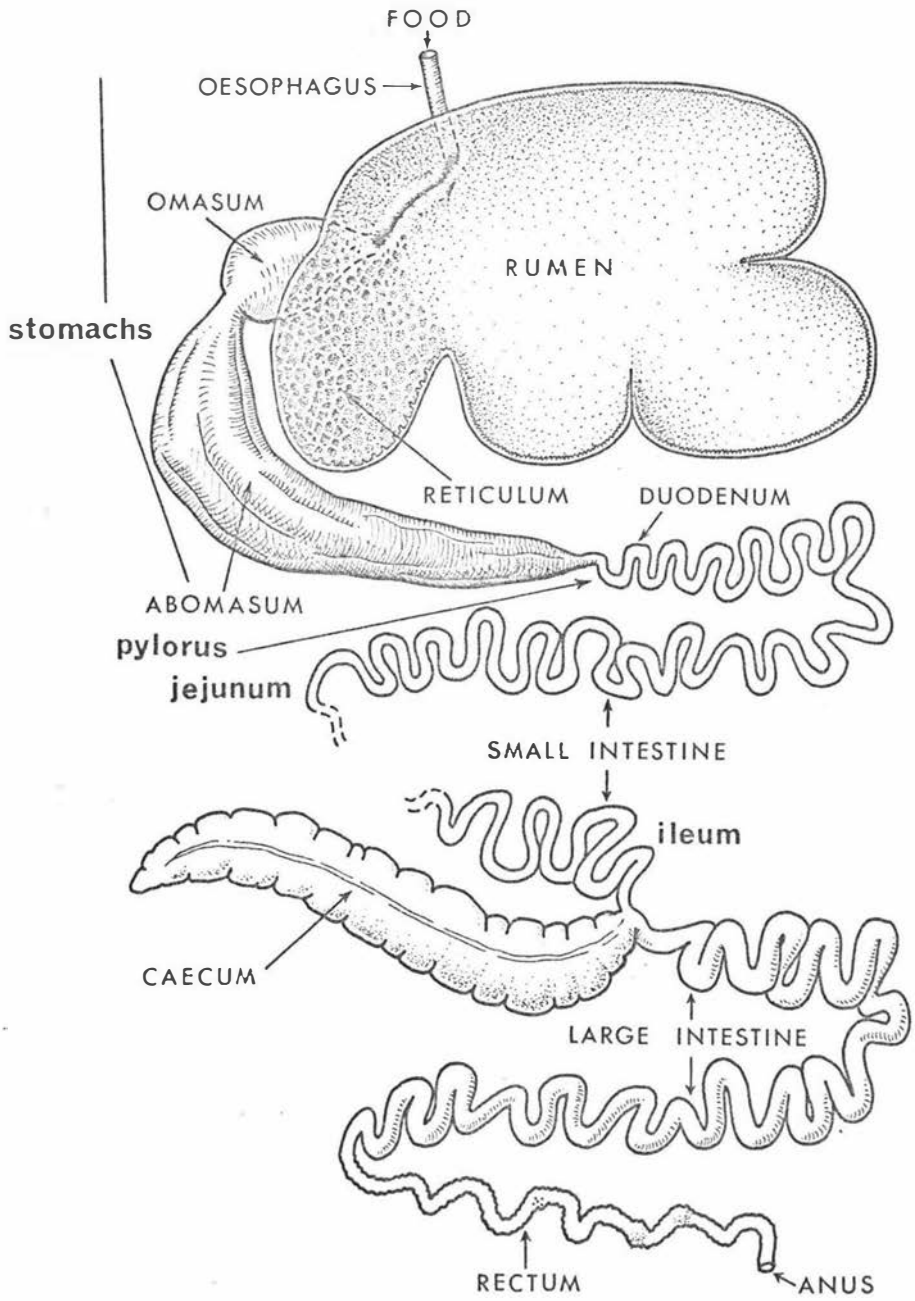
(where M and L are the metal and ligand species, respectively, and ionic charges are ignored; x and n are integers and n is the number of donor groups per molecule of ligand).

In aqueous solutions, such as intestinal fluid, such metal ions will be hydrated and the ratio e^2/r_{hyd} is likely to be a better determinant of the degree of chelation, particularly for the alkali and alkaline-earth metals where chelate bonding is primarily ionic. Thus an organic ligand and a cation exchange resin are similar in their cation sequestering function and, hence, such cations in a physiological electrolyte solution (ignoring all other metabolic variables) would be expected to be preferentially chelated in the same selectivity order as that for a cation exchange resin, ie., with an increase in the ratio e^2/r_{hyd}, to give a decrease in chelation strength of Ba> Sr> Ca> Mg (see section 3.2.).

The decrease in net charge on the metal chelate and the simul-

taneous loss of water of hydration tends to convert a highly hydrophilic metal to a more lipophilic structure. Cell membranes, including the assymetric system of an epithelial membrane such as the intestinal wall, appear to have a general backbone system made up of a bimolecular leaflet of lipid with associated layers of protein (Curran, 1963). Most transport, however, seems to be associated with specialised regions of the membrane which occupy only a very small fraction of the total area. It is possible, therefore, that the decreased charge, decreased hydration and increased lipotrophic character of some alkaline earth chelates may permit the metal ion in this form to approach the charged membrane and become available for transport. Alternatively, if the membrane is the site of the alkaline earth-binding group, such chelation may permit the passage of a charged ion through the lipid barrier.

Most authors agree that calcium and magnesium absorption in adult ruminants occurs mainly in the small intestine, with little or no net absorption from the stomachs of large intestines (see diagram). A common transport system for the absorption of magnesium and calcium by facilitated diffusion from the ileum of sheep was postulated by Care and van't Klooster (1965), whereas calcium absorption from the duodenum is considered to be an active process which is sensitive to vitamin D (Care, 1967). The role of the jejunum and ileum as the major sites of net calcium absorption in cows has been confirmed also by van't Klooster (1967) and Rogers and van't Klooster (1969). The latter authors, however, made the startling finding that net Mg absorption occurred mainly from the stomachs with little or no absorption between the pylorus and the anus. While this finding was recognised by these authors as being contrary to the large body of evidence from esperiments based on perfusion of intestinal loops <u>in vivo</u>, it does seem that their fistulation technique may give more accurate results. Therefore,



SCHEMATIC REPRESENTATION OF THE DIGESTIVE ORGANS OF A RUMINANT (kindly reproduced, with amendments, from Davey, A. W. F., 'Dairy cattle nutrition.' Dairyfarming Annual, 1970, 32-38.)

they suggest that the omasum may be an organ of considerable importance in ruminant Mg absorption and that such absorption is very unlikely to be a passive process.

The physico-chemical activity of calcium and magnesium in the small intestine of a ruminant The findings of Rogers and van't Klooster (1969) regarding Mg absorption will doubtless stimulate considerable research into the physico-chemical status of Mg in the ruminant stomachs; at present there is nothing in the literature of this nature. Since, hitherto, Ca and Mg were considered to be absorbed from the intestinal tract, the few investigations carried out concerned the state of these elements in this organ only. The duodenal absorption of Ca by active transport is considered to depend upon the ionic concentration of the ion in the digesta (Schachter, Dowdle and Schenker, 1960; Lengeman, 1959). It is not known in which form magnesium must be to be absorbed but it seems a reasonable assumption that, like calcium, it must be in an ionic form to pass into the blood through the intestinal villi. The only significant investigation of the physico-chemical activity of calcium and magnesium in ruminant intestinal digesta is that of van't Klooster (1967). By employing an ion exchange resin method, he concluded that the activity (see discussion of 'activity', section 3.1.2.) of both ions in duodenal ultrafiltrates was about 90% of the activity of these elements in pure salt solutions with the same concentrations of these cations. In ileal ultrafiltrates, however, these percentages had dropped to 63% and 78% respectively. These differences between duodenal and ileal ultrafiltrates could be partly explained by large pH differences (duodenal, 4.7; ileal, 8.2) and the proportions of anions in these fluids. However, van't Klooster did not exclude the possibility that a small part of the calcium in the ileal ultrafiltrates was in a chemically bound form. Furthermore, he considered that these results supported the hypothesis that

calcium and magnesium are absorbed in the ionic state. The importance of the availability of these elements in the feed of the animals is highlighted by his finding, for both sheep and cows, that the Mg concentration in the duodenal contents was so low on a diet consisting of grass only that hardly any net absorption of Mg could be expected.

1.3. UNFAVOURABLE PASTURE CHEMICAL COMPOSITION HYPOTHESES

The overwhelming body of circumstantial evidence implicating the chemical composition of fresh grass in the grass tetany syndrome has naturally enough given rise to a proliferation of hypotheses which consider hypomagnesaemia to be brought about by:

(a) a deficiency of Mg in the grass or,

(b) some plant chemical factor which acts to reduce the availability of the plant Mg to the animal.

The difficulty of deciding exactly what level constitutes a 'low' level of Mg in the plant has already been discussed in section 1.1. and the agronomic problem of increasing the Mg content of the plant - which is undoubtedly correlated with an increased Mg intake in the animal (Lomba, Paquay, Bienfet and Lousse, 1968) - is considered outside the scope of this discussion. Suffice to point out that a large amount of European work has stemmed from the classical survey of dairy herds in Holland by Kemp (1960) who demonstrated a significant positive correlation between the serum Mg levels of lactating cows and the magnesium content of the herbage; as a consequence, a variety of workers have derived arbitrary 'safe' herbage Mg levels and almost as many investigators have shown that this 'safety margin' is still subject to a number of other variables in the pasture, the animal and the quality of management. This whole field of adequate mineral nutrition, including herbage Mg, Ca, K, Na levels, Ca/P and K/Ca + Mg ratios, and specific competitive cationic relationships, is very important but also very comp-

licated owing to conflicting findings and is best reviewed by Metson, Saunders, Collie and Graham (1966) and Allcroft and Burns (1968).

1.3.1. Protein/carbohydrate imbalance It has been proposed that there is a relative imbalance between protein and carbohydrate in tetany-producing grasses, protein levels being high, carbohydrate levels low (Dishington, 1965; Metson, Saunders, Collie and Graham, 1966). Most New Zealand pastures which give rise to outbreaks of grass tetany have high 'crude protein' (N% x 6.25) and potassium levels (up to 39% and 4% dry weight respectively, Metson, et al, 1966). Such high levels of nitrogen are characteristic of rapidly-growing spring pastures and were early implicated in the grass tetany syndrome in Europe (Sjollema, 1930). Cows ingesting such high protein pasture have been estimated (Dishington, 1965) to consume three times as much protein as they need in order to fulfill their energy requirements.

A direct effect of dietary nitrogen on 'Mg availability' has been shown by Kemp, Deijs, Hemkes and van Es (1961) who found that such availability decreased markedly with an increase in the crude protein content of the feed. By extrapolating their data, Metson et al (1966) found that the availability of Mg in typical New Zealand 'tetany' pastures may be as low as 5 - 10%. Such high levels of nitrogenous compounds (protein and non-protein) are rapidly metabolised to ammonia in the rumen and Head and Rook (1955) attributed the incidence of hypomagnesaemia in ruminants turned out to graze such new spring grass to these high levels of rumen ammonia. Later, the same authors (Head and Rook, 1957) associated high rumen ammonia levels with a marked decrease in the concentration of ultrafilterable Mg in the small intestinal digesta of sheep. Simesen (1959) suggested that this ammonia could contribute to the precipitation of Mg in the intestinal tract as the magnesium ammon-

ium phosphate complex; such a process would be aided by the increase in pH due to the ammonia present. However, Simesen (1963) was later unable to demonstrate any significant changes in either ruminal or abomasal pH of a cow fed on such high-protein grass despite increases in ammonia concentrations in both ruminal and abomasal fluids. Neither he, nor Storry (1961), found the magnesium ammonium phosphate complex to be present in the ruminant intestine. On the basis of studies on the digesta of both the duodenum and mid-ileum of sheep, Care (1965) has also refuted the hypothesis that grass-induced hypomagnesaemia is related to a reduction in the acidity of the digesta.

The 'non-protein' nitrogen of 'tetany' pastures seems to be maintained at a fairly constant level of 16 - 22% of the total nitrogen (Metson et al, 1966) and this 'soluble' nitrogen has been found to be highly correlated with low values of serum Mg (Larvor and Guegen, 1963). Although this non-protein nitrogen is probably quickly utilised for the production of ammonia in the rumen, this correlation probably only parallels that for total nitrogen - which was not investigated by these workers.

While high pasture nitrogen levels certainly seem to be implicated in the onset of hypomagnesaemia, the causative mechanism is not clear. It could well be a simple energetic shortcoming through low carbohydrate levels as a consequence of high pasture protein content. However, the role of low pH in maintaining Ca and Mg in an ultrafilterable form during the passage of the digesta through the abomasum and duodenum has been clearly demonstrated by Storry (1961). Yet the role of pasture carbohydrate/protein balance in maintaining favourable pH levels is far from proven and such high protein/hypomagnesaemia correlations may be falsely paralleling a number of other relationships which represent the causative mechanisms, eg, that with pasture higher fatty acid content.

1.3.2. Higher fatty acids (HFA) The importance of fat in the magnesium balance of cattle was initially reported by Brouwer, Dijkstra and Frens (1943), and a linear relationship between pasture higher fatty acids and nitrogen was later found by Brouwer (1944). Subsequently, many Dutch workers have investigated the role of pasture higher fatty acids in the production of hypomagnesaemia in cattle. The common C_{18} unsaturated fatty acids (oleic, linoleic and linolenic) in pasture lipids are mainly hydrogenated to stearic acid in the rumen and these saturated, long-chain acids do not appear to be absorbed, nor metabolised by rumen micro-organisms, during their passage from the rumen through the true stomachs to the small intestine which is the predominant site of their absorption (Garton, 1967). Consequently, the finding of Rogers and van't Klooster (1969) that the omasum is the site of Mg absorption from the alimentary tract assumes a new significance since there will probably be quite a high concentration of unabsorbed long-chain fatty acids capable of forming insoluble Ca and Mg soaps in the digesta in this organ. An increase in pH with passage along the alimentary tract from the omasum to the small intestine would be expected to enhance the formation of such soaps, yet Storry (1961) obtained a considerable decrease in ultrafilterable Ca and Mg upon indreasing the pH of unfractionated abomasal digesta but found no concomitant fall in the concentration of free fatty acids.

It has been shown that New Zealand spring pastures contain high levels of HFA (Hawke, 1963); close correlation has also been observed between herbage levels of HFA and protein nitrogen (Immink, Geurink and Deijs, 1965; Kemp, Deijs and Kluvers, 1966), a relationship also confirmed for New Zealand pastures by Molloy and Metson (unpublished work). Furthermore, the addition of fat supplements to the diet of cattle has been shown to result in the increased excretion of calcium and magnesium soaps plus a slight depression

of serum Mg levels (Wind, Deijs and Kemp, 1966). A recent large scale statistical analysis of the relationship between the Mg status of 162 cows and 75 nutritive factors in their 55 different experimental diets has convincingly highlighted this relationship between the nitrogen and the 'fat' content of the feed (Lomba, et al, 1968). A highly significant correlation between faecal magnesium and nitrogen intake and, to a lesser extent, with fat intake was found.

Most of the investigations outlined above were carried out with only a very limited number of animals, and with little or no attempt at eliminating genetic variations; a more significant series of trials were performed by Wilson, Reid, Molloy, Metson and Butler (1969) with a twinned herd of 24 animals grazing a 'tetany-prone' pasture in three groups of eight, with the following supplementation:

(a) control - no supplementation

(b) starch (= energy)

(c) peanut oil (= HFA)

Marked depression of plasma Mg was obtained for the peanut oil supplementation although no clinical cases of tetany occurred, possibly because plasma Ca levels remained within the normal physiological range. Faecal levels of HFA and FA soaps closely correlated with depression of plasma Mg levels in mature cows, but were less closely correlated in younger animals which were able to markedly increase their concentration of faecal fatty acid soaps (and free fatty acids). These results certainly give strong support to the HFA hypothesis but these investigators considered that if a relationship does exist between dietary HFA intake and plasma Mg concentration, then it is not a simple one.

1.3.3. Organic acids Organic acids (water soluble, carboxylic) were implicated in the grass tetany syndrome with the finding (Burau and Stout, 1965) of large accumulations of the tricarboxylic acid, trans-aconitic acid, in the spring range grasses of central Calif-

ornia which give rise to seasonal outbreaks of tetany. They hypothesised that large concentrations of trans-aconitic acid in the pastures may inhibit the Krebs cycle conversion of citrate to iso-citrate in the animal (see 2.6.2.) thereby leading to an accumulation of citrate in the tissues and a consequent chelation of calcium and magnesium (Burt and Thomas, 1961). Subsequently, Bohman, Lesperance, Harding and Grunes (1969) were able to experimentally induce a tetany which resembled field cases by force-feeding cattle very large doses of KCl plus either citric or trans-aconitic acid.

However, considerable doubts about the role of trans-aconitate inthe hypomagnesaemia syndrome were raised by the sheep-feeding experiments of Kennedy (1968). Under the conditions of his experiment, little or no effect on plasma calcium and magnesium was obtained from the addition of relatively large supplements (3.5 and 7.0% of the dry weight) of trans-aconitate to the diet, or the intravenous injection of sodium trans-aconitate at 1.0 m.mole/kg bodyweight. A similar negative result was obtained with single doses of trans-aconitate at 10% of the daily dry matter intake for sheep (Wright and Wolff, 1969).

The role of KCl in precipitating the hypomagnesaemic condition was not delineated by Bohman et al (1969), who found that the separate administration of the two acids or KCl did not produce tetany. Although they found that the administration of KCl alone over an extended period only slightly reduced plasma Mg, there is ample evidence that such massive doses of KCl (up to 194 gm per 100 kg body weight) are sufficient to kill the animal from potassium poisoning alone (Ward, 1966). The primary role of the KCl in the mixture is further substantiated by findings of Lomba, Fumiere, Chauvaux, Binot and Bienfet (1969) who introduced mixtures of citric acid, trans-aconitic acid and KCl into the rumen of cows. In spite of important modifications of pH, Ca, Mg and K in the rumen,

they found no modification of blood Ca and Mg while blood K reached such values that they considered the death of one cow to be due to potassium poisoning.

The massive doses of these acids and electrolytes fed to the cattle in the series of American feeding trials are obviously a very gross approximation to the gradual intake of acid which an animal receives under normal field conditions. Furthermore, there is no mechanistic reason why other plant acids may not be involved, and in this respect it is interesting to note that oxalic acid has been found to be far more toxic than citric or trans-aconitic acid (Lomba, Chaubaux, and Bienfet, 1968). Moderate doses of these metabolites seem to be utilised fairly rapidly by rumen micro-organisms and this would be expected under normal field conditions (see Discussion, Section 4).

The conclusion drawn from the above work is that while pasture organic acids may be of minor importance in contributing towards hypomagnesaemia under conditions of marginal Mg supply, they are very unlikely to be the prime cause of grass tetany.

1.3.4. Inorganic anions Since electroneutrality must be maintained within the plant, the inorganic anion content is related to the cation and organic acid content by the following relationship:

$$\sum \text{cations} = \sum \text{organic anions} + \sum \text{inorganic anions} + \sum \text{polymeric anionic groups (uronides)}$$

all expressed in milliequivalents (see definition, section 3.2.). The major inorganic anions in the pasture plant are Cl^-, SO_4^{--}, $H_2PO_4^-$ and NO_3^-, and there is a large amount of work relating these anion levels to different forms of fertilisation and the nature and concentration of the organic anions; however, comparatively little is known of the role of these inorganic anions in regulating Ca and Mg absorption.

Over a number of years Norwegian workers (Dishington, 1965;

Dishington and Tollersrud, 1967) have considered sulphate and phosphate contents of pasture as factors contributing to outbreaks of grass tetany. A more convincing case for the binding of Ca and Mg by inorganic phosphate in the ileal contents of ruminating calves was given by Smith and McAllan (1966). Above a pH of 6.5 they found Ca and Mg precipitation to depend upon the concentration of inorganic phosphate; Ca precipitation appeared to be solely dependent upon ileal Ca and inorganic phosphate content while Mg precipitation seemed to depend upon a variety of other factors (such as ammonia) besides Mg and inorganic phosphate concentration.

1.3.5. Histamine This hypothesis proposes critical pasture histamine levels as tetany 'triggering'agents in animals already suffering from hypomagnesaemia (Fowler, 1963; O'Sullivan, 1968). These Irish workers consider that histamine acts as a 'sensitiser to potassium' and thereby further contributes to the action of potassium in increasing 'neuromuscular excitability' (tetany). They claim a relationship between climatic factors and pasture histamine levels and further speculate how this histamine precipitates outbreaks of tetany. This hypothesis is far from proven when it is based on such speculative evidence; histamine has been fed to adult sheep without any adverse effect (McDonald, MacPherson and Watt, 1963) and a variety of other chemicals including guanidine, adenosine, uracil, urea and thiocyanate can act as sensitisers towards potassium.

1.3.6. Conclusions These five general hypotheses accounting for the decreased availability of Ca and Mg to animals feeding on fresh grass have stemmed from a wide variety of experimental situations, and it certainly would be an oversimplification to expect a direct relationship between decreased Ca and Mg availability and any one particular plant factor. The significance of any possible Mg- or Ca-complexing agent is unlikely to be determined solely by its concentration in the feed; rather, its importance would be expected to

depend upon the absolute level of Mg and Ca in the herbage and a variety of factors related to:

- the rate of digestion of the complexing agent itself,
- its site of digestion (and/or absorption) relative to Ca and Mg,
- the pH of the digestive tract,
- the concentrations of all other ions in the digesta and their effect upon the chemical and physiological potency of the complexing agent.

Again, there is no reason why all these 'binding hypotheses' may not be acting in concert at any particular time to bring about hypomagnesaemia or hypocalcaemia or, if this is already present (eg. by simply underfeeding), to precipitate tetany.

At the risk of stressing the obvious, it is necessary to accept that these hypotheses are merely attempts to account for the variables inherent in the second of the auxiliary criteria (ie., 'the chemical composition of the herbage', bottom, p.2) manifest in the complex hypomagnesaemia syndrome.

1.4. OUTLINE OF PRESENT INVESTIGATION

There is a limited amount of evidence that undigested plant cell wall components may be involved in the binding of Ca and Mg in the digesta of ruminants. In an investigation of the effect upon Ca and Mg distribution of a reduction of the acidity of abomasal digesta in sheep, Storry (1961) found that considerable quantities of these ions were bound to 'suspended material'. While he did not elucidate which components in the complex and heterogeneous suspended material were resposible, he did consider that the binding was due to surface absorption phenomena.

The Ca, Mg and phosphate in the non-ultrafilterable fraction of cow faeces were found by van't Klooster (1967) to be almost completely present as insoluble compounds. From his experiments, he

calculated that 25 - 30% of the Ca and Mg in the faeces could be absorbed to the undigested (organic) feed residues, especially to the fibrous particles.

Consequently, it seemed worthwhile to undertake an investigation of organic compounds in a typical pasture grass and their ability to bind Ca and Mg in an in vitro situation approximating that in the ruminant digestive tract. The grass chosen was Yorkshire fog since it is widespread in wetter New Zealand pastures and has never been subjected to chemical investigation although it has gained an undesirable nutritional reputation. The project fell into two natural categories:

1. The isolation of these plant organic fractions and their chemical and structural analysis. While cell wall polymers were considered of prime interest because of their known resistance to digestion, it was considered important to compare these with the water-soluble organic acids in the plant.
2. The ability of these isolated fractions to bind Ca^{++} and Mg^{++} in solutions of ionic composition approximating that of the intestinal tract of a ruminant. If appreciable complexing were to occur then it would be of interest to see if binding ability of a particular fraction was related to its structure.

It must be stressed that the project was not undertaken in an attempt to extrapolate these findings to the in vivo situation. Rather, it was considered a simple chemical approach to a particular problem of universal biological and chemical significance - not just to intestinal absorption in ruminants.

PART II THE COMPREHENSIVE ANALYSIS OF THE ORGANIC FRACTIONS OF YORKSHIRE FOG

2.1. INTRODUCTION

The perennial grass Yorkshire fog is widely represented in pastures of diverse type in New Zealand, especially in plant communities of the more humid and less fertile regions. Ecotypic development of plants from seed sources in Europe has ensured that New Zealand has in fact become a new centre of diversity of this species (Jacques, 1962) with favoured habitats being the wetter, warmer regions of the North Island, especially Manawatu, Taranaki and Waikato. Metson et al (1966) have noted the preponderance of Yorkshire fog in many North Island pastures which have given rise to outbreaks of grass tetany in beef cattle.

The plant material investigated was a synthetic strain of Yorkshire fog, Holcus lanatus, 'Massey Basyn', developed by the Agronomy Department of Massey University. Samples were obtained at six-monthly intervals from grazed plots (six ewes/acre) at Tuapeka field station (soil type: Tokomaru Silt Loam).

2.2. DRYING AND STORAGE OF GRASS

The sampling and preservation of plant material in a form suitable for analysis is a problem of considerable importance in herbage analysis. The ideal method must rapidly arrest chemical change while preserving all metabolites intact. Forced air drying (21°C) causes extensive respiratory losses of carbohydrates (Melvin and Simpson, 1963) and non-volatile organic acids (Melvin, 1965), although losses are smaller with a forced hot air draught at 70°C (Hirst and Ramstad, 1957). Freeze-drying was proposed by Davies, Evans and Evans (1948) as a more suitable drying method and has been found to be less damaging than oven-drying (Bathurst and Allison, 1949; Raguse and Smith,

1965). While this method avoids the deleterious effects of high temperatures it should be realised that the freeze-drying process does not inactivate all the enzymes originally present in the tissue and probably preserves micro-organisms intact on the leaf surface.

The erratic changes in carbohydrates and amino-acids during the storage of freeze-dried herbage has been pointed out by Perkins (1961). Since some enzymes can function in the presence of the residual 5 - 10 percent of water in freeze-dried tissues it is advisable to analyse samples for readily respired constituents, such as soluble carbohydrates, amino acids and organic acids, as soon as possible. With precautions such as storage under nitrogen, at low relative humidity and at temperatures as low as -20°C it is possible to minimise these chemical changes.

Little information is available upon the effect of freeze-drying on the composition of structural carbohydrates in herbage tissues. However, Czerkawski (1967) found no difference in the cellulose and lignin contents of grasses dried at 50°C and 100°C but noted a considerable increase in these constituents when samples were stored at relative humidity as high as 80%.

The conclusion of most investigators of this problem is that no universal method of drying plant tissue can be relied on for consistent results, since the appropriate drying temperature depends upon the chemical and physical composition and enzyme content of the plant.

In this present investigation, freeze-drying was used, primarily to preserve the non-volatile organic acids intact; extractions and analyses were then carried out within two weeks of drying. Samples of Yorkshire fog (3" - 5") were collected from the plots with hand-shears and immediately frozen in solid

carbon dioxide prior to freeze-drying. Any dead leaves were removed as the frozen leaves were being spread on the freeze-dryer tray. The dry plant material was then ground in a Wiley mill to pass a 1mm sieve and stored under vacuum with silica gel at -5°C.

2.3. EXTRACTION AND PURIFICATION PROCEDURE

The extraction scheme for the fractionation of polysaccharides and lignin from the dry grass is outlined in Fig. 1. This exhaustive procedure, using mild extractants, was used to isolate the structural polymers with the minimum of modification or degradation. Furthermore, the scheme gave a comprehensive chemical analysis of the grass. (See Table 9). In order to avoid oxidation and the onset of 'horniness', the residues after each extraction step were not air-dried, except for the initial benzene/ethanol extract. The loss in weight with each extraction step was determined separately on 10g of the dry grass with each residue being dried overnight in an air drying cabinet at 55°C.

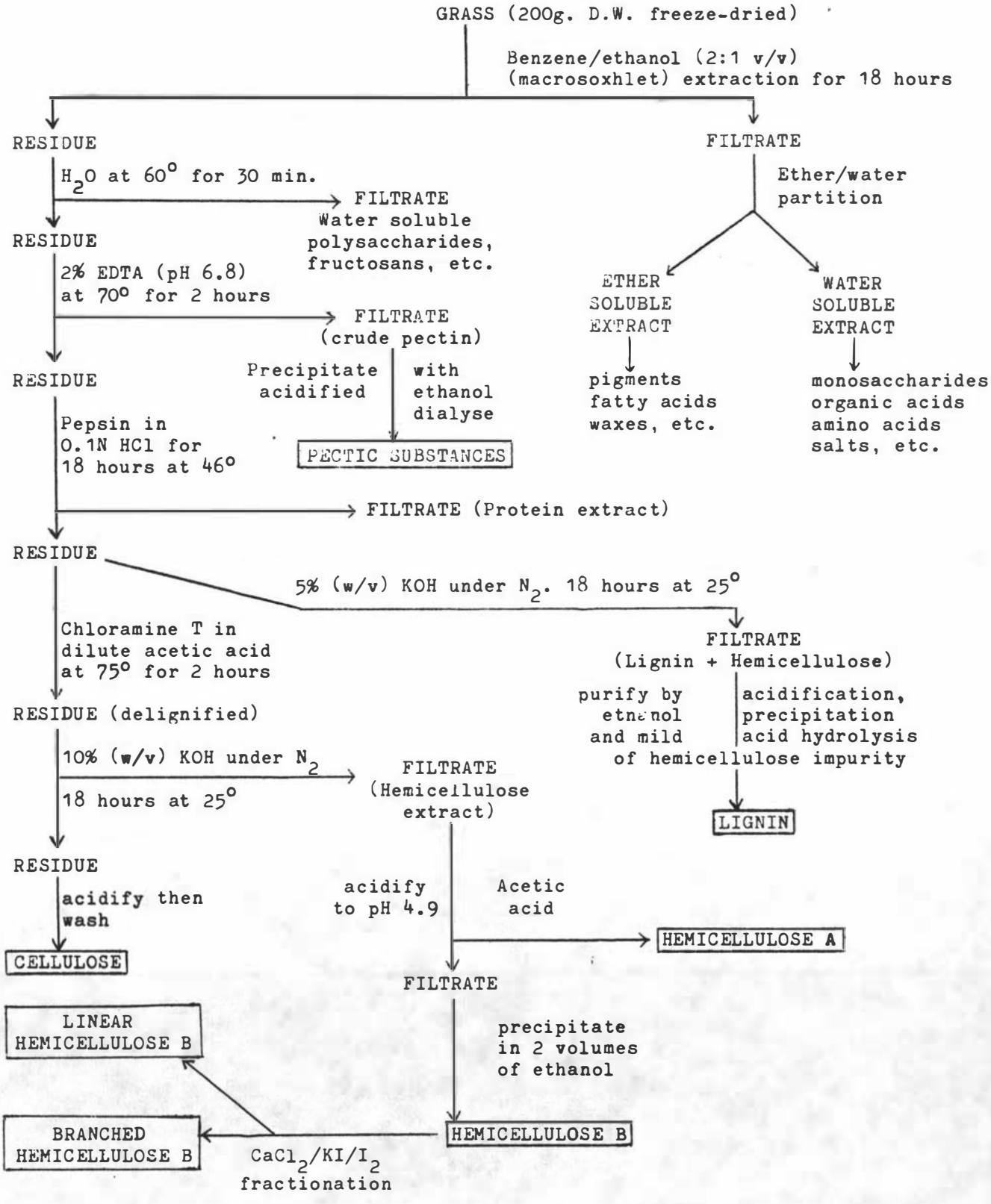
2.3.1. Preliminary extractions

The freeze-dried plant material (200g) was extracted in a macrosoxhlet apparatus with an azeotropic mixture of benzene/ethanol (2:1 v/v, 4 litres) for 18 hours. The residue was air dried and then extracted with water (2 x 1.5 litres) at 60°C for 30 minutes to remove water soluble polysaccharides. An aliquot of the filtrate (250 ml) was taken to dryness in a rotary evaporator (temperature below 40°C) and then partitioned between ether and water in a liquid/liquid extractor for 18 hours. The aqueous portion was combined with an aliquot of the 60°C water filtrate and the 'combined water extract' analysed (section 2.5. and Table 10).

2.3.2. Pectic substances

The classical ammonium oxalate (Weihe and Phillips, 1947)

FIG. 1 Scheme for Fractionation of Polysaccharides in Grass



extractant for pectic substances has been known to cause ammonia contamination of the residue (Whistler and Smart, 1953) although thorough washing with hot water (Waite and Gorrod, 1959 b) avoids this retention. Solutions of oxalic acid, ammonium citrate, fluorides, arsenates and phosphates have been employed as extractants for pectic substances but the extractant used in this investigation was a 2% (w/v) solution of E.D.T.A. (Na salt) at pH 6.8 (Aspinall and McGrath, 1966). The residue was extracted (25 cm^3/g) three times for two hours at 70°C and the combined filtrates concentrated by rotary evaporation to a suitable volume. This crude pectin extract was dialysed overnight against tap water to remove the bulk of the impurities and then poured into one volume of 95% ethanol. The precipitate of impure pectin was collected on a nylon gauze filter and dissolved in hot water (70°C) to give an approximately 1% solution. The pale brown solution was cooled, brought to pH 1.5 by the addition of a few drops of c. HCl and then dialysed overnight in distilled water (dialysis against tap water caused flocculation of the pectin, probably through interference from Ca^{++}). The dialysate was checked for chloride content (silver nitrate solution) and EDTA-nitrogen (micro-Kjeldahl) before the pure pectin was precipitated in two volumes of 95% ethanol. The filtered 'pectin' was soaked overnight in un-acidified absolute ethanol, dispersed in deionised water and freeze-dried. The product was a very light porous, white polymer which was stored under vacuum.

2.3.3. Lignin

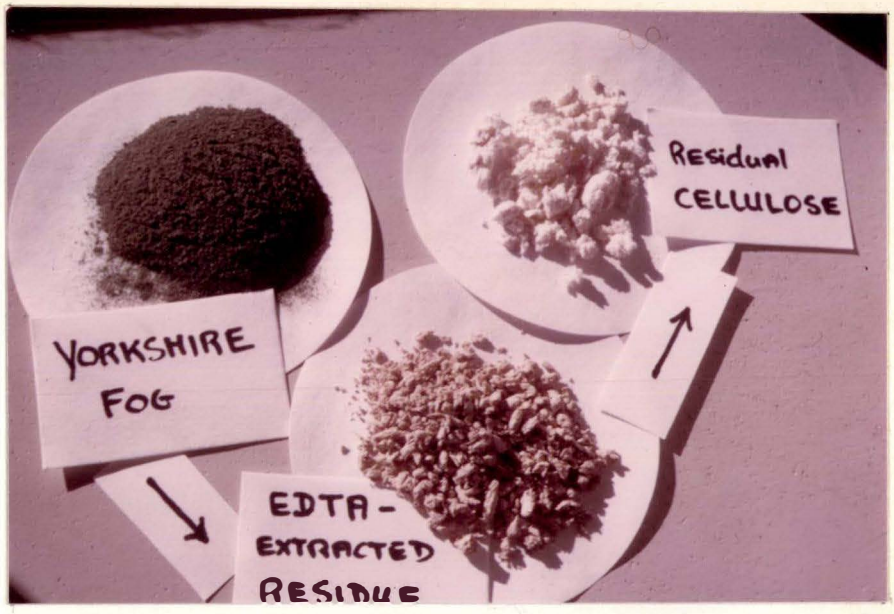
The lignin content of a portion of the residue from the EDTA extraction was estimated by polysaccharide digestion with 72% (w/v) H_2SO_4 (Gailliard and Nijkamp, 1968). The bulk of the residue from the pectin extraction was deproteinated by digestion

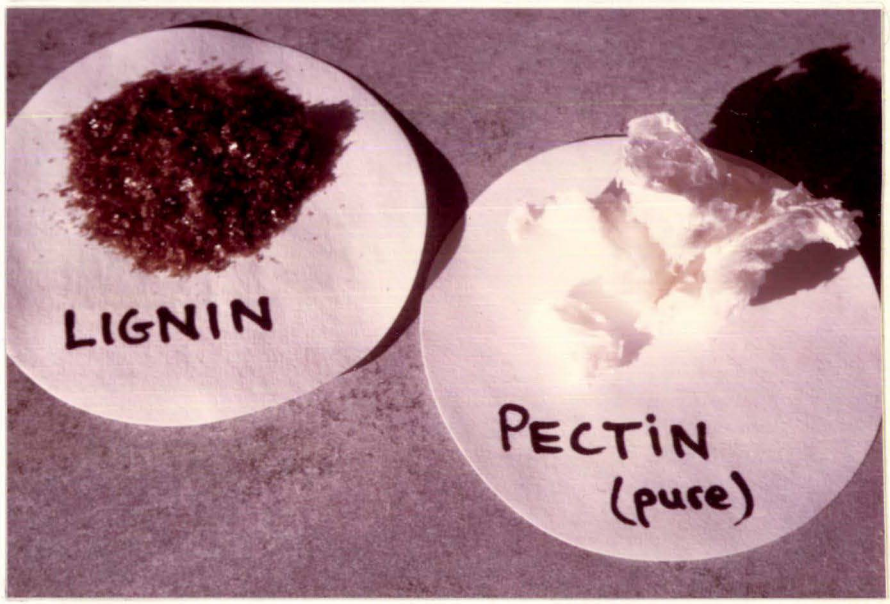
with a 0.5% solution of pepsin in 0.1M HCl at 46°C for 18 hours (Waite and Gorrod, 1959 b). The residue was then extracted (under nitrogen deoxygenated by Fieser's solution) by aqueous 5% KOHat 25°C for 18 hours. The extracted lignin, contaminated with hemicellulose, was precipitated by acidifying with 5N H_2SO_4. The crude lignin was redissolved in warm aqueous 1.25N NaOH and the bulk of the hemicellulose precipitated by pouring the solution into four volumes of 95% ethanol. The ethanolic lignin solution was finally purified by hydrolysing contaminating hemicelluloses by boiling the acidified solution (pH 4.0) for one hour (Bondi and Meyer, 1948). An aqueous suspension of the lignin was dialysed against distilled water and freeze-dried. The products were brown powders or flakes.

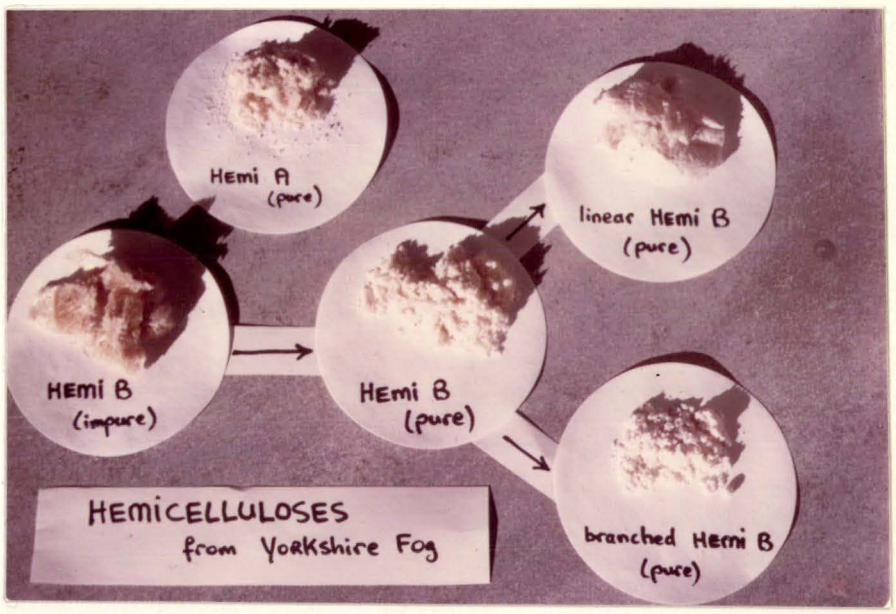
2.3.4. Hemicelluloses

Hemicelluloses are usually extracted from delignified plant tissue (holocellulose) by alkaline reagents. The classical use of chlorine dioxide generation for delignification of wood (Wise et al, 1946) has been modified by Whistler, Bachrach and Bowman (1948) who found that 90% of the lignin in corn cobs was removed by the sodium chlorite/acetic acid reagent within one hour. Since young grasses contain only 4% - 6% dry weight of lignin it was considered safer to use the slower but milder delignification of Gailliard (1958) because acidified sodium chlorite has been shown to cause slight hemicellulose degradation. Reducing end groups can be oxidised to aldonic acids (Jeanes and Isbell, 1941) and slight depolymerisation and oxidation of 2,3 glycol groups may occur (Becker, Hamilton and Lucke, 1965).

Portions of the deproteinised residue (30 g) were suspended in 750 cm^3 of distilled water and heated on a boiling water bath to 80° - 85°C. Chloramine T (10 g) and glacial acetic acid


YORKSHIRE FOG
Residual CELLULOSE
EDTA-EXTRACTED


LIGNIN
PECTIN (pure)


Hemi A (pure)
linear Hemi B (pure)
Hemi B (impure)
Hemi B (pure)
branched Hemi B (pure)
HEMICELLULOSES from Yorkshire Fog

(5 cm^3) were stirred into the suspension and the mixture left, with occasional stirring, for two hours. The residue was filtered off on a large glass filter and the procedure repeated twice more. The residue was then left in contact with a boiling 3% ethanolic solution of ethanolamine (twice) and exhaustively washed with ethanol and water (Gailliard, 1958).

The filtered residue was extracted, under deoxygenated nitrogen, with 10% KOH at 25°C for 18 hours. Hemicellulose A was precipitated by acidifying the extract to pH 5.0 by 50% aqueous acetic acid and hemicellulose B by pouring the filtrate into two volumes of 95% ethanol (Whistler and Feather, 1965). The precipitate (pale brown in colour) was collected on a nylon gauze and dissolved in water to give a 4% solution. Not all the precipitate would redissolve in water, so the water-insoluble fraction (designated hemicellulose B(I)) was redissolved in 0.5M KOH, dialysed until the polysaccharide flocculated out, and freeze-dried. The aqueous hemicellulose B solution was centrifuged (2000 rpm) and poured, with vigorous stirring, into five volumes of 95% ethanol slightly acidified with acetic acid. The white precipitate was redissolved in water, dialysed overnight against distilled water, and freeze-dried. The freeze-dried hemicellulose B was a white, porous compound while hemicellulose B(I) was pale brown and was probably contaminated with lignin, (section 2.4.2.).

The hemicellulose B was further fractionated into linear and branched hemicellulose B by the iodine/calcium chloride method of Gailliard (1965). The fractions were freed from iodine contamination by titration with sodium thiosulphate solution, then purified and dried as outlined above for hemicellulose B.

Most hemicelluloses extracted from plant tissues are mixtures

of different polysaccharides which can be differentiated in terms of 'polymolecularity', 'polydispersity' and 'polydiversity' (Reid and Wilkie, 1970 a). Methods for the fractionation of hemicelluloses mixtures into homogeneous polysaccharides include copper complex formation (Jones and Stoodley, 1965), ethanol precipitation (Whistler and Sanella, 1965) and quaternary ammonium salt formation (Scott, 1955). The homogeneity of such mixtures can be determined by ultracentrifugation (Adams, 1960), free-boundary electrophoresis (Northcote, 1954) or high-voltage zone electrophoresis (Northcote, 1965).

In the present investigation, hemicelluloses separated by the iodine/$CaCl_2$ fractionation were further fractionated by precipitation with ethanol. All polysaccharide fractions were also checked for homogeneity with high voltage ionophoresis on glass-fibre paper in borate buffer (Briggs, Garner and Smith, 1956). Polysaccharide samples (100 - 150 µg) in 0.05M Na tetraborate buffer (20 µl) at pH 9.2 were applied to the centre of glass fibre strips (Whatman GF 81) of 20 cm x 40 cm dimension in a Pherograph Model 64 high voltage electrophoresis unit. A potential gradient of 35 v/cm (40 mA current) was applied for one hour, the electropherograms dried and sprayed with an acidified α-naphthol solution (90 cm^3 n-butanol, 1 g α-naphthol, 8 cm^3 H_2O and 2 cm^3 conc. H_2SO_4) and heated at 110°C for 20 minutes. Polysaccharides appeared as purple bands which turned brown with time. The nitrobenzene-p-sulphonate ion was used as a standard for rate of migration while 2,3,6-tri-O-methyl-D-glucose was a suitable non-migrating marker to correct for electro-osmotic flow (Frahn and Mills, 1959).

2.3.5. Cellulose

The residue from the 10% KOH extraction was washed with 0.1M

HCl, distilled water and then freeze-dried. It was designated 'cellulose'.

2.3.6. Organic acids

Portions of freeze-dried grass (1.5 g) were shaken with water (50 cm^3) containing a few drops of toluene (to suppress microbial growth) for six hours, filtered, and the residue similarly re-extracted for a further 16 hours. The combined aqueous extracts of non-volatile acids were purified by acidification, centrifugation and passage through a cation exchange resin column (Molloy, 1969). The organic acid content of aliquots was quantitatively estimated, after gradient elution from the anion exchange resin Dowex 1, by the method of Hulme and Wooltarton (1958).

2.4. CHEMICAL ANALYSIS OF CARBOHYDRATE FRACTIONS

2.4.1. Analytical methods

Hydrolysis of polysaccharides. Samples of hemicellulose and the pectic fraction were hydrolysed in 0.125M H_2SO_4 (10 cm^3) for 22 hours in sealed tubes at 100°C. Increasing acid strength, up to 0.5M, gave little improvement in hydrolyses as measured by the optical rotation of the solution and often gave a marked discolouration of the hydrolysate. Cellulose samples were hydrolysed by 72% (w/v) H_2SO_4 at room temperature for 24 hours.

All hydrolysates were neutralised with saturated aqueous $Ba(OH)_2$, and the centrifugates decationised by rotation with a small portion of Dowex 50-X8 (H^+ form) cation exchange resin. Solutions were concentrated in a rotary evaporator and made up to 2 cm^3 prior to paper chromatographic identification of 10 ul aliquots.

Paper chromatography. Carbohydrate solutions were chromatographed on Whatman No. 1 paper in a descending solvent of ethyl

acetate/pyridine/water, 12:5:1 (v/v) for 18 hours. This fast solvent, which was allowed to drip off the bottom of the chromatogram, gave an excellent separation of glucose and galactose while uronic acids hardly moved from the origin. An acidic solvent of ethyl acetate/acetic acid/formic acid/water, 9:1.5:0.5:2 (v/v) for 18 hours was used when uronic acids, galactose and sucrose were to be separated. The Rfs of the sugars in these two solvents are given in Table 1.

TABLE 1

$R_{rhamnose}$ VALUES OF SUGARS IN TWO CHROMATOGRAPHIC SOLVENTS.

Sugar	EtAc/Pyr./H_2O 12 : 5 : 1	EtAc/HOAc/HCOOH/H_2O 9 : 1.5: 0.5 : 2
	$R_{rhamnose}$	$R_{rhamnose}$
Rhamnose	100	100
Xylose	74	58
Arabinose	66	49
Fructose	50	42
Glucuronic acid	2	38
Galacturonic acid	6	30
Glucose	38	29
Galactose	31	27
Sucrose	20	24

Sprays

Indicator sprays used were:

aldoses and uronic acids: p-anisidine HCl (Hough, Jones and Wadman, 1950).

ketoses: urea - H_3PO_4 (ibid.)

differentiation of reducing and non-reducing sugars: silver nitrate/acetone/NaOH (Trevelyan, Procter and Harrison, 1950).

Quantitative estimation of sugars.

(a) Spectrophotometric. Sugar unknowns were chromatographed with standard sugars and guide strips and eluted from the paper

with deionised water (Laidlaw and Reid, 1950). Duplicate 1 cm^3 aliquots of the eluate (3 cm^3) were analysed by the Bath method (Bath, 1958). The optical densities of the solutions were measured in a Unicam SP 500 spectrophotometer at the following wavelengths: fructose, galactose, glucose and sucrose, 322 nm; arabinose, 287 nm; xylose, 316 nm.

The standard concentration/optical density curves for four of the sugars are given in Fig. 2. The 'eluted' curves are those obtained by eluting standard amounts of the sugars from paper chromatograms while the 'non-eluted' curves are those corresponding to standard aqueous sugar solutions. Despite exhaustive analytical precautions, only glucose gave a satisfactory standard curve upon elution (r^2 = 0.994). For all eluted sugars there was a marked decrease in optical density indicating that there had been incomplete elution of the sugar from the paper. The coefficient of multiple determination, r^2, for the regression lines shown in Fig. 2 indicated the considerable variability inherent in the elution technique and its limitations in accurately determining the sugar content of an unknown.

Apart from incomplete elution, there are probably many reasons for the variation in readings - contamination from marker spots and cellulose from the filter paper, excessive manipulation, etc. Coupled with the inherent fastidiousness and tedium of the method, these unsatisfactory results necessitated the use of gas-liquid chromatography for accurate quantitative analyses.

(b) Gas-liquid chromatographic. Samples of polysaccharides (20 mg) were hydrolysed as outlined above and Me-α-D-glucopyranoside (10 mg) was added as an internal standard after neutralization. Such hydrolysates are equilibrium mixtures of α- and β-anomers of the free aldoses and chromatography of such mixtures gives a large number of peaks. This problem was avoided

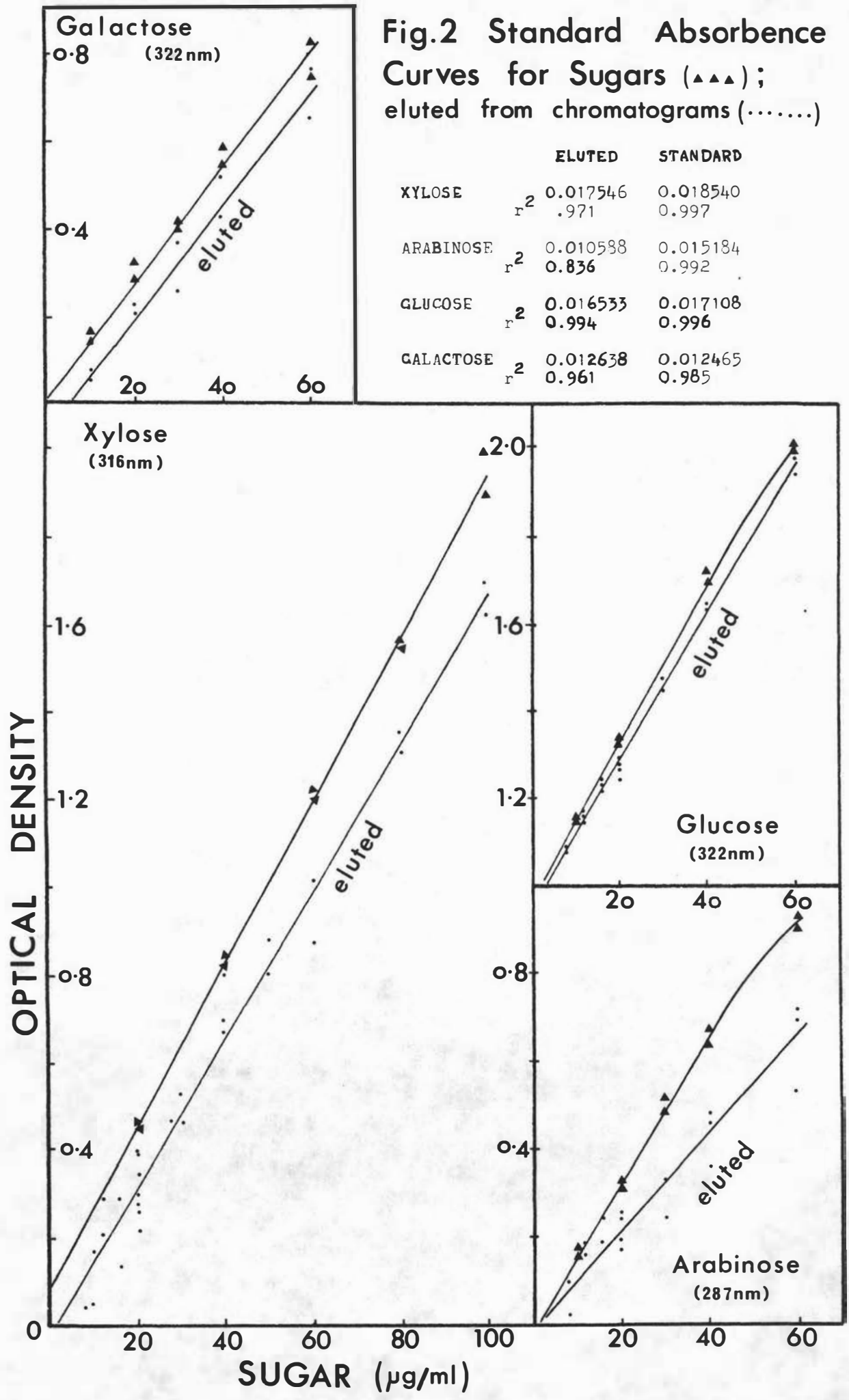
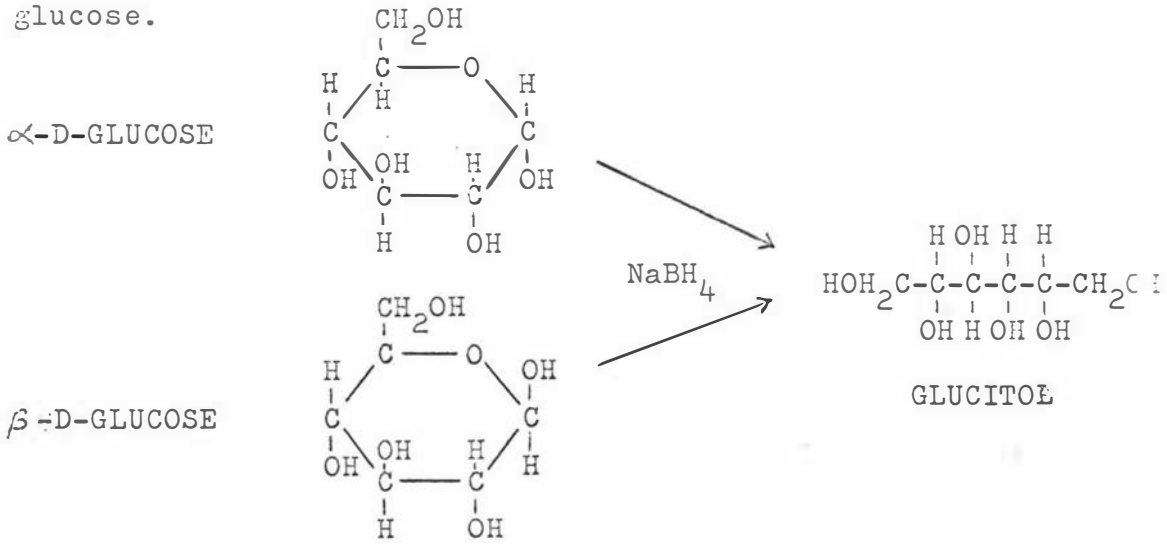


Fig.2 Standard Absorbence Curves for Sugars (▲▲▲); eluted from chromatograms (·····)

		ELUTED	STANDARD
XYLOSE		0.017546	0.018540
	r^2	.971	0.997
ARABINOSE		0.010588	0.015184
	r^2	0.836	0.992
GLUCOSE		0.016533	0.017108
	r^2	0.994	0.996
GALACTOSE		0.012638	0.012465
	r^2	0.961	0.985

by reducing the aldoses to the corresponding open-chain alditol by reduction with sodium borohydride (10 mg), as shown below for glucose.



After standing overnight at room temperature, excess $NaBH_4$ was decomposed by gently shaking the solution with an excess of cation exchange resin (0.5g, Dowex 50 - X8) and the solution taken to dryness in a rotary evaporator. Borate ion was removed as volatile methyl borate by three co-distillations with methanol (20 cm^3).

Initially trimethylsilyl derivatives were formed by the method of Sweeley, Bentley, Makita and Wells (1963) but, as in their investigation, it was found that arabitol/xylitol and galactitol/glucitol mixtures could not be separated on an SE - 52 column (Silicone gum rubber, phenyl). Consequently, alditol mixtures were acetylated with a mixture of pyridine/acetic anhydride 1:1 v/v (4 cm^3) in a boiling water bath for 10 minutes (Bjorndal, Lindberg and Svensson, 1967). The mixture was evaporated under reduced pressure to a syrup which was dissolved in ethyl acetate (2 cm^3) for injection into the gas chromatograph.

Quantitative determination of the acetates was performed on a Varian Aerograph 1740 (flame ionisation detector) Gas chromatograph using an $\frac{1}{8}$" x 6' column of SE - 30 (Silicone gum rubber, methyl) on Aeropak 30 and a nitrogen carrier gas flow of 30 cm^3 min.

Peak areas were measured by planimetry (mean of six measurements) and by weighing. With temperature programming from 144°C to 170°C after 20 minutes, an adequate separation of the monose derivatives was obtained except for glucitol- and galactitol-hexa-acetate. A typical chromatogram of a hemicellulose hydrolysate is given in Fig. 3 with the retention times and peak area/weight ratios of the alditol acetates given in Table 2. Rhamnose, ribose and 3-OMe-glucose were unsuitable as standards since 3-OMe-glucitol penta-acetate interfered with the hexitol acetates and rhamnitol- and ribitol penta-acetates with the pentitol acetates.

It has been claimed (Gunner, Jones and Perry, 1961) that a relationship exists between the retention times of the alditol acetates and their stereochemical structure as described in the preferred zig-zag conformations. For alditol acetates of the same molecular weight, the greater the number of acetoxy groups on non-terminal carbon atoms which are arranged on one side of the molecule, the greater the affinity of the compound for the liquid phase and hence the longer its retention time. Furthermore, they postulate that for alditol acetates of the same molecular weight and the same number of acetoxy groups arranged on one side of the molecule, the closer these acetoxy groups are to each other the greater is the affinity of the compound for the liquid phase and hence the greater the retention time. These empirical rules also predict the present difficulty of separating glucitol- and galactitol hexa-acetates, a problem which was also encountered by Gunner et al with the three column mixtures they employed but was not discussed by them.

Galactitol hexa-acetate (I) in the zig-zag conformation has two acetoxy groups in close proximity on either side of the molecule while in glucitol hexa-acetate (II) there are three

TABLE 2

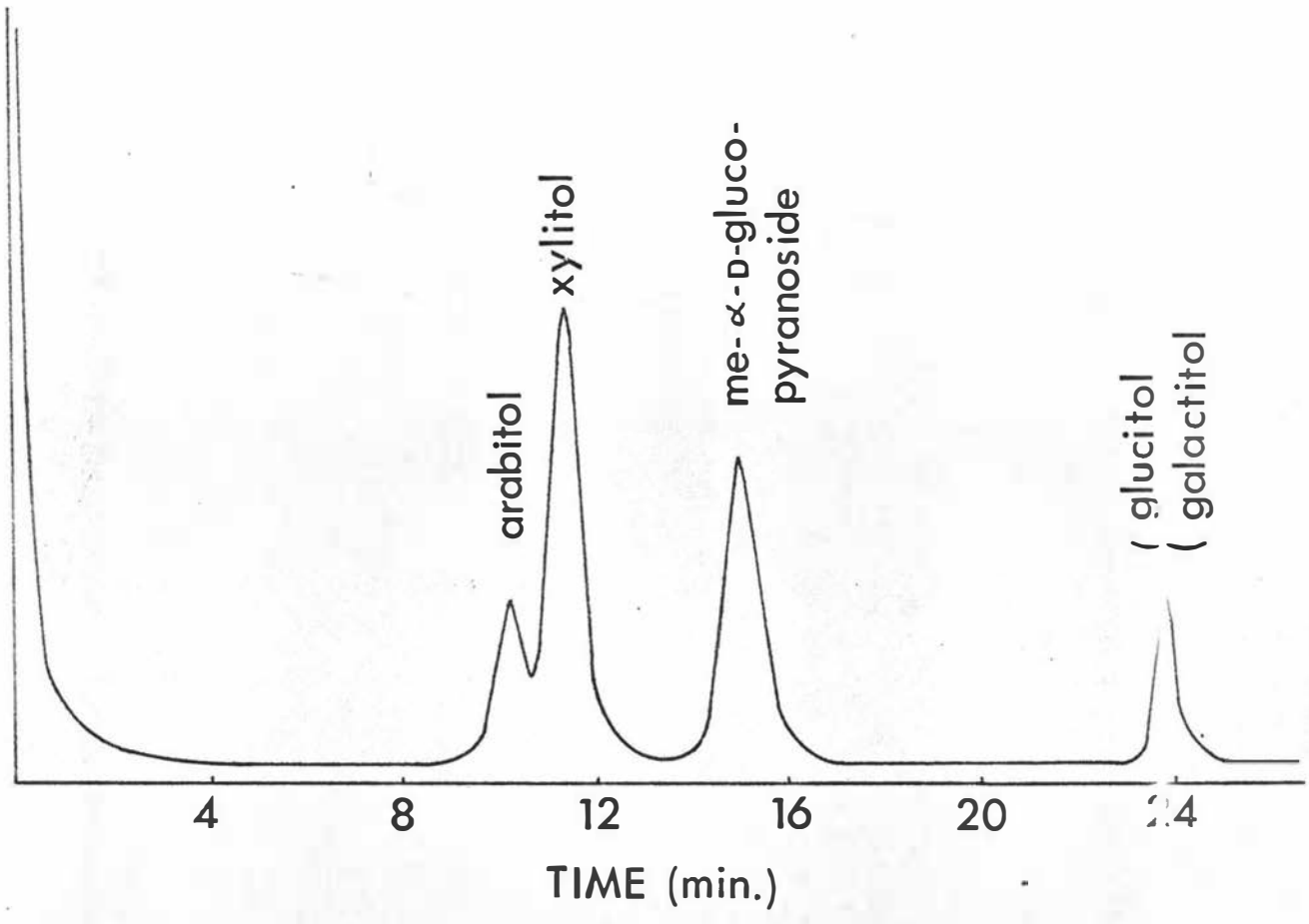
RETENTION TIMES AND PEAK AREA/WEIGHT RATIOS OF ALDITOL ACETATES FROM POLYSACCHARIDES.

Alditol acetate	Retention time (min.)	peak area / weight ratio *
Temperature 144°C		
Arabitol penta-acetate	10.0	1.35
Xylitol penta-acetate	11.0	1.29
Me-2,3,4,6-tetra-O-acetyl- α -D-glucopyranoside	14.6	1.00
Temperature 170°C at 20 min.		
Glucitol hexa-acetate	24.0	1.23
Galactitol hexa-acetate	24.3	1.21

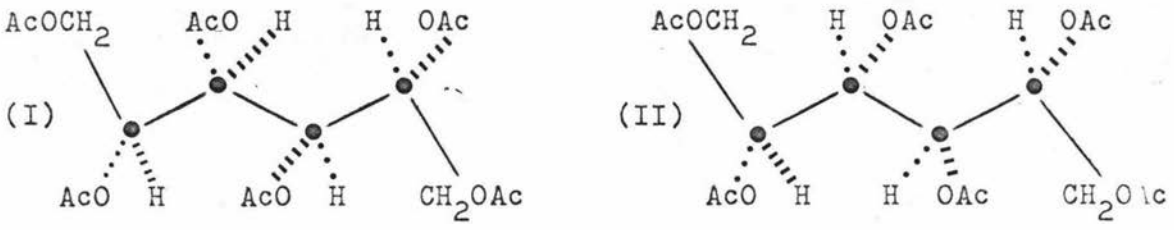
* Relative to ratio for Me-2,3,4,6-tetra-O-acetyl- -D-glucopyranoside = 1.00

* * * *

FIG. 3 Separation of alditol acetates from Hemicellulose B (linear) on SE - 30.



consecutive acetoxy groups on one side of the molecule.



On the basis of these 'rules' of Gunner et al, it is reasonable to expect the affinity of each acetylated molecule for the liquid phase to be roughly similar, hence their difficulty of resolution.

In the present analytical work the hexitol hexa-acetate peak was designated 'glucose', 'galactose' or both, on the basis of paper chromatographic analysis of an aliquot of the original hydrolysate. However, since the peak area/weight ratios for both derivatives were so similar it was possible to calculate the glucose plus galactose content of an unknown by treating the hexose peak as an entity. (Tables 4 and 5).

Comparison of spectrophotometric and gas-liquid chromatographic methods. Duplicate samples of linear hemicellulose B and branched hemicellulose B were hydrolysed and analysed for monosaccharide composition by both the elution/spectrophotometric method and the gas-liquid chromatographic method. The results are given in Table 3. The standard errors of the means for all monosaccharides by the gas chromatographic method were vastly superior, and this method also gave greater total recoveries of monosaccharides. The results indicate that low estimations of xylose are obtained with the paper chromatographic elution method while glucose plus galactose values are high, possibly through cellulose contamination. Coupled with the variation in the standard curve for each monosaccharide when estimated by the elution/spectrophotometric technique (Fig. 2), it was clear that the gas-liquid chromatographic technique of analysis was preferable. Consequently, it

was used for the monosaccharide analysis of all the polysaccharide fractions.

TABLE 3

COMPARISON OF METHODS OF ANALYSIS OF MONOSACCHARIDE COMPOSITION OF HEMICELLULOSES

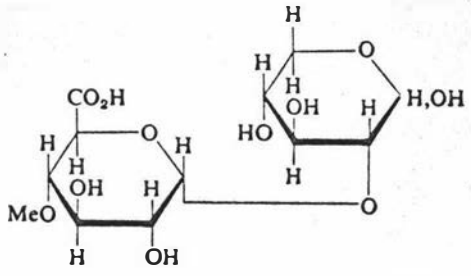
	Elution from paper chromatograms and spectrophotometric estimation (Bath, 1948)		Gas-liquid chromatography of alditol acetates on SE-30 column.	
	Mean * (% D.W.)	SE_{mean}	Mean (% D.W.)	SE_{mean}
Linear hemicellulose B				
Xylose	32.7	± 1.7	50.15	± 0.37
Arabinose	11.7	± 0.7	13.20	± 0.25
Glucose	15.8(8)	± 0.6	15.72	± 0.17
Galactose	3.5	± 0.4		
Total	63.7	± 1.3	79.07	± 0.24
Branched hemicellulose B				
Xylose	31.2(8)	± 1.8	36.90	± 0.32
Arabinose	18.8	± 1.1	20.05	± 0.77
Glucose	4.1	± 0.8	12.58	± 0.24
Galactose	9.1	± 0.5		
Total	63.2	± 2.2	69.53	± 0.81

* = mean of 4 values except where indicated in parentheses.
% D.W. = percentage dry weight
SE_{mean} = standard error of the mean.

Uronic acid analysis of polysaccharides

The uronic acids, 4-O-methyl-α-D-glucopyranosyluronic acid (woody plants) or α-D-glucopyranosyluronic acid (annual plants), are generally attached to C-2 or C-3 of the xylan chain of hemicelluloses (Whistler and Richards, 1970). This linkage is resistant to hydrolysis in H_2SO_4 up to a strength of 0.5M and such acid

hydrolysis gives rise to an aldobiuronic acid, usually 2-O(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose.



2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose

Such aldobiuronic acids do not move from the origin with paper chromatography in the solvents used since their Rfs are lower than those of free uronic acids. Furthermore they do not form suitable derivatives for gas-chromatographic separation and are probably lost as the barium salts in the neutralisation of the hydrolysate.

Consequently, the uronic acid content of the hemicellulose and pectic (polygalacturonic acid) fractions was determined directly on the free polymers by the spectrophotometric carbazole method of Dische (1947, 1963). Since hexoses, and to a lesser extent pentoses, contribute to the colour formation blanks were used containing the proportions of xylose, arabinose, glucose and galactose in the hemicellulose or pectic compound. Sample absorbences were measured at 530 nm in a Unicam SP 500 spectrophotometer and contained 10 - 100 µg uronic acid and a sugar concentration no greater than 0.02% w/v, a level which gave only a minor contribution to the total absorbence.

It should be noted, however, that the carbazole method does not give accurate determinations of absolute hexuronic acid content since the colour intensity varies slightly with the type of polysaccharide investigated (Dische, 1947). Nevertheless, the polysaccharide fractions studied were broadly similar in monosaccharide content and were free of interfering proteins and

sulphydryl-containing compounds so the method was valuable for determining the comparative uronic acid contents.

General polysaccharide purification and analyses

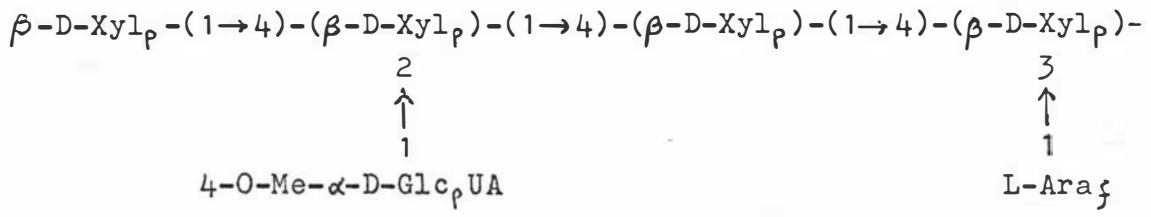
All fractions were checked for nitrogen content by a micro-Kjeldahl procedure using a $K_2SO_4/HgO/H_2SO_4$ catalyst (Steyermark, 1961), and mineral contamination was determined by ashing the polymers to 550°C in a muffle furnace.

The -OMe ether content of some fractions was determined by the Microanalytical Laboratory, University of Otago.

Infrared spectra of plant fractions were obtained with a Perkin Elmer Model 137 I.R. Spectrophotometer. Samples (2 mg) were dispersed in KCl (170 mg) by grinding by hand for 10 minutes and pressed discs prepared as described by Farmer (1957). To obtain uniform sample distribution, discs were ground and repressed several times until a clear disc was obtained.

2.4.2. Results and Discussion

The hemicelluloses of grasses generally consist of D-xylan backbones with L-arabinofuranose attached to C-3 of xylose and D-glucuronic or 4-O-Me-D-glucuronic acid residues attached to C-2 of xylose, usually as single unit sidechains (Aspinall, 1959).



The 4-O-methyl ether of glucuronic acid is the most common side-chain in the xylans of higher plants, while many annual plants contain only the unmethylated D-glucuronic acid sidechain. L-arabinose is usually found as a non-reducing end group but non-terminal L-arabinofuranose residues have been found in perennial ryegrass (Aspinall, Cairncross and Ross, 1963) and many cereals.

In such side-chains, L-arabinose is usually in the furanose form and the L-arabinosyl groups in the side-chain are often terminated by 4-O-methyl- -D-glucopyranosyluronic acid or D-xylopyranosyl groups (Whistler and Richards, 1970). On the other hand, all side-chain substituents may be absent as in esparto grass xylan (Chanda et al, 1950) which is a true D-xylan, composed exclusively of D-xylose residues.

Glucose and galactose have long been known to be present in grass hemicellulose hydrolysates (Gailliard, 1959b; Waite and Gorrod, 1959a), but the nature of their structural incorporation was not clear. Terminal, non-reducing galactose residues have been found in a xylan from perennial ryegrass (Aspinall, Cairncross and Ross, 1963) and on the basis of methylation studies Ehrenthal, Montgomery and Smith (1954) have claimed that glucose is an integral part of the xylan chain in wheat straw. Gailliard has found indications of a neutral glucan and an acidic, highly branched galactoarabinoxylan in perennial ryegrass (Gailliard, 1965; Bailey and Gailliard, 1965). Small amounts of a β-glucan, resembling cellulose in properties, were separated from corn stalks (Gramera and Whistler, 1963) as well as an acidic galactoarabinoxylan and a neutral arabinoxylan. Recently, a neutral glucan (Fraser and Wilkie, 1970) and an acidic galactoarabinoxylan with both terminal and non-terminal galactose residues (Reid and Wilkie, 1969b) have been isolated in a pure form from young oat leaves.

The chemical analysis of the polysaccharides from a sample of Yorkshire fog (collected 5/3/1968) is given in Table 4. The 'pectin' was a polygalacturonic acid with a low -OMe content (presumably the methyl ester) and also contained small amounts of arabinose and galactose.

The hemicelluloses obtained from Yorkshire fog were basically arabinoxylans although varying amounts of hexose were present.

Table 4

CHEMICAL ANALYSIS OF POLYSACCHARIDES FROM YORKSHIRE FOG (5/3/68)

	% Dry Weight									
	Arabinose	Xylose	Hexose*	Anhydro-uronic acid	Aldobiuronic acid xylose+	-OMe	NX6.25	Ash	Insoluble residues after hydrolysis	Total
Pectin	4.2	-	3.0 (Gal.)	87.4	-	1.9	1.2	0.4	-	98.1
Hemicellulose A	13.5	49.1	21.3 (Glu.)	5.6	4.3	N.D.	-	-	4.0	97.8
Hemicellulose B (linear)	13.2	50.1	15.7 (Glu. + Gal.)	7.9	6.1	0.6	-	-	3.2	96.8
Hemicellulose B (branched)	20.0	36.9	12.6 (Glu. + Gal.)	12.9	9.9	0.8	-	-	-	93.1
Hemicellulose B(I)	7.5	56.0	11.0 (Glu.)	3.2	2.5	N.D.	-	-	16.3	96.5
Cellulose	-	4.7	92.3 (Glu.)	-	-	-	0.8	0.6	2.8	101.2

* Glucose + Galactose. Glu. = Glucose, Gal. = Galactose

+ Anhydrobiuronic acid x0.77

Aldobiuronic acid xylose (section 2.4.1.) was calculated by assuming a 1:1 ratio of xylose to glucuronic acid and thereby multiplying uronic acid content (carbazole method) by 0.77. Hemicellulose A was present in only small quantities (see Table 9) in Yorkshire fog, unlike perennial ryegrass (Gailliard, 1965), while linear and branched hemicellulose B plus hemicellulose B(I) comprised over 95% of the fractionated hemicellulose (Table 9). Furthermore, the monosaccharide composition of Yorkshire fog hemicellulose A is quite different from that of perennial ryegrass (Gailliard, 1965) in that it contains glucose and has a higher uronic acid content. In this respect, its composition is similar to that of linear hemicellulose B and it may simply be a small part of that fraction which slowly precipitated on standing after acidification of the extract.

Iodine/$CaCl_2$ fractionation of Yorkshire fog hemicellulose B gave a differentiation of the polymers in uronic acid content (Table 4) but the hexoses were not clearly separated into glucose (linear) and galactose (branched) as was found by Gailliard (1965). An attempt at copper complex formation with fehlings solution (Jones and Stoodley, 1965) gave no further fractionation of these 'linear' and 'branched' hemicellulose B fractions (Lee, 1970).

The hemicellulose B fraction which was insoluble in water but soluble in dilute KOH (hemicellulose B (I)) was incompletely hydrolysed by 0.125 M H_2SO_4. This fraction is virtually a pure xylan (Table 4) with only low amounts of uronic acid and in this respect bears a similarity to the composition of the hemicellulose A isolated by Gailliard from perennial ryegrass. The infra-red spectrum of this polymer (Fig. 6) was typical of a hemicellulose except for the shoulder at 1550-1580 cm^{-1}, which is an indication of the C=C skeletal vibrations of the aromatic nucleii of lignin.

The analyses given in Table 4 indicated that 'linear' and 'branched' hemicellulose B of Yorkshire fog, obtained by iodine/ $CaCl_2$ fractionation, were unlikely to be pure polysaccharides. Glass fibre electrophoresis gave further evidence of heterogeneity in some cases but was mostly inconclusive through streaking, (possibly due to high rates of endosmotic flow in alkaline buffers as a result of the loose matrix of the glass fibre paper). An attempt was made to further fractionate these mixtures by ethanol precipitation (Whistler and Sanella, 1965). Aqueous 2% solutions were titrated with 95% ethanol and when turbidity was reached the suspension was centrifuged at 2000 rpm. The centrifugates were dissolved in deionised water, freeze-dried and weighed. On the basis of this treatment, linear hemicellulose B appeared to consist of two fractions, and branched hemicellulose B of three fractions (Fig. 4).

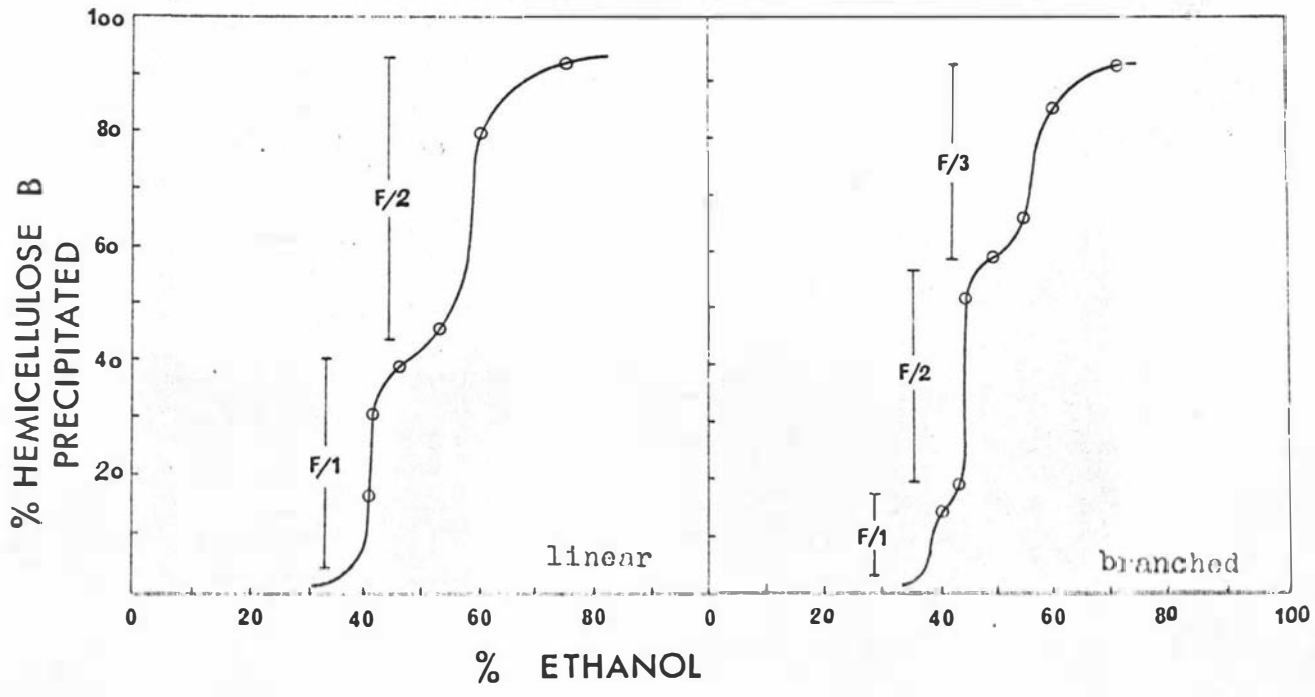


Fig. 4. Ethanol fractionation of linear and branched Hemicellulose B

The individual freeze-dried precipitates were analysed for their monosaccharide composition and the average composition of the grouped fractions is given in Table 5.

The hexose of linear hemicellulose B, F/1 was entirely glucose and the overall composition was very similar to that of the previously-mentioned linear hemicellulose B isolated from perennial ryegrass by Gailliard. Branched F/3 contained only galactose and was similar in composition to Gailliard's branched hemicellulose B while the remaining three fractions contained both glucose and galactose in approximately equal amounts. The composition of linear F/2 was very similar to that of branched F/2.

The monosaccharide composition of the hemicellulose fractions given in Table 5 are consistent with the presence of three pure polysaccharides - a neutral glucan, a weakly acidic arabinoxylan and a more acidic galactoarabinoxylan. All fractions except branched F/3 were of doubtful homogeneity on electrophoretic evidence. The acidic galactoarabinoxylan hypothesis is consistent with the observation that all fractions rich in galactose and arabinose were also higher in uronic acid content. Gailliard (1965) found that the linear hemicellulose B fractionated from ryegrass gave evidence of being a mixture of a neutral glucan and an arabinoxylan. The composition of linear F/1 from Yorkshire fog is consistent with such a mixture while the composition of linear F/2 and branched F/2 and F/3 are consistent with mixtures of these two polysaccharides with varying amounts of the acidic galacto-arabinoxylan (branched F/3).

In view of the resistance of these hemicellulose mixtures to resolution and the tenuous relationship of such fine structure to the problem of complexing of Ca^{++} and Mg^{++} (Part 3) by hemi-

Table 5

MONOSACCHARIDE ANALYSIS OF ETHANOL-FRACTIONATED LINEAR AND BRANCHED HEMICELLULOSE B.

	Weight (mg.) fractionated from 500 mg	% Dry Weight					
		Arabinose	Xylose	Hexose*	Anhydro-uronic acid	Aldobiuronic acid Xylose+	Total
Hemicellulose B (linear)							
Ethanol F/1	235	9.3	62.3	15.8 (Glu.)	4.5	3.5	95.4
Ethanol F/2	250	17.7	41.7	11.6 (Glu. + Gal.)	12.0	9.3	92.3
Hemicellulose B (branched)							
Ethanol F/1	70	13.0	48.2	16.5 (Glu. + Gal.)	8.9	6.9	93.5
Ethanol F/2	175	19.6	39.9	10.3 (Glu. + Gal.)	13.6	10.5	93.9
Ethanol F/3	230	26.6	35.4	10.5 (Gal.)	13.4	10.4	96.3

* Glucose + Galactose.

Glu. = Glucose, Gal. = Galactose

+ Anhydrouronic acid x 0.77

cellulose B this problem was not investigated further. However, this investigation does indicate that the $I_2/CaCl_2$ fractionation procedure of Gailliard, as well as other procedures such as copper complex formation, may only be separating the polysaccharides in the mixture on the basis of molecular weight (polymolecularity) rather than structure (polydiversity) (Reid and Wilkie, 1969).

2.5. CHEMICAL ANALYSIS OF LIGNIN

2.5.1. Analytical methods

Nitrobenzene/alkali oxidation of lignin Samples of lignin (220 mg) extracted from Yorkshire fog (collected 5/3/1968) were heated at 180°C in a bomb calorimeter with 2M KOH (15 cm^3) and redistilled netrobenzene (1.5 cm^3) for two hours. The aromatic aldehydes were purified from the nitrobenzene degradation products in the reaction mixture and finally extracted by continuous liquid/liquid extraction with ether (Pepper, Manolopoulo and Burton, 1962).

Chromatography of oxidation products The aldehydes were separated by thin-layer chromatography on silica gel (Merck G) in a solvent of chloroform/ethyl acetate 90:10 (v/v). The plates were sprayed with an acidic solution of 2:4 dinitrophenyl hydrazine (Reitsema, 1954) to identify carbonyl compounds and with methanolic KOH followed by diazotised sulphanilic acid (Ames and Mitchell, 1952) and acidic hydrazine sulphate (Deshusses and Desbaumes, 1957) to identify the aldehydes by specific colour reactions.

The aldehydes were estimated quantitatively by gas-liquid chromatography (Varian Aerograph 1740 gas chromatograph) on a $\frac{1}{8}$" x 6' column of 3% XE-60 (Silicone GE, nitrile gum) on Aeropak 30. A temperature of 160°C and a carrier gas of dry nitrogen at 65 p.s.i. with a flow rate of 30 cm^3/minute was employed.

m-Hydroxybenzaldehyde was used as an internal standard.

Spectrophotometric analyses Infra-red spectra and O-Me-analyses were carried out on lignin as outlined for polysaccharides.

The ultra-violet absorption spectra of lignin were obtained on a Unicam SP 500 spectrophotometer for solutions (50 ppm) in water, 0.1M NaOH and concentrated H_2SO_4 which had been diluted from stock lignin solutions (200 mg/100 cm^3). To calculate ε, the molecular extinction coefficient, lignin concentration was based on methoxyl equavalent weight (grams of total solids per 31.02g of methoxyl).

2.5.2. Results and Discussion

Studies on the fine structure of the cell wall show that lignin is intimately associated structurally with the cellulose microfibrils and, with the non-cellulosic carbohydrates, it constitutes the amorphous material in which the microfibrils are embedded. (Wardrop and Bland, 1958). Lignin is difficult to define chemically, however, since its composition varies with plant species (especially the angiosperm lignins) and is modified to different degrees by methods of extraction from the plant. Despite this heterogeneity, it is clear that 'lignins' are a tri-dimensional system of polymers derived from coniferyl alcohol and other guaicylpropane monomers; generally they are insoluble in water, most organic solvents and strong sulphuric acid. There is an exhaustive list of physical and chemical properties which serve as 'lignin criteria' (Kratzl, 1965) for the absolute identification of a material as 'lignin'. Again, these are of limited use since exceptions have been found by investigators to some of these rigid criteria.

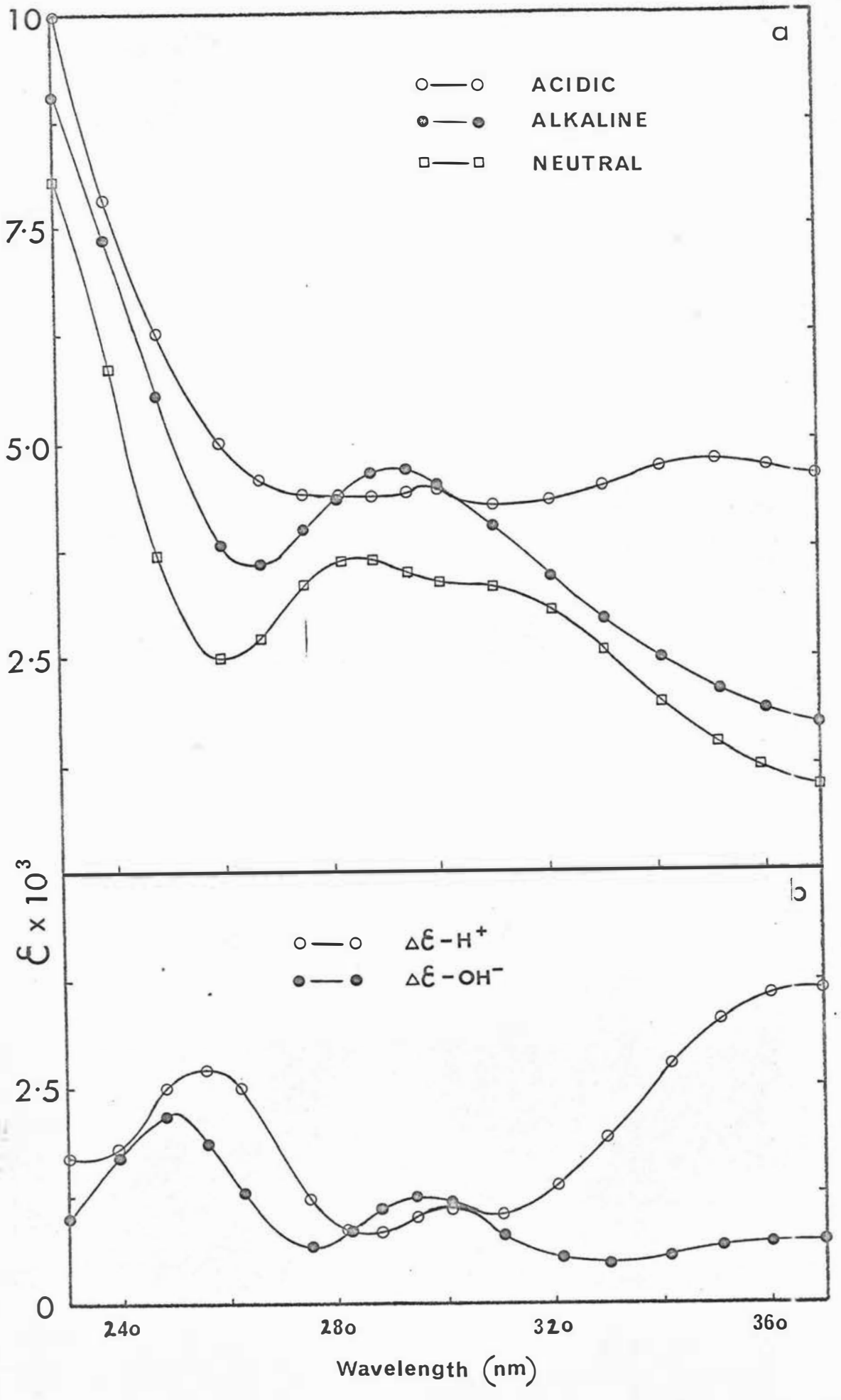
The purpose of this present investigation was to determine whether the isolated 'lignin' from Yorkshire fog (section 2.3.5.)

was actually lignaceous and, if so, its degree of purity and chemical composition. The three criteria employed were: ultraviolet and infra red spectroscopy and alkaline nitrobenzene oxidation.

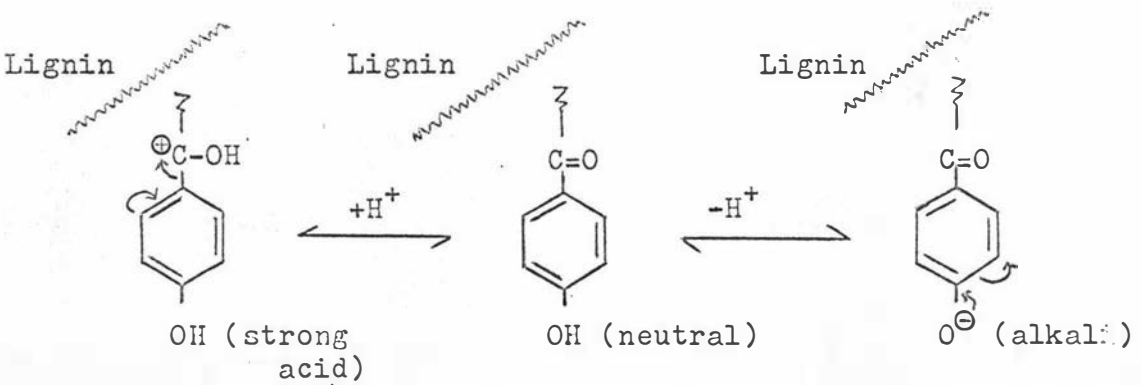
Ultraviolet spectroscopy The ultraviolet absorption spectra of most lignins show a strong absorption maximum in the region of 210 nm and the characteristic weaker absorption shoulder in the 280 nm region (Pearl, 1967). The procedure used in this investigation is similar to that originally used by Aulin-Erdtman (1958) in her exhaustive study of lignins and depends upon the difference in ultraviolet absorption of phenols and their corresponding phenoxide ions. The absorption maxima due to the phenoxide ions are generally higher and are located at longer wavelengths than those given by the non-ionised phenol. Consequently, if the lignin were to contain a large proportion of phenolic groups then an increase in pH of the solution would be expected to give a significant shift of these maxima. The lack of such a significant shift would be a clear indication that most of the chromophores present were nonphenolic.

The absorption spectra for the lignin from Yorkshire fog are given in Fig. 5a. The spectrum under alkaline conditions (pH 12.5) represents the absorption due to the non-ionisable chromophores plus the changed absorption due to the ionisable chromophores. Since the absorptions of these two chromophore groups occur in similar regions, even under these alkaline conditions, it is only possible to study the characteristics of ionisable chromophores by subtracting the absorption curve of the neutral solution (non-ionisable) from that of the alkaline solution (ionisable + non-ionisable); this was termed the '$\Delta\varepsilon$ curve' by Aulin-Erdtman (1958), and is solely dependent upon the ionisable chromophores.

FIG. 5 DIRECT (a) AND DIFFERENCE (b) ULTRA-VIOLET SPECTRA OF LIGNIN



The spectra of lignins in concentrated acid solutions have also been found to give band displacements towards longer wavelengths (Goldschmid, 1953) and the spectrum for lignin in 98% H_2SO_4 in Fig. 5a shows such a broad maximum at 345-365 nm. Doub and Vandenbelt (1949) have shown that the U.V. absorption bands in benzoid compounds can be explained by considering substituent groups (ie, -OH, -OMe, and α-carbonyl groups) to shift the secondary (250 nm), first primary (200 nm) and second primary (180 nm) absorption bands of benzene to longer wavelengths. Similar band displacements can arise, in both strongly acidic and basic solutions, from ionisation of benzenoid compounds containing opposing electron-releasing and electron-attracting groups in a para- position. Extremes of pH in such lignin solutions would enhance electron transfer between α-carbonyl groups (on the propyl- sidechain) conjugated through the benzene ring with a phenolic hydroxy- group in the para- position and would give rise to typical absorption shifts to higher wavelengths:



The actual '$\Delta\varepsilon$ curves' for the lignin from Yorkshire fog (Fig. 5b) show two maxima (250 nm and 295 nm) for the alkaline solution and three for the strongly acidic solution (255, 300, and ~370 nm). On the basis of difference spectra obtained from model guaicyl- compounds and lignins (Goldschmid, 1953; Aulin-Erdtman and Hegborn, 1958; Wexler, 1964) it is possible to assign these

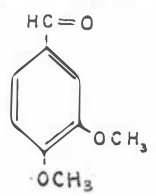
bands to different features in the lignin molecule.

(a) The strong absorption band at 250-255 nm This absorption in both curves can not be definitely ascribed to any particular group but is probably the sum of two effects: a first primary shift (200 nm $\longrightarrow$ 250 nm) of guaicyl- nucleii with no side-chain unsaturation conjugated through the benzene ring plus a second primary shift (180 nm $\longrightarrow$ 235 nm) of guaicyl- nucleii conjugated with side-chain α-carbonyl groups.

(b) The absorption band at 290-305 nm The peak with $\lambda_{max.}$ at 295 nm in the alkaline solution is due to ionised phenolic hydroxyl groups but a slight shoulder is also apparent in the strongly acidic solution and probably results from the partial displacement of the secondary bands of guaicyl nucleii with no side-chain unsaturation (Goldschmid, 1953).

(c) The broad absorption in the region 350-370 nm The absence of this band in the alkaline solution can be interpreted as an absence of phenolic hydroxyl groups conjugated through the ring with an α-carbonyl group in this lignin. However, there is a marked absorption in the strongly acidic solution indicating that α-carbonyl groups on the side-chain are present and are probably conjugated with etherified phenolic groups in the para- position. Such etherified phenolic groups (as in veratraldehyde) would not ionise in alkaline solution and there would, therefore, be no shift in the primary band to the 350-370 nm region.

veratraldehyde



Calculation of phenolic hydroxyl content of lignin On the basis of studies with model compounds containing non-conjugated guaicyl units, Goldschmid (1954) and Wexler (1964) were able to relate the absorptivity, A, (A = optical density/concentration in

g/litre x unit light path length) at spectra maxima to the content of phenolic hydroxyl in the lignin. Although the per cent increase with ionisation in the 280 nm maximum of the direct spectrum was found to be roughly proportional to the phenolic hydroxyl content, Wexler (1964) found the absorptivity of the difference spectrum at 250 nm a more useful index. Goldschmid (1954) used the absorptivity of the 300 nm peak in the difference spectrum but Wexler (1964) has shown that conjugated carbonyl compounds and other unidentified impurities can contribute appreciably to the absorbence at this wavelength and, consequently, the difference spectrum at 250 nm seems the more reliable index.

Since the direct and difference spectra for lignin from Yorkshire fog are very similar to those of 'native lignins' and lignosulphonates investigated by these workers it seemed reasonable to use their data in calculating the percentage phenolic hydroxyl in the Yorkshire fog lignin, particularly as it seemed free of vanillin-like conjugated α-carbonyl compounds (absence of peak in 350-370 nm region for $\Delta\varepsilon$-OH^-). The values obtained (Table 6) indicate a molar methoxyl/phenolic hydroxyl ratio of approximately 4:1 since the upper estimated % phenolic hydroxyl content of 1.49 is probably too high because of the contribution of impurities to the absorption band at 295 nm as discussed above.

TABLE 6

PHENOLIC HYDROXYL CONTENT OF LIGNIN FROM YORKSHIRE FOG AS CALCULATED FROM $\Delta\varepsilon$ (OH^-) CURVE OF FIG 5b.

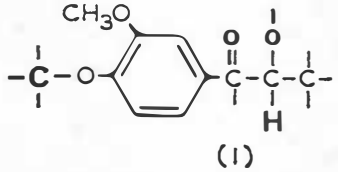
Estimation method	nm	Difference spectra absorptivity ($l.g^{-1}.cm^{-1}$)	factor	% phenolic hydroxyl content	molar ratio of methoxyl* to hydroxyl
Goldschmid (1954)	295	3.6	0.414	1.49	3.6
Wexler (1964)	250	6.7	0.192	1.29	4.1

* -OMe content = 9.9% dry weight of lignin

Infrared spectroscopy The infrared spectra for the whole grass, pectin, lignin, hemicellulose B(I) and hemicellulose B (branched) of Yorkshire fog are given in Fig. 6. The spectra for hemicellulose B (linear) and cellulose are virtually identical with that shown for branched hemicellulose B. It is clear that the structural polysaccharides are responsible for most of the absorption of the grass, viz., the broad carbohydrate band from 1180-930 cm^{-1} and characteristic peak at 900 cm^{-1}. Lignin makes little contribution to the spectrum of the whole grass and the typical aromatic absorption of the 1500-1550 cm^{-1} region is submerged in the total absorption.

All the spectra show strong hydroxyl group stretching vibrations at 3400 cm^{-1}, the broadness of which probably indicates hydrogen bonding. The shoulder at 2900 cm^{-1} in all samples arises from C-H stretching (either methoxyl groups or side chain C-H). The bands at 1720-1740 cm^{-1} in the pectin and hemicellulose samples are probably aliphatic ester carbonyl stretching bands.

The spectrum for lignin gives no indication of carbohydrate contamination in the sample and gives two distinct aromatic stretching bands at 1505 cm^{-1} and 1595 cm^{-1}. The strong C=O stretching band at 1645-1655 cm^{-1} has been attributed (Hergert, 1960) to a ketone carbonyl alpha to the aromatic ring which has the para- position etherified and with an oxygen atom (as a hydroxyl group or etherified) in the two position on the side chain, as in (1).



(I)

The presence of such a grouping is supported by the high U.V.

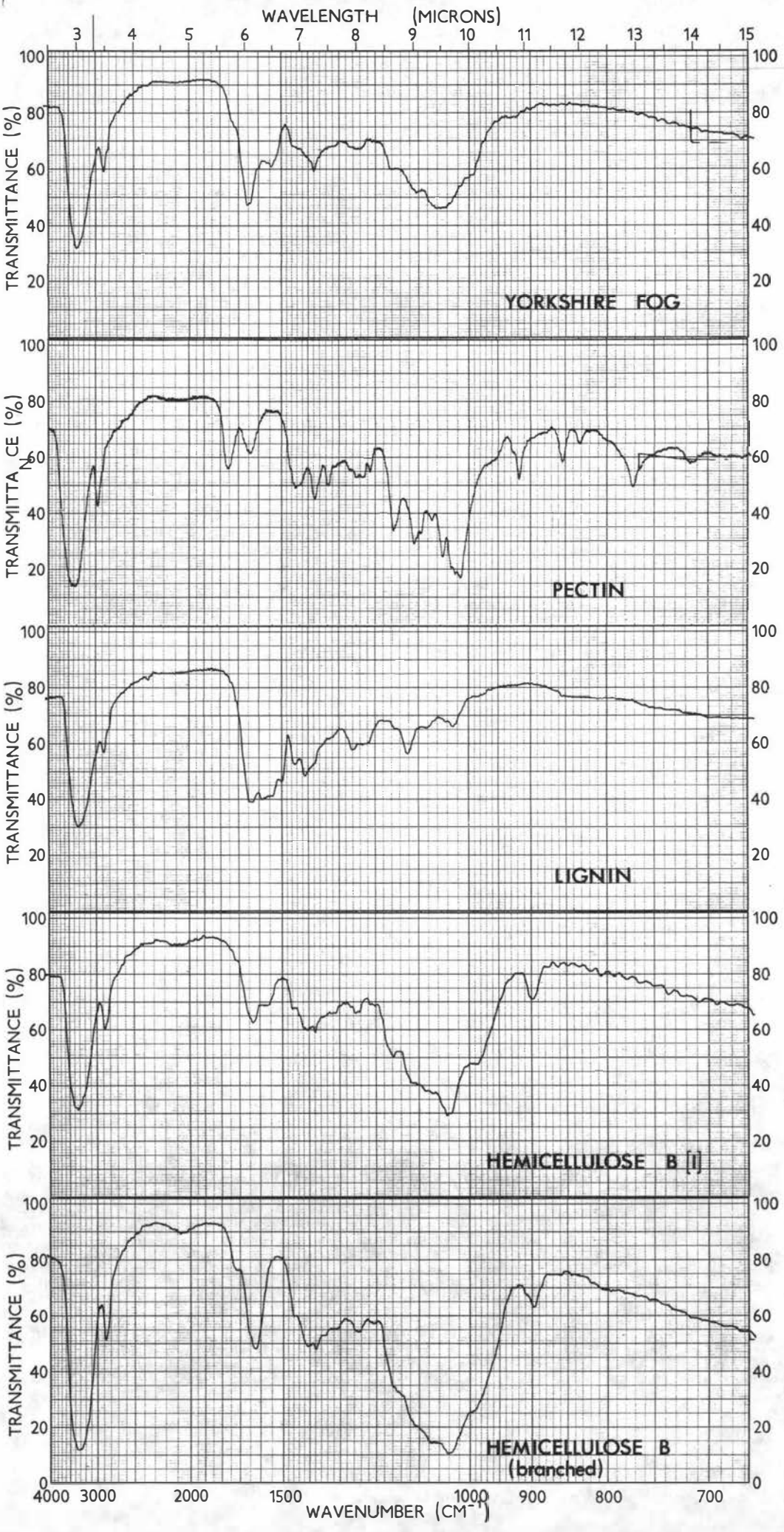
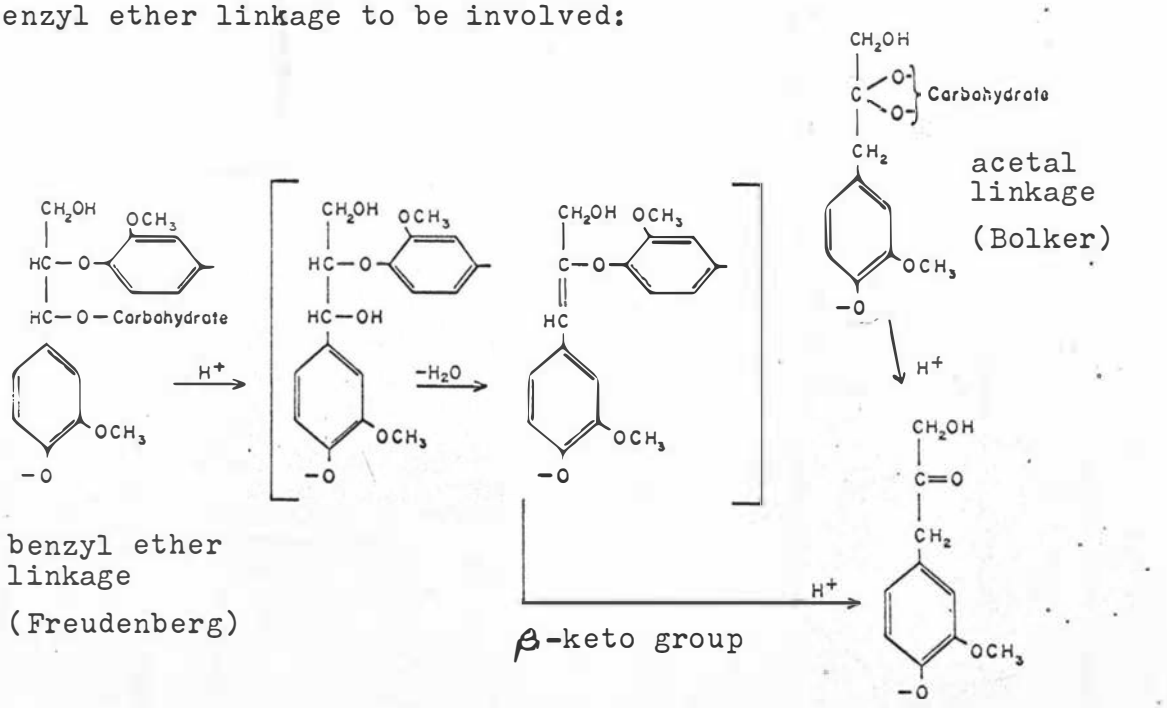


FIG.6 INFRA RED SPECTRA OF PLANT FRACTIONS

absorbence at 370 nm in the strong acid difference spectrum ($\Delta\varepsilon$-H^+) of the lignin as pointed out in the previous section.

Most workers have attributed any lignin infrared peak at 1710-1715 cm^{-1} to the presence of a non-conjugated ketone carbonyl group in the β-position of the phenyl propane side-chain (Hergert, 1960; Bolker, 1963). Since this 1710 cm^{-1} band was present in all of the 35 isolated lignins studied by Bolker (1963), yet absent from the spectra of the 15 wood and pulp lignins investigated, he postulated that the carbonyl group arose through the breaking of the lignin-carbohydrate bond during extraction of the lignin. Bolker (1963) considers these carbonyl groups of lignin to be linked to hydroxyl groups of the holocellulose carbohydrates by acetal or hemiacetal bonds while Freudenberg (1968) considers a benzyl ether linkage to be involved:



The infrared spectrum of lignin from Yorkshire fog, however, shows only a very slight shoulder at 1710 cm^{-1} but this can be explained by the fact that the alkaline extraction of this lignin probably destroyed most of the ketone carbonyl through oxidative cleavage, with the consequent formation of a carboxyl group.

Alkaline treatment of such model compounds as guaicyl acetone and the keto- form of β-hydroxy coniferyl alcohol has indeed eliminated this 1710 cm^{-1} band (Hergert, 1960).

The Yorkshire fog lignin bands between 1460 cm^{-1} and 1370 cm^{-1} are typical of C-H deformations (symmetric and assymmetric); 1260-1220 cm^{-1} from aromatic C-O stretching (methoxyl and phenol); and 1100-1020 cm^{-1} from C-O deformations (primary and secondary hydroxyl and methoxyl, Hergert, 1960).

Summing up, the infrared spectrum of the lignin isolated from Yorkshire fog indicated that the fraction appeared to be free of hemicellulose contamination and in most respects gave evidence of functional groups identified in other lignins.

Alkaline nitrobenzene oxidation The lignin content of monocotyledenous angiosperms, especially the grasses, is quite low compared with woody plants and consequently they have not been investigated as extensively as softwood (gymnosperms) or hardwood (dicotyledenous angiosperms) lignins. The lignins of such monocotyledenous angiosperms differ from the other two categories in that they contain polymers of all these dihydro- analogues of the following cinnamyl alcohols:

(1) coniferyl- (guaicyl propane or 4-hydroxy-3-methoxyphenylpropane monomers);

(2) sinapyl- (3,5 dimethoxy-4-hydroxyphenylpropane monomers),

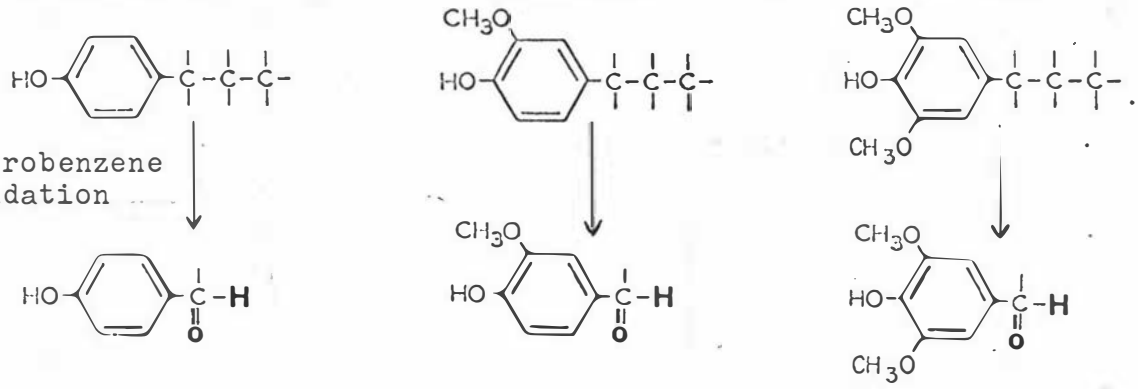
(3) p-coumaryl- (4-hydroxyphenylpropane monomers)(Pearl, 1967). However, Freudenberg (1968) has pointed out that yields of aromatic aldehydes obtained by alkaline nitrobenzene oxidation are of limited value in determining the proportions of these phenylpropane monomers in the lignin polymer. As shown below, the increasing strength in condensation of the phenylpropane units in the lignin polymer is inversely related to the actual yields

of aromatic aldehydes.

STRENGTH OF CONDENSATION IN LIGNIN

dihydro-cinnamyl alcohol grouping

p-coumaryl- > coniferyl- > sinapyl-



p-hydroxybenzaldehyde < vanillin < syringaldehyde

aromatic aldehydes

YIELD OF ALDEHYDE

The three aromatic aldehydes, vanillin, p-hydroxybenzaldehyde and syringaldehyde were identified by their Rfs (Table 7) and colour reactions after thin-layer chromatography on silica gel. Vanillin and p-hydroxybenzaldehyde were confirmed by their molecular weight with mass spectrometry (Prof. R. Hodges) in an AEI MS9 high resolution mass spectrometer after elution from fluorescent plates (Merck $G_{254}F$ silica gel) where the aldehydes could be outlined through their quenching of the fluorescent background.

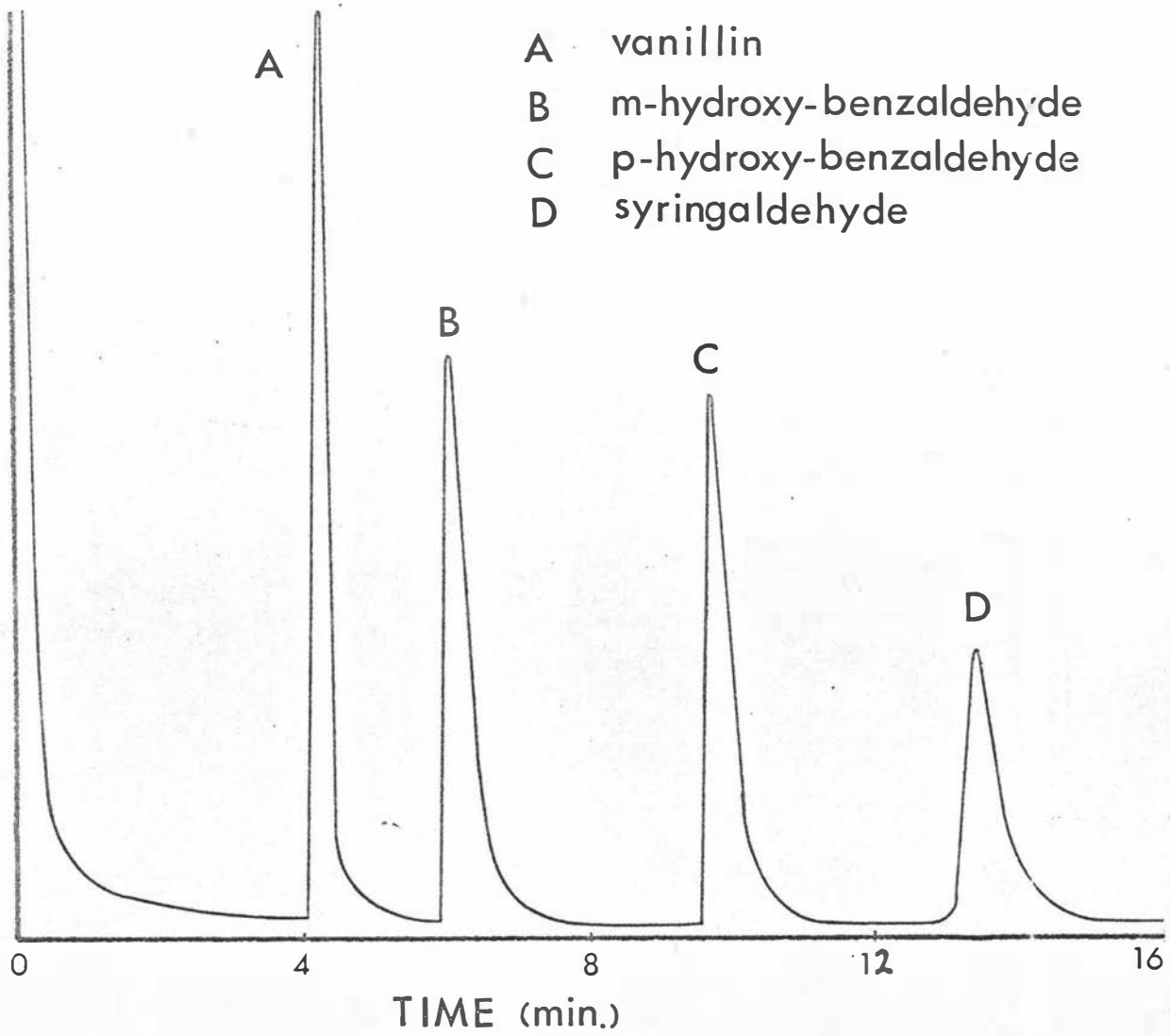
The yield of aldehydes, determined by gas-liquid chromatography, totalled 17% dry weight of the original lignin sample, with vanillin being the major product (Table 7). A typical chromatogram is shown in Fig. 7 and relevant chromatographic data in Table 7. As stated above, the proportion of these aromatic aldehydes can only be interpreted as chemical evidence for the lignaceous nature of the compound and not as conclusive evidence for the proportions of guaicylpropane units in the lignin, which had also probably been modified to some extent during extraction from the plant with alkali. The mode of lignin extraction has

TABLE 7

CHROMATOGRAPHIC DATA ON AROMATIC ALDEHYDES FROM ALKALINE NITROBENZENE OXIDATION OF LIGNIN

	TLC	GLC		
	Rf in solvent $CHCl_3$/EtAc 90:10 v/v	Retention time (min.) T = 160°C	peak area / weight factors	% dry weight of lignin
vanillin	46	4.1	1.11	11.5
p-hydroxy-benzaldehyde	24	9.6	1.01	3.2
m-hydroxy-benzaldehyde	32	6.0	1.00	-
syringaldehyde	57	13.1	0.97	2.3

FIG. 7 SEPARATION OF AROMATIC ALDEHYDES FROM LIGNIN



been found to be of considerable importance in determining the yield of aromatic aldehydes after alkaline nitrobenzene oxidation of different plant lignins (Wildung, Chesters and Behmer, 1970). These workers found that an extraction with aqueous 5% NaOH gave the highest yield of aromatic aldehydes but there is little information in the literature on such yields for monocotyledonous species, regardless of their method of extraction.

The yield of aromatic aldehydes from the mature tissues of grasses and cereals such as corn stalks (Creighton and Hibbert, 1944), wheat straw (Pepper, Manolopoulo and Burton, 1962) and rye straw (Wildung, Chesters and Behmer, 1970) has generally been in the order syringaldehyde $\geqslant$ vanillin $\gg$ p-hydroxybenzaldehyde. The only information on a young grass is that of a 5% NaOH-extracted lignin from prairie grass (Wildung et al, 1970) which yielded only vanillin and syringaldehyde in low, but approximately equal, yields. In explanation, they suggested that p-hydroxybenzaldehyde was either destroyed or else the p-hydroxybenzaldehyde-yielding lignin units were modified during the extraction process. In the present investigation, however, syringaldehyde was the oxidation product obtained in lowest yield from Yorkshire fog lignin, while p-hydroxybenzaldehyde was present in small amounts. While these aldehyde yields may indicate a true generic difference in lignin structure they are more likely to be due to extraction variations. Since this whole field of taxonomic classification of lignin oxidation products is very little understood as yet, it is not wise to draw any conclusions about the phenyl-propanoid nature of the lignin of Yorkshire fog and its chemical relationship to the largely uninvestigated lignins of other monocotyledons.

Conclusions. In the absence of an investigation of model

lignin compounds in this study, and with inadequate literature data on other grass lignins for comparison, it is not possible to reach definite condlusions on the chemical nature of the lignin isolated from Yorkshire fog. The ultraviolet and infrared spectrometry indicate the purity of this lignin as well as the presence of typical 'lignin' functional groups. The sample has a moderate phenolic hydroxyl and -OMe content and, like most other monocotyledonous lignins, gave yields of syringaldehyde, vanillin and p-hydroxybenzaldehyde on alkaline nitrobenzene oxidation. The outstanding features of this lignin seem to be its appreciable content of etherified hydroxyl groups in the 4- position of the aromatic ring (U.V.) and the low recoveries of syringaldehyde.

2.6. ANALYSIS OF ORGANIC ACIDS

2.6.1. Analytical methods

Anion exchange chromatography Aliquots (5 cm^3) of the purified aqueous extract of organic acids (section 2.3.6.) were added to a column (10 cm x 1 cm) of the strongly basic anion exchange resin Dowex 1-X8 (3g, 200-400 mesh) prepared in the acetate form (Hulme and Wooltorton, 1958). The acids were separated by gradient elution with 1M acetic acid (100 cm^3), 6M acetic acid (50 cm^3) and 6M formic acid (200 cm^3) and the eluates collected as 5 cm^3 fractions by a mechanical fraction-collector. To obtain a reproducible separation of the acids a fixed volume mixing-chamber, similar to that employed by Hulme and Wooltorton, was used. The eluate fractions were evaporated to dryness by holding them in polythene flasks in a water bath at 60°C under a stream of warm air. Boiled, deionised water (10 cm^3) was added to each flask plus 3 drops of Bromothymol blue (40 mg in 200 cm^3 CO_2-free water plus 0.1M NaOH until neutral) and the solution of non-volatile organic acids titrated with 0.0112M barium hydroxide

solution.

Identification of acids by paper chromatography. Pooled eluate peaks were reduced to a small volume in a rotary evaporator and chromatographed on unwashed Whatman No. 1 in a descending solvent of the organic phase of tert-amyl alcohol/iso-amyl alcohol/ 90% formic acid/H_2O, 48:32:10:40 (v/v) for 24 hours. Organic acids were outlined with a spray of bromocresol green (0.04% w/v) in 95% ethanol (pH 6.7). Acids were identified by their Rf (Molloy, 1969) and the following spray reactions: Sulphanilamide/ β-naphthol/$NaNO_2$ (Schmidt, Fischer and McOwen, 1963) - all acids; 10% (v/v) acetic anhydride in pyridine (Buch, Montgomery and Porter, 1952) - citric acid (U.V.) and trans-aconitic acid (daylight and U.V.);
Na metaperiodate/nitroprusside/piperazine (Cartwright and Roberts, 1955) - quinic and shikimic acids;
K ferrocyanide/ferric ammonium sulphate (Martin, 1955) - oxalic acid;
Benedict's solution (Hasegawa, Johnson and Gould, 1966) - chlorogenic acid (also U.V. before spraying).

Trans-aconitic acid was determined quantitatively by the absorbence of the complex at 370 nm with a mixture of pyridine and acetic anhydride. Spots of trans-aconitic acid were outlined on the chromatograms under U.V. ($\lambda_{max.}$ = 254 nm) and eluted from the paper for six hours with 95% ethanol/pyridine/acetic anhydride (1:0.4:3.6 v/v) and the absorbence read in a Unicam SP 500 spectrophotometer. An excellent standard curve was obtained from known amounts of the acids (10 - 100 μg) which were chromatographed and eluted along with the unknown plant extracts.

Inorganic phosphate on the chromatograms was identified by an ammonium molybdate spray (Hanes and Isherwood, 1949)

2.6.2. Results and discussion

A typical ion-exchange resin elution spectrum of non-volatile organic acids from Yorkshire fog (sample 5/3/1968) is given in Fig. 8. Recoveries after elution of standard organic acid mixtures from the anion exchange column were as follows: shikimic (94.6%), quinic (95.1%), malic (98.4%), citric (91.2%) and trans-aconitic (45.7%), Low recoveries of trans-aconitic acid from ion-exchange columns have previously been reported (Busch, 1952) and, furthermore, the trans-aconitic acid fraction from the grasses was found to elute from the column with $H_2PO_4^-$. Consequently, levels of trans-aconitic acid were determined separately by paper chromatography of an aqueous extract of the grass and subsequent spectrophotometric estimation as a complex.

The levels of organic acids in several Yorkshire fog samples are given in Table 8 as percentage dry weight. They show a consistent pattern with trans-aconitic acid, as determined spectrophotometrically, providing the bulk of the acidity. Trace amounts of malonic, oxalic and chlorogenic acids were found by paper chromatography of the extracts but they could not be estimated quantitatively. Malonic acid eluted with the citric acid fraction and oxalic acid could not be eluted as a sharp peak from the anion-exchange column.

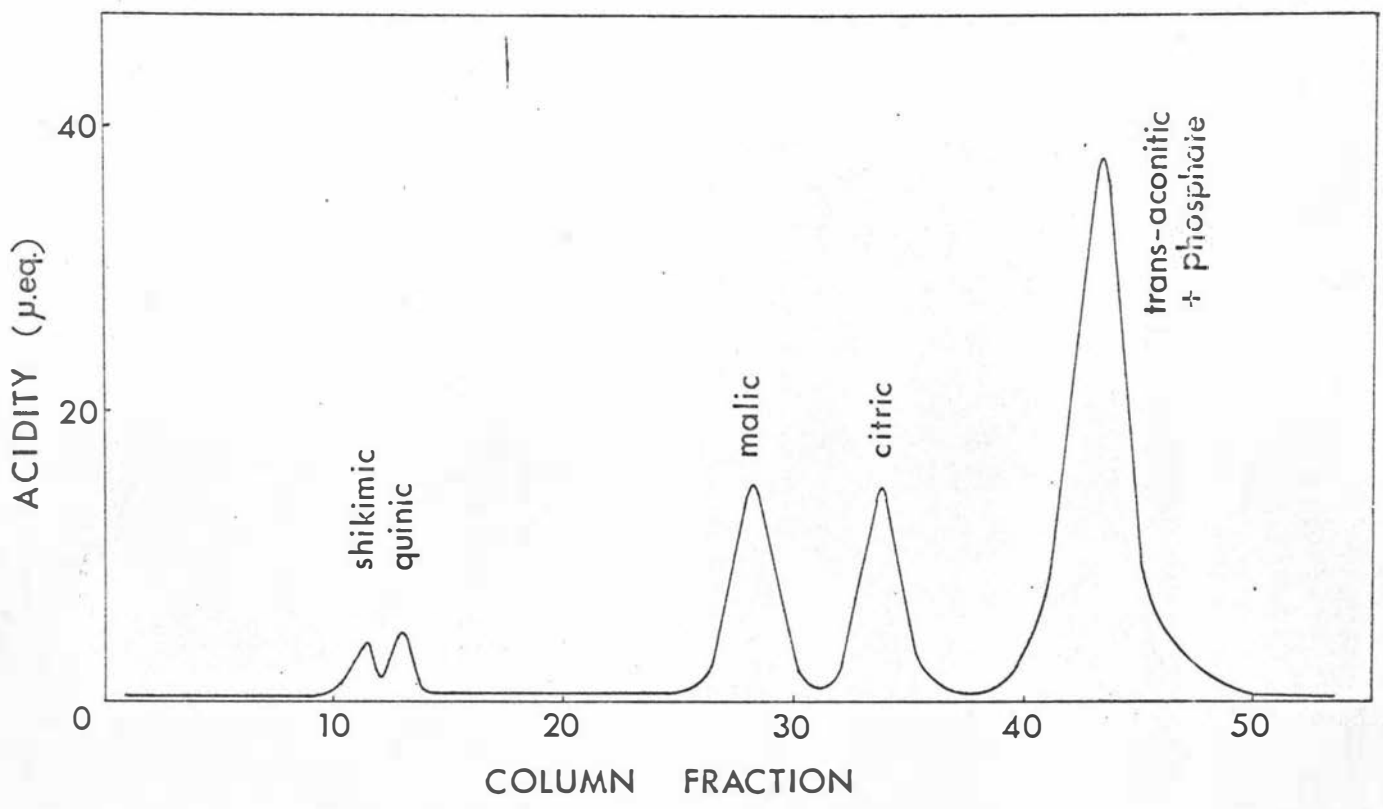
The organic acids occupy a central, dynamic position in the carbohydrate, protein and fat metabolism of plant cells. "In addition to functioning in catabolic changes, the organic acids produced by the dark fixation of carbon dioxide apparently act as important intermediates in photosynthesis. Organic acids may be accumulated in quantity as end products of metabolism; furthermore, the oxidation of organic acids has a close connection with growth." (Thimann and Bonner, 1950). Most organic acids in plants are

TABLE 8

NON-VOLATILE ORGANIC ACID CONTENT (% DRY WEIGHT) OF FOUR SEASONAL SAMPLES OF YORKSHIRE FOG

Acid	5/3/1968	26/10/1968	6/11/1968	10/5/1969
Shikimic	0.24	0.33	0.06	0.08
Quinic	0.21	0.49	0.07	0.08
Malic	0.35	0.39	0.33	0.20
Citric	0.30	0.35	0.36	0.33
trans-Aconitic	1.20	1.32	1.24	1.72
Malonic	-	trace	trace	-
Oxalic	trace	trace	trace	trace
Chlorogenic	trace	trace	trace	trace
cis-Aconitic	trace	trace	trace	trace

FIG. 8. ELUTION SPECTRUM OF NON-VOLATILE ORGANIC ACIDS FROM ANION EXCHANGE RESIN, DOWEX 1



metabolised through the Tricarboxylic acid (TCA or Krebs) cycle but little is known of the metabolism of acids not participating in the cycle. The TCA cycle functions not only as a respiratory pathway but as the means of providing intermediates for many biosynthetic reactions. It follows that carbon dioxide fixation by the plant is necessary to allow the formation of these intermediates while maintaining a steady state concentration of these cycle acids (Davies, 1959). The inverse relationship between carbohydrate and organic acids condentration and their diurnal fluctuations in the plant are well known. Carbon dioxide is incorporated into organic acids, usually malate, in the dark while light appears to prevent the photosynthetic carbon from entering the TCA cycle. In some special cases carbohydrates may be formed in the dark from the synthesised organic acids (Davies, 1959). Furthermore, the extent of organic acid formation is dependent upon mineral nutrition and can be related to the excess of soil cations (generally expressed as ΣCa^{++}, Mg^{++}, Na^{+} and K^{+}) over soil anions (generally expressed as ΣNO_3^{-}, SO_4^{--}, $H_2PO_4^{-}$, Cl^{-} and HCO_3^{-}) incorporated into the plant root (De Wit, Dijkshoorn and Noggle, 1963). The incorporation of nitrogen as either NO_3^{-} or NH_4^{+} can also have a marked effect upon the organic acid content of pasture plants.

The accumulation of relatively large quantities of non-volatile organic acids in plant tissues seems to depend upon the 'compartmentation' of these metabolites into 'non-metabolic pools' which are in equilibrium with 'turnover pools' actively involved in metabolism (MacLennan, Beevers and Harley, 1963). The proportion of organic acid in the 'non-metabolic pool' increases in progressively older plants; since vacuolation increases with age these workers postulated the vacuole as being the site of organic

acid 'compartmentation' in the leaf. Acids such as trans-aconitic acid accumulated to a large extent in the older tissues of corn plants (MacLennan and Beevers, 1964), and the 'compartmentation' hypothesis seems a reasonable explanation for the occurrence, in quantity, of such an unusual metabolite.

The cis- stereoisomer of aconitic acid only is involved in TCA cycle metabolism and trans-aconitic acid is known to be a competitive inhibitor of the enzyme Aconitic hydratase (Saffron and Prado, 1949) which converts citrate to iso-citrate via cis-aconitate. Consequently, significant levels of trans-aconitic acid can cause a block in the TCA cycle and lead to an accumulation of citrate in the tissue. It was this inhibitory metabolic function that led Burau and Stout (1965) to implicate the acid in the aetiology of hypomagnesemic tetany in ruminants. Notwithstanding this inhibitory function, trans-aconitic acid is found in a wide variety of plant tissues; in some plants it is the major organic acid while in most it is only present in trace amounts (Buch, 1960).

The major organic acids in pastures such as ryegrass, meadow foxtail and meadow fescue are quinic, citric and malic acids (Hulme and Richardson, 1954; Hirst and Ramstad, 1957) although trace amounts of shikimic acid (Richardson and Hulme, 1955) and succinic, malonic, glycollic, glyceric, glyoxylic, pyruvic, oxalic, fumaric, α-ketoglutaric and chlorogenic acids have been reported in grasses by these and other workers. That the organic acid composition of grasses varied with species was noted by Hirst and Ramstad (1957) who found lower contents of quinic, malic and citric acids, but large amounts of trans-aconitic acid in creeping soft grass (Holcus mollis). In an extensive investigation of 94 species in the foothill pastures of the western slopes of the

Sierra Nevada Mountains in California (Stout, Brownell and Burau, 1967), accumulators of trans-aconitic acid ($\geqslant$ 1% dry weight) were more frequently found among the gramineae (14 out of 30 species) with Phalaris tuberosa, Bromus rigidus and Bromus catharticus containing higher than 3.5% dry weight. They considered that the accumulation of trans-aconitate depended firstly upon species and secondly upon climatic and environmental factors. Under New Zealand grazing conditions the acid was found to accumulate in Prairie grass (Bromus unioloides) and Barley grass (Hordeum murinum) but was only present in trace amounts in Cocksfoot (Dactylis glomerata) and ryegrass species (Molloy, 1969).

Cis-aconitic acid is the less stable of the two stereo-isomers (Krebs and Eggleston, 1944) and the trans-aconitate reported in some of these analyses may have arisen through extremes of temperature and pH during extraction. However recent investigations have conclusively shown that approximately 95% of aconitate in 'accumulator' plants is in the trans- form (MacLennan and Beevers, 1964; Stout, Brownell and Burau, 1967; Molloy, 1969). In the present investigation only trace amounts of cis-aconitic acid were found under U.V.

The physiological basis for the accumulation on non-TCA cycle organic acids such as trans-aconitic acid in grasses, oxalic acid in spinach and rhubarb and fluoroacetic acid in the South African pasture plant, Dichapetalum cymosum, is of considerable interest. Do such acids exist in the vacuole in the free state or as a salt?; how does the absorbing tissue sense a preferential absorption of cations and respond by synthesising organic acids?; are such acids metabolic 'end products' deposited in vacuole 'compartments' after their involvement in transportation of cations from the root to the meristematic portions of the plant?; indeed,

are these organic acid accumulations the cause or the result of cation uptake?

In view of the earlier discussion on the diurnal fluctuations in plant organic acid levels, and in the absence of soil and climatic data from this investigation, it is of little value to discuss the variations in organic acid levels for the different samples in Table 8. Suffice to say that Yorkshire fog appears to be an accumulator of trans-aconitic acid while the 'normal' organic acids, citric and malic, are present in considerably lower quantities. The alicyclic acids, quinic and shikimic, are present in small quantities, the latter probably as an intermediate in the biosynthesis of phenylpropanoid amino acids and the guaicyl precursors of lignin (section 2.5.2.).

2.7. CONSTITUENT CHEMICAL ANALYSIS OF YORKSHIRE FOG

2.7.1. Results and discussion

Table 9 outlines a gross chemical analysis of the three seasonal samples of Yorkshire fog, late summer (5/3/1968), early spring (pooled 26/10/1968 and 6/11/1968) and autumn (10/5/1969). The left hand column gives the percentage weight loss in the residue with each extraction step, while the right hand column gives the purified polysaccharide or lignin from the respective extracts, as a percentage of the original dry weight of the grass.

Purified 'pectin' only made up approximately 15% of the weight of the EDTA extract, which also contained calcium, carbohydrate and nitrogen-containing compounds. However, purified hemicelluloses totalled about 90% of the 10% KOH extract, giving a fairly constant ratio of 1:3 with the cellulose residue. Apart from the crude protein levels, the proportions of the various fractions show no marked seasonal variations. This high crude protein level is typical of New Zealand pastures (White, Thompson

TABLE 9

CONSTITUENT ANALYSIS OF THREE SEASONAL SAMPLES OF YORKSHIRE FOG IN % DRY WEIGHT

Extract	Summer (5/3/1968)		Spring (26/10/1968 + 6/11/1968)		Autumn (10/5/1969)	
	Ext[d]	Purif.	Ext[d]	Purif.	Ext[d]	Purif.
benzene/ethanol	15.7		18.1		18.6	
water, 60°C	22.1		28.3		21.1	
EDTA (pectic substances)	7.0	1.1	5.9	1.0	6.2	0.9
pepsin	16.0		20.6		23.6	
5% KOH (lignin)		2.3		0.8		1.8
chloramine T	3.8		2.2		3.0	
10% KOH	8.6		5.4		5.9	
hemicellulose A		0.2		0.1		-
hemicellulose B (branched)		2.0		1.8		2.3
hemicellulose B (linear)		3.0		1.6		1.6
hemicellulose B(I)		2.8		1.2		1.7
cellulose residue	26.0		18.9		20.8	
Total	99.2		99.4		99.2	

Proximate analyses

Nitrogen	2.94	4.54	5.12
Crude protein (N x 6.25)	18.50	28.38	32.00
Lignin (Gailliard and Nijkamp, 1968)	4.5	3.3	4.1

TABLE 10

WATER-SOLUBLE CONSTITUENTS OF YORKSHIRE FOG (5/3/68)

Yorkshire fog		100g	
Ethanol/benzene extract		15.7g	
ether-soluble portion		= 5.8g	
water-soluble portion		= 9.9g	
Water extract, 60°C		= 21.1g	
total water-soluble extract		= 30.0g	
		(fructose	12.5
		(glucose	6.4
	% total	(galactose	4.0
	water-soluble	(sucrose	13.6
	extract	(protein (N x 6.25)	18.5
		(organic acids	8.1
		(ash	30.4
		Total	93.5

and Brice, 1948; Metson, Saunders, Collie and Graham, 1966) and this is reflected in the high values for the pepsin, water and benzene/ethanol extractives. Sample 5/3/1968 was collected after a very dry summer when most other grasses had dried off and this accounts for its low N content. The only other point of note is the variability of the branched to linear ratio of hemicellulose B with seasonal sample. In view of the inability to obtain pure polysaccharide fractions with the iodine/$CaCl_2$ fractionation procedure (section 2.4.2.) this variation was considered of little significance and was not investigated further.

The composition of the water-soluble material from the combined 60°C aqueous extract and the ether/water partition of the ethanol/benzene extract is given in Table 10. The carbohydrates were identified by the paper chromatographic/spectrophotometric method (section 2.4.1a). The values are of no particular significance and are similar to those reported for other pasture grasses (Waite and Gorrod, 1959b).

PART 3 THE COMPLEXING OF Ca^{++} AND Mg^{++} BY THE ORGANIC CONSTITUENTS OF YORKSHIRE FOG

3.1.1. Introduction

This section outlines an investigation of the ability of the organic fractions isolated from Yorkshire fog (see Part 2) to complex Ca^{++} and Mg^{++} in a solution of ionic strength and composition similar to that of the small intestine of a ruminant (van't Klooster, 1967). This simplified aqueous solution contained the cations Ca^{++}, Mg^{++}, Na^{+}, K^{+}, NH_4^{+} as chlorides, and thereby avoided the anionic complexity of physiological solutions. Even so, the thermodynamic equilibria of solutions of such simple, mixed electrolytes are extremely complex, involving a large number of degrees of freedom which reflect a multiplicity of interactions occuring in the electrolyte solution - long-range electrostatic attractions and repulsions, short-range Van der Waals forces, ionic hydration and ion dipole effects, ionic size effects, ion pair formations, etc. The stability constants of metal ion - organic ligand complexes can be obtained for simple binary electrolyte solutions but are extremely difficult to determine in mixed electrolytes. This is due not only to the large number of cation-ligand equilibria but also to the inadequacies of present theoretical descriptions of mixed electrolyte interactions.

Because of the complexity of these electrolyte solutions, it was considered sufficient for the purpose of this investigation to determine:

(1) Whether the organic fractions isolated from Yorkshire fog gave any evidence of cation complexing.

(2) If such complexing did occur, the comparative complexing ability of the various fractions, regardless of the structure of the cation-ligand complex.

Since most of the plant fractions studied were insoluble cell

wall polymers of unknown molecular weight, an experimental method which avoided this problem was necessary. Ultrafiltration and dialysis have been used extensively in studying the physico-chemical activities of cations in intestinal digesta (Smith and McAllan, 1966; van't Klooster, 1966; Storry, 1961) but these techniques can be severely criticised since they are non-equilibrium processes. Specific calcium and magnesium electrodes could be used but they are easily poisoned in biological solutions. Cation exchange resins have been used in the study of ionic and complexed cations in milk (van Kreveld and van Minnen, 1955) and ruminant intestinal ultrafiltrates (van't Klooster, 1967). This method has been used in the present investigation since it is an equilibrium process whereby the equilibrium between a salt solution and the potential complexant is reflected in the cationic composition of the resin. The distribution of cations on the resin at equilibrium can then be interpreted to gain information on the activities of the individual cations in the solution.

3.1.2. Electrolytes and the concept of ionic activity

As mentioned in the previous section, it is difficult to give a complete theoretical account on the thermodynamic properties of even a simple 1:1 electrolyte solution since this requires a knowledge of the long-range interionic forces as well as the short-range interactions between ions and solvent molecules. The five salts used in this investigation are all strong, or 'non-associated', electrolytes and can be considered to dissociate completely into ions in aqueous solution.

For a simple, binary electrolyte such as NaCl, the chemical potential of the solute, $\bar{G}$, can be thermodynamically described by:

$$\bar{G} = \bar{G}^{o} + RT \ln m + RT \ln \gamma_{\pm} \qquad .....(3.1.1.)$$

$$\text{or } \bar{G} = \bar{G}^{o} + RT \ln a \qquad .....(3.1.2.)$$

$$\text{where } a = m \times \gamma_{\pm} \qquad .....(3.1.3.)$$

and $\bar{G}^{o}$ = the standard chemical potential of the solute at any chosen standard state, usually a 1 molal ideal solution

m = concentration of the solute as molality

R = gas constant T = absolute temperature

$\gamma_{\pm}$ = mean molal activity coefficient of the electrolyte and $\gamma_{\pm} \rightarrow 1$ as $m \rightarrow 0$.

a = activity of the electrolyte.

The term $RT \ln \gamma_{\pm}$ arises from the effect of all those factors such as ion-ion interaction or ion-solvent interaction which are considered not to be present in the ideal solution.

The contribution of ion-ion interaction to the activity coefficient has been predicted with considerable success by the Debye-Huckel theory and its various extensions. A form of the Debye-Huckel equation which holds for many single electrolytes up to an ionic strength of about 0.1 is:

$$\log f_{\pm} = \frac{-A|Z_1 Z_2|\sqrt{I}}{1 + Ba^{o}\sqrt{I}} \qquad .....(3.1.4.)$$

$f_{\pm}$ = mean rational activity coefficient for an electrolyte

A,B = constants involving the absolute temperature and dielectric constant of the solvent

$\underline{a}$ = a parameter equal to the mean effective diameter of the ions or their distance of closest approach

The numerator in eqn. (3.1.4.) gives the effect of the long-range coulombic forces, while the denominator allows for the short-range interactions between the ions.

The effect of ion-solvent interaction on the activity of the electrolyte is more difficult to account for in terms of a model. The entropy of a system is related in part to the degree of spatial disorder in the system. The introduction of charged particles into water would be expected to cause a considerable entropy loss, owing to 'ordering' of the water molecules because of the

intense electrical field of the ions in the solution. However, Frank and Evans (1945) have suggested that the ionic charges from dissociated electrolytes could also tend to increase the entropy of the system by increasing the disorder in the solvent beyond the primary solvation layer. This 'structure breaking entropy' for alkali- and alkaline-earth halides is greatest for the largest ions and corresponds to a considerable increase in disorder. From the Gibbs-Helmholz equation,

$$\Delta G = \Delta H - T\Delta S \qquad .....(3.1.5.)$$

where ΔG = change in free energy

ΔH = change in enthalpy

ΔS = change in entropy

it is clear that such an increase in entropy would bring about a decrease in the free energy of the system, which from eqn. (3.1.1.) would lead to a corresponding decrease in the activity coefficient of the solute as compared with a solution containing only non-charged particles.

A further aspect of ion-solvent interaction is that the short-range forces between the ions and and the solvent dipoles will tend to hold the solvent in solution with a consequent decrease in solvent vapour pressure from the ideal value and a corresponding increase in the activity coefficient of the solute. Since the effect of such forces is negligible at very low solute concentrations, electrostatic attractions can account for the bulk of the non-ideality of dilute systems and the Debye-Huckel limiting law can be used to calculate activity coefficients in these cases. However, at higher concentrations of solute, (ca 1m), the short-range effects, which increase linearly with concentration, may be paramount. This behaviour accounts for the characteristic variation of electrolyte activity coefficients from $\gamma_{\pm} < 1$ to $\gamma_{\pm} > 1$ at moderately low and high concentrations respectively, while both effects

are of comparable magnitude at concentrations of the order of 1 molal.

It has not been possible to account for these ion-solvent effects quantitatively in terms of a model. However, some success in predicting the observed behaviour of single electrolytes up to at least 1 molal concentration has been achieved by adding an empirical term linear in ionic strength to the Debye-Huckel equation to give

$$\log f_{\pm} = \frac{-A|Z_1 Z_2|\sqrt{I}}{1 + Ba^{\circ}\sqrt{I}} + bI \qquad .....(3.1.6.)$$

This equation contains two adjustable parameters a and b. A modification which has been widely adopted is to put $aB = 1$ (ie. $a^{\circ} = 0.304$ nm for all electrolytes at 298K), so that eqn. (3.1.6.) becomes

$$\log f_{\pm} = \frac{-A|Z_1 Z_2|\sqrt{I}}{1 + \sqrt{I}} + bI \qquad .....(3.1.7.)$$

The value of the adjustable parameter b is chosen to give the best fit to experimental results. Davies (1938) has shown that the relationship $b = 0.1|Z_1 Z_2|$ gives a useful guide to the best value of b, and can be used to estimate activity coefficients when no experimental results are available.

The discussion so far has considered the effects of ion-ion interaction and ion-solvent interaction in a solution of a single electrolyte. A further factor which must be taken into account in mixed electrolyte solutions is the difference in the interactions which may occur between a particular anion (for example) and two different cations, or between a particular cation and two different anions. This effect is commonly referred to as 'specific ion interaction' in contrast to the general ion interaction already considered in the Debye-Huckel theory. Guggenheim (1935) has shown that it is possible to take account of specific ion inter-

action in mixed electrolyte solutions by an extended Debye-Huckel equation of the form

$$\log \gamma_{\pm} \text{ (for electrolyte MX)} = \frac{-A/Z_M Z_X/\sqrt{I}}{1 + \sqrt{I}} + \frac{V_+}{V_+ + V_-} \sum_{X'} B_{M,X'} m_{X'} + \frac{V_-}{V_+ + V_-} \sum_{M'} B_{M',X} m_{M'} \quad .....(3.1.8.)$$

where V = number of moles of ions from one mole of dissociated electrolyte, MX

m = molality

M',X' = other cations or anions present apart from M and X

B = empirical interaction coefficient for the specified cation-anion pair

It should be noted that the so-called specific ion interaction terms in eqn. (3.1.8.) replace the bI term in eqn. (3.1.7.), and must therefore include the effect of ion-solvent interaction as well as that of specific ion interactions.

Standard salt solution The mixed electrolyte solution (standard salt solution) used in this investigation contained the following molal concentrations: $CaCl_2$, 0.00615; $MgCl_2$, 0.00360; NaCl, 0.07980; KCl, 0.02620; NH_4Cl, 0.01090. The ionic strength, I ($= \sum m_i z_i^2$) was 0.146. Since $[Ca^{++}]$ and $[Mg^{++}]$ comprise only 13.3% of the ionic strength of the standard salt solution, the monovalent ions effectively act as a 'swamping' electrolyte. In such a situation, large percentage changes in $[Ca^{++}]$ and $[Mg^{++}]$ cause only small changes in solution ionic strength with expected small changes in ionic activity coefficients. For example, a 50% decrease in $[Ca^{++}]$ and $[Mg^{++}]$ in the standard salt solution only decreases the ionic strength by 6.67% to 0.136. If the assumption can be made that this small change in ionic strength produces only

a negligible change in the activity coefficients of the ionic species involved, then to a good first approximation all calculations can be done in terms of concentrations, which can be readily determined by experiment.

A sufficiently good estimate for this purpose of the changes in the activity coefficients with change in total ionic strength from 0.146 to 0.136 can be obtained by using eqn. (3.1.7.) in the form suggested by Davies, ie., with $b = 0.1/Z_1Z_2/$. Use of this equation ignores specific ion interaction effects but these cannot yet be predicted accurately in solutions as complex as the present standard salt solution, and in any case they will make only second order corrections to the changes in activity coefficients predicted by eqn. (3.1.7.).

The calculated activity coefficients at the two ionic strengths are shown in Table 11. It is apparent that there are only small changes in the values of $\gamma_{\pm}$ when the ionic strength changes from 0.146 to 0.136 (which is equivalent to a 50% decrease in both Ca^{++} and Mg^{++} concentration in the standard salt solution).

TABLE 11

ACTIVITY COEFFICIENTS CALCULATED FOR ELECTROLYTES IN STANDARD SALT SOLUTION

Electrolyte in standard salt solution	Molality of electrolyte	Calculated activity coefficients*	
		$\gamma_{\pm}$ at I = 0.146	$\gamma_{\pm}$ at I = 0.136
$CaCl_2$	0.00615	0.559	0.566
$MgCl_2$	0.00360	0.570	0.578
NaCl	0.07980	0.746	0.751
KCl	0.02620	0.740	0.744
NH_4Cl	0.01090	0.743	0.747

* $\gamma_{\pm}$ calculated from Davies (1938) modification of Debye-Huckel equation (section 3.1.2.), $\log f_{\pm} = \dfrac{-A/Z_1Z_2/\sqrt{I}}{1 + \sqrt{I}} + bI$ where

$b = 0.1/Z_1Z_2/$ and $\gamma_{\pm}$ the mean molal activity coefficient is

calculated from $f_{\pm}$, the mean rational activity coefficient, by the relationship $\gamma_{\pm} = \frac{f_{\pm}}{1 + 0.001 v W_A m}$ (Robinson and Stokes, 1959)

Since these fluctuations in activity coefficients are virtually negligible, the activity of any particular ion in the solution will be proportional to its concentration from the relationship of eqn. (3.1.3.), $a = m \times \gamma$. Furthermore, it is reasonably well-established that the exchange affinities of cation exchange resins reflect the activities of the cations in the equilibrium solution (Boyd, Schubert and Adamson, 1947; Bonner, Argersinger, and Davidson, 1952). Hence, under the present experimental conditions of nearly constant 'swamping' ionic strength, the concentrations of the divalent cations Ca^{++} and Mg^{++} in the solution should be proportional to their load on the resin at equilibrium.

3.2. ION EXCHANGE METHODOLOGY

3.2.1. Cation exchange equilibria

Extensive attempts have been made to formulate ion exchange equilibrium relationships by assuming either (1) a simple adsorption process of the Langmuir type or (2) the application of the Law of Mass Action (Kunin, 1958). Each approach is successful only to a first approximation, with most difficulties being those inherent to all mixed electrolyte systems - the precise determination of ionic activities in the equilibrium solution as well as in the solid exchangers. The interpretation of equilibrium exchange data is further complicated by the heterogeneous nature of the functional groups in some exchangers as well as the marked influence of the degree of crosslinking (percentage divinylbenzene, % DVB) in the resin. As mentioned above, the distribution, or 'loading', of cations in a particular cation exchange resin depends primarily upon the activity of the particular cations in the equi-

librium solution. However, the selectivity of a resin for any particular cation depends upon a variety of factors:

(a) the type of functional group in the resin and the degree of cross-linking,

(b) the degree of hydration of the particular cations,

(c) the pH of the solution,

(d) the ionic strength of the solution, etc.

Notwithstanding these variables, the total resin cation load (in milliequivalents*/gram dry resin) will always be a constant which is an individual characteristic of the resin type (van Kreveld and van Minnen, 1955). This total load serves as an important check upon the accuracy of the analytical techniques used to determine the cation concentrations in the resin eluates.

Since the activity coefficients of the typical non-associated electrolytes used in this study vary with concentration (bottom p.66), it follows that similar electrolyte activities could be obtained at different electrolyte concentrations - and thereby different solution ionic strengths. Consequently, similar resin cation loads could be caused by solutions of widely differing ionic strengths. As already pointed out, however, large decreases in calcium and magnesium concentration only caused slight reductions in the ionic strength of the standard salt solution. In this case, the technique of cation exchange is ideal since the concentrations of the cations on the resin are proportional to their concentrations in the solution at this nearly constant ionic strength. Furthermore, as the resin beads constitute a completely immiscible phase, the technique is equally applicable to water soluble (organic acids and most hemicelluloses) and insoluble (lignin and cellulose) plant fractions which may bind calcium and magnesium in either a soluble or insoluble non-ionic form.

* One equivalent of an ion is defined as one mole (mol) divided by the ion charge number (z), ie., eq = mol/z

3.2.2. Resin equilibration with standard salt solution

The standard salt solution (pH 6.5, ionic strength 0.146) contained Ca^{++} 12.3 meq/l, Mg^{++} 7.2 meq/l, Na^{+} 79.8 meq/l, K^{+} 26.2 meq/l and NH_4^{+} 10.9 meq/l, all as chlorides and corresponded to the concentration of these cations in the duodenal ultrafiltrate of a cow. (van't Klooster, 1967). The solution was made from A.R. grade NaOH, NaCl, KCl, NH_4Cl, $CaCO_3$ and Mg ribbon all stored under vacuum over anhydrous $CaSO_4$. Appropriate weights of $CaCO_3$ and freshly acid-cleaned Mg ribbon were dissolved in a slight excess of 1M HCl, the pH adjusted with pellets of NaOH and the remainder of the cations added as the chlorides before diluting the solution to the appropriate volume with deionised water. A few drops of chloroform were added to inhibit microbial growth and the solution stored at $4^{o}C$ before use. New batches of the solution were prepared every three days.

A large volume of standard salt solution (20-30 l) was passed through a column of Dowex 50-X12 (12% DVB), a strong cation exchange resin of cross-linked polystyrene type with $-SO_3H$ as sole functional group (50g, 100-200 mesh), until the effluent had the same concentration of cations as the original standard salt solution. The resin bed equilibration dynamics, with passage of standard salt solution are illustrated in Fig. 9. The rapid uptake of Na^{+} is no doubt a reflection of the high concentration of this cation in the solution. However, equilibrium is gradually reached, with K^{+} and NH_4^{+} displacing the 'Na^{+}-saturated resin' while they, in turn, are gradually replaced by the divalent cations Ca^{++} and Mg^{++}, until the column effluent has the composition of the standard salt solution. The affinity of the resin for calcium is evident from the cationic load of the resin at equilibrium (Table 12). The final column of 'selectivity coefficients' in Table 12 is an index of the affinity of Dowex 50-X12 for various

FIG. 9 UPTAKE OF CATIONS BY EQUILIBRATING RESIN AS INDICATED BY EFFLUENT CATION CONCENTRATION

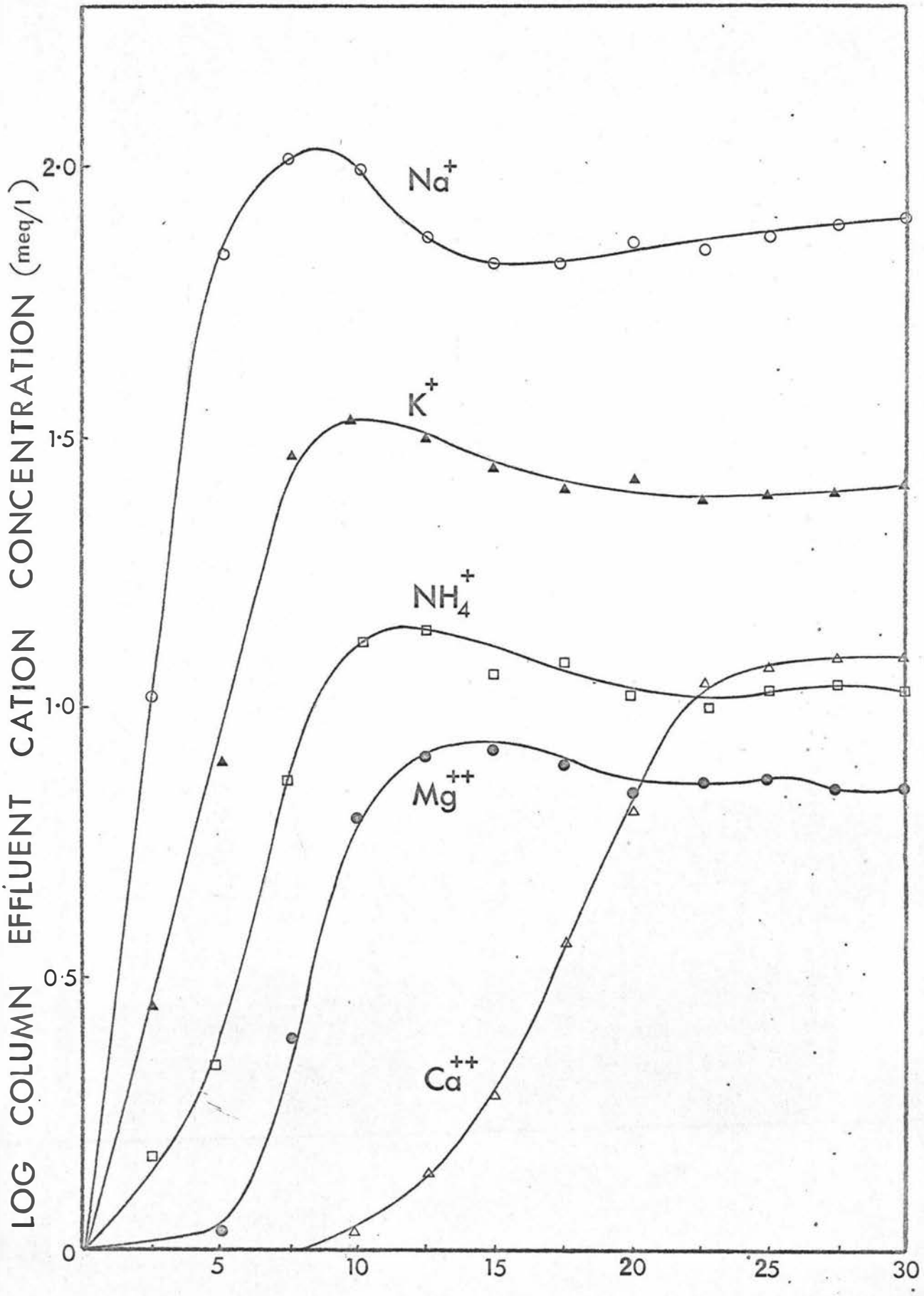


TABLE 12

CATIONIC LOAD OF RESIN IN EQUILIBRIUM WITH STANDARD SALT SOLUTION

Standard salt solution concentration (meq/l)		Individual cations as % of total solution cation concentration	Resin cation loading* (meq/g) $\pm$ SE_{mean}	Individual resin cation load as % of total	Cation selectivity coefficient, K^{m}_{100-m}
Ca^{++}	12.3	9.02	2.330 $\pm$ 0.008	52.54	11.155
Mg^{++}	7.2	5.28	0.430 $\pm$ 0.007	9.70	1.927
K^{+}	26.2	19.21	0.662 $\pm$ 0.005	14.93	0.738
NH_4^{+}	10.9	7.99	0.225 $\pm$ 0.010	5.07	0.615
Na^{+}	79.8	58.50	0.788 $\pm$ 0.010	17.77	0.153
Total	136.4	Total	4.435 $\pm$ 0.011		

* Mean of 6 individual values (2 duplicate dilutions from 3 resin eluates)

SE_{mean} = standard error of the mean

TABLE 13

IONIC CHARGE/RADII RELATIONSHIPS

	r^{*}_{cryst}	e^2/r_{cryst}	r^{**}_{hyd}	e^2/r_{hyd}
Na^{+}	0.98	1.02	7.9	0.13
K^{+}	1.33	0.75	5.3	0.19
NH_4^{+}	1.43	0.70	5.37	0.18[5]
Mg^{++}	0.82	4.88	10.8	0.37
Ca^{++}	1.17	3.42	9.6	0.41
Sr^{++}	1.34	2.98	9.4	0.42
Ba^{++}	1.49	2.68	8.8	0.45

* Wiklander (1946) ** Jenny (1932)

cations. For this mixed electrolyte solution, a selectivity coefficient, K^{m}_{100-m} has been defined as follows:

$$K^{m}_{100-m} = \frac{(m)_R}{(100-m)_R} \times \frac{(100-m)_S}{(m)_S}$$

where R = resin phase

S = solution phase

m = concentration (in meq per unit weight or volume) of any cation, M^{n+}, as % of total cation concentration in either phase.

100-m = concentration (in meq per unit weight or volume) of remainder of cations, 100-m, as % of total cation concentration in either phase.

These selectivity coefficients clearly show that the resin exchange affinity for the various ions is in the order $Ca^{++} > Mg^{++} > K^{+} > NH_4^{+} > Na^{+}$, as has already been shown in a qualitative way by the replacement dynamics illustrated in Fig. 9.

This difference in ion exchange resin cation affinity is best explained by the ionic hydration theory. Because of the high dielectric constant of water, ions in aqueous solutions of electrolytes are hydrated to an extent which depends upon the charge and size of the ion. This degree of ionic hydration has been shown to increase with increasing ionic charge and decreasing crystallographic radius - indicating a simple electrostatic attraction between the ion and the water molecule dipole. The increasing affinity of an ion exchange resin for cations of decreasing <u>hydrated</u> ionic radius and increasing charge is shown in Fig. 10, which is from the data of Nachod and Wood (1945). Furthermore, Boyd, Schubert and Adamson (1947) have correlated ion exchange affinity with a^{o}, the Debye-Huckel ion size parameter in eqn. (3.1.4) which itself is an index of ionic hydration. The expression e^2/r has been extensively used in relating ion exchange selectivity to

ionic charge and radius and this charge/radius ratio for these cations is presented in Table 13 with $r_{crystallographic}$ and $r_{hydrated}$ from a variety of sources. It is clear that for the five cations in the standard salt solution, e^2/r_{hyd} has the order $Ca^{++} > Mg^{++} > K^+ > NH_4^+ > Na^+$, which is identical to the order found for their resin selectivity coefficients (Table 12). Thus, as a working approximation for strong cation exchange resins of the Dowex 50 type, the exchange potentials of equivalent ions in aqueous solution are related directly to their crystallographic radius and inversely to their hydrated radius.

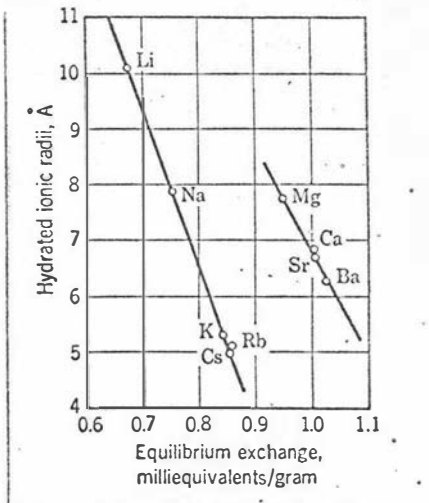


Fig. 10 Effect of ionic radius on ion exchange in a carbonaceous zeolite. Data of Nachod and Wood.

3.2.3. Preparation of resin eluates and cation analyses

The equilibrated resin (designated 'standard resin') was also stored at 4°C under a small volume of standard salt solution containing a few drops of chloroform until required for use.

Resin eluates were prepared by pipetting 0.7 cm^3 (= 0.33g dry resin) of wet resin onto a sintered glass filter under suction and washing quickly with deionised water (2 x 10 cm^3). This washing step was necessary to remove exterior salt solution and provided it was not prolonged, did not cause any exchange of cations from the resin beads. The washed resin was then transferred to another flask and shaken with 20 cm^3 of 2M HCl for 15 minutes. The contents of the flask were then transferred to a weighed, sintered-glass filter and washed with 1M HCl (20 cm^3), 0.1M HCl (20 cm^3), deionised water (20 cm^3) and the filtrate made up to 100 cm^3 with deionised water. Aliquots (5 cm^3) of the filtrate were diluted to 50 cm^3 with 0.1M HCl for cation analysis.

The filter containing the washed resin was heated overnight

at 108°C and the weight of resin recorded to enable cation loads to be expressed on a dry weight of resin basis (meq/g).

Resin eluate dilutions were initially stored in glass flasks prior to analysis but this was found to give a slight increase in $[Na^+]$ and $[K^+]$ over 24 hours. Problems were also encountered with polyvinyl chloride containers which functioned as reasonably efficient ion exchangers, probably because of the presence of phthalate plasticisers. The problem was traced to the use of the bottles for storing the initial resin eluate (x 10 conc.) before dilution. If this was done, and the bottle used for a subsequent diluted solution, then calcium and magnesium were gradually exchanged off the vessel walls over a 24 hour period. Magnesium was particularly suspect since it was analysed at a low concentration of the order of 1 - 2 parts per million (ppm or $\mu g/cm^3$). Provided the plastic bottles were used only for the diluted solutions and the solutions were analysed for magnesium within 6 - 8 hours of dilution no problems were encountered.

Calcium and magnesium (4 - 24 and 0.2 - 2.4 ppm respectively) were determined in duplicate resin eluate dilutions by atomic absorption spectrophotometry (Willis, 1960a and b) in a Techtron A.A.5 spectrophotometer with a hollow cathode Ca/Mg lamp. Sodium (2 - 10 ppm) was similarly determined by flame emission spectrophotometry in a Techtron A.A.3 spectrophotometer while potassium (2 - 12 ppm) was determined by flame emission photometry in a Gallenkamp Na/K flame photometer. Stock standard solutions of Ca^{++} (1000 ppm), Mg^{++} (50 ppm), K^+ (500 ppm), Na^+ (250 ppm) and NH_4^+ (100 ppm) were made up in 0.1M HCl each week and stored at 4°C. Appropriate duplicate dilutions were made with 0.1M HCl (in deionised water) for standard curves for each batch of analyses. Excellent curves with linearity over the concentration ranges indicated were invariably obtained under the operating conditions

outlined in Table 14.

Ammonium ion was determined as ammonia by a slight variation of the Yuen and Pollard (1952) modification of the Nessler reaction. Because of the high concentration of the interfering metallic cations, the addition of 2 cm^3 of 10% Na/K tartrate was found to be necessary to suppress flocculation of the complex. A linear standard curve was obtained when 2 cm^3 of undiluted resin eluate (containing 10 - 50 μg of NH_3), 2 cm^3 of Na/K tartrate solution and 2 cm^3 of Nessler reagent (KI/HgI_2 mixture in NaOH solution) were diluted to 50 cm^3 and allowed to stand in diffused light for 30 minutes before reading the absorbence of the orange complex. Optical densities over this concentration range of 0.2 - 1.0 ppm NH_3 were read at 435 nm in an SP500 U.V./visible spectrophotometer; under these conditions flocculation did not occur until approximately 90 minutes after adding the reagent.

Because of the necessity of measuring small, but possibly significant, variations in cation concentration, an evaluation of the precision of the analytical and instrumentational procedure was required. Ten separate dilutions, generally at six standard curve concentration levels, of the stock salt solution were analysed for each cation. The coefficients of variation ($= \frac{\text{standard deviation}}{\text{mean}} \times 100$) as a percentage is given for each cation at different concentration levels in the lower part of Table 14. The precision of determination by atomic absorption spectroscopy was $Mg \gg Na \geqslant Ca$. The precision of determination for K, as determined by flame photometry, was not as good as that for Ca. This was probably due to small fluctuations in coal gas pressure which sould not be controlled. However, the precision of determination for these metallic cations, especially for Mg, was considered to be highly satisfactory and is certainly superior to classical titrimetric and gravimetric methods. On the other hand,

TABLE 14. ATOMIC ABSORPTION OPERATING CONDITIONS AND COEFFICIENTS OF VARIATION AS AN INDEX OF PRECISION OF DETERMINATION FOR 5 CATIONS

	Ca		Mg		Na		K		NH_3	
Acetylene flow (litres/min.)	0.6		0.6		0.6					
Air flow (litres/min.)	4		4		4					
Lamp current (mA)	4		4		-					
Slit width (μ)	25		50		100					
Wavelength (A^o)	4227		2852		5893					
	ppm*	% c.of v.	ppm	% c.of v.	ppm	% c.of v.	ppm	% c.of v.	ppm	% c.of v.
Coefficients of variation+ (% c.of v.) at points on standard curve (each point mean of 10)	4	5.67	0.4	2.23	2.0	2.93	2.0	4.52	0.4	12.52
	8	3.22	0.8	1.32	3.0	1.63	4.0	3.68	0.6	11.55
	12	2.45	1.2	0.79	4.0	1.92	6.0	3.12	0.8	9.80
	16	2.37	1.6	0.45	6.0	0.53	8.0	2.81	1.0	14.58
	20	2.56	2.0	0.62	8.0	0.71	10.0	2.09	-	-
	24	1.31	2.4	0.45	10.0	1.02	12.0	2.05	-	-

*. Concentration of standard in parts per million ($\mu g/cm^3$)

+ % coefficient of variation = (standard deviation/mean x 100)

the coefficients of variation for the ammonia analyses were considered to be rather high and, consequently, in such determinations triplicate dilutions were analysed for both standard curves and unknowns. Since NH_4^+ provided only approximately 5% of the total resin load (Table 12), this moderate variability in determination was considered to be of little importance.

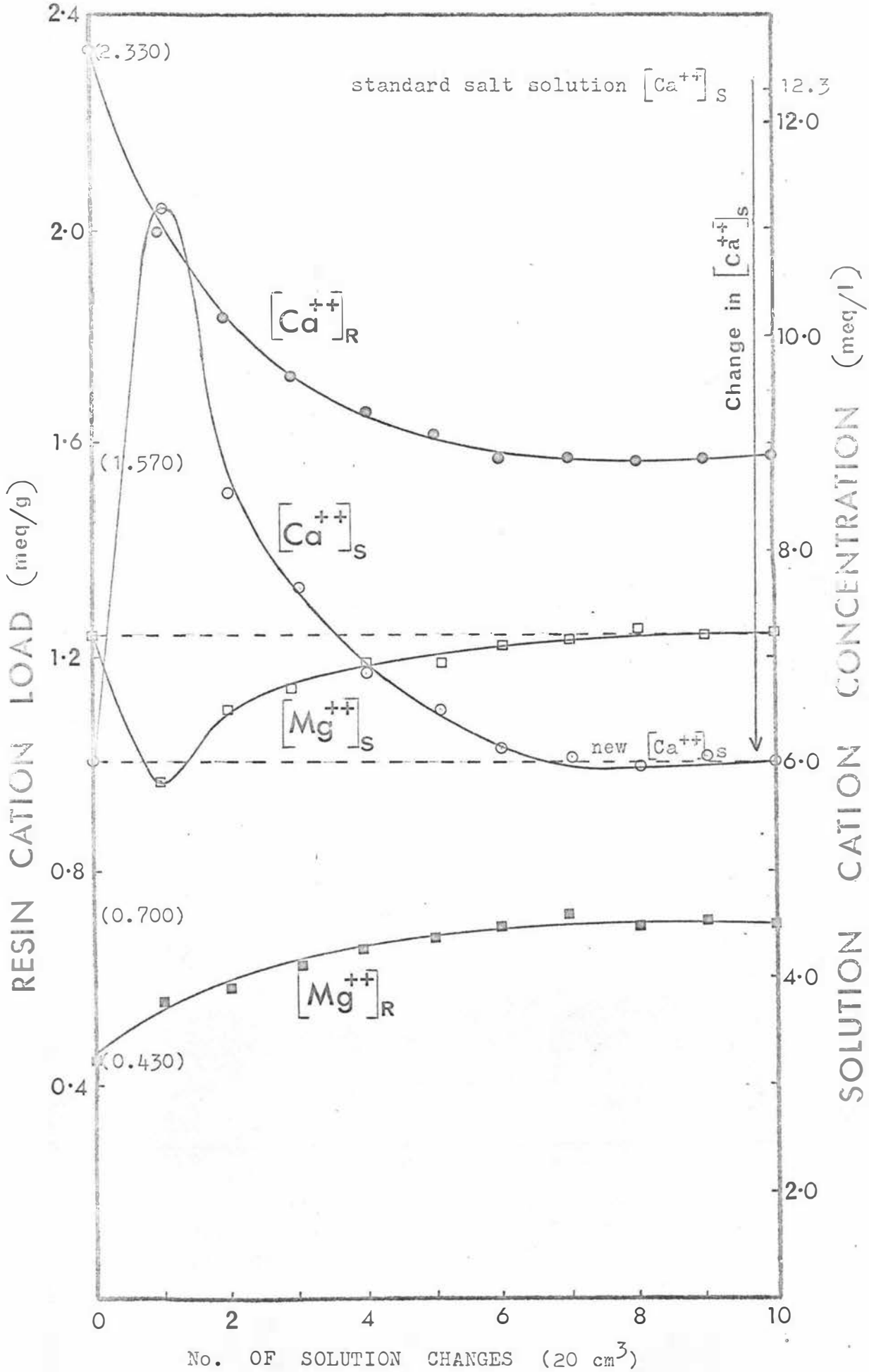
3.2.4. Resin calibration

To calibrate the relationship between resin cation loads and equilibrium solution cation concentrations, portions of standard resin were re-equilibrated with calibration solutions, each with the same Mg^{++}, Na^+, K^+ and NH_4^+ concentrations as the standard salt solution but decreasing in Ca^{++} concentration from 12.3 meq/l (standard value) to 5.9 meq/l. The calibration solutions (31) were passed through micro-columns (6 cm x 0.6 cm) containing 2g of standard resin. Simple linear and multiple regressions were fitted to relate variation in resin bound Ca^{++} and Mg^{++} to changes in the ionic composition of the salt solutions. The regression relationships were used as calibration equations to determine the amounts of bound and ionic Ca^{++} and Mg^{++} in solutions in equilibrium with complexing ligands (see section 3.3.4.).

3.2.5. Equilibration with complexing ligand

Mixtures of standard salt solution (20 cm^3), standard resin (0.7 cm^3) and complexant (50 mg of organic acid, lignin of polysaccharide) were gently shaken for 15 minutes. The change in the activities of Ca^{++} and Mg^{++} in the solution, on the addition of the complexant, was reflected in the resin load which adjusted to a new equilibrium value. However, the solution now was no longer the 'complexed standard salt solution' but one reflecting the adjustment of the resin to the new situation. This type of 'false equilibrium' is illustrated in Fig. 11. which is a plot of the experimental data obtained when 0.7 cm^3 of standard resin was

FIG. 11 PROGRESSIVE RESIN ADJUSTMENT TO NEW EQUILIBRIUM BY SUCCESSIVE EQUILIBRATIONS WITH A NEW SOLUTION



equilibrated with a solution containing only 6.0 meq/l of Ca^{++} (ie., simulating a solution where the $[Ca^{++}]$ in the standard salt solution (12.3 meq/l) had been reduced to this value through the effect of an added complexant). While this single equilibration with the solution containing only 6.0 meq/l of Ca^{++} does reduce the calcium concentration on the resin it is clear that the process needs to be repeated a number of times (eight appeared to be sufficient for this change in concentration) until the true equilibrium resin loading is reached. Fig. 11 is the result of ten separate equilibration experiments in which solution calcium $[Ca^{++}]_S$ and magnesium $[Mg^{++}]_S$ as well as resin calcium $[Ca^{++}]_R$ and magnesium $[Mg^{++}]_R$ were analysed after consecutive 15 minute equilibrations with 20 cm^3 portions of the new solution (6.0 meq/l in $[Ca^{++}]_S$). As the standard resin $[Ca^{++}]_R$ (= 2.330 meq/g) decreased to the new equilibrium value (1.570 meq/l), the calcium concentration in the equilibrating solution rose rapidly to an initial maximum of 11.15 meq/l before gradually dropping back to the new (equilibrium) value of 6.0 meq/l. This behaviour was merely a reflection of the complementary displacement of resin Ca^{++} by solution Mg^{++} (and the other univalent cations) in their adjustment to the new equilibrium. Consequently, $[Mg^{++}]_R$ increases to the new equilibrium value of 0.700 meq/g along with corresponding increases in $[Na^+]_R$, $[K^+]_R$ and $[NH_4^+]_R$ (which are not shown in Fig. 11 in order to avoid confusion). However, it must be borne in mind that the total resin cation load of approximately 4.435 $\pm$ 0.011 meq/g (Table 12) is constant (see section 3.2.1.).

In view of the equilibrium adjustment illustrated in Fig. 11, a simplified procedure was adopted whereby a bulk mixture of standard salt solution (200 cm^3) and complexant (500 mg) was made up and allowed to stand for one hour to equilibrate before successive 20 cm^3 portions were shaken with the resin. In making up the

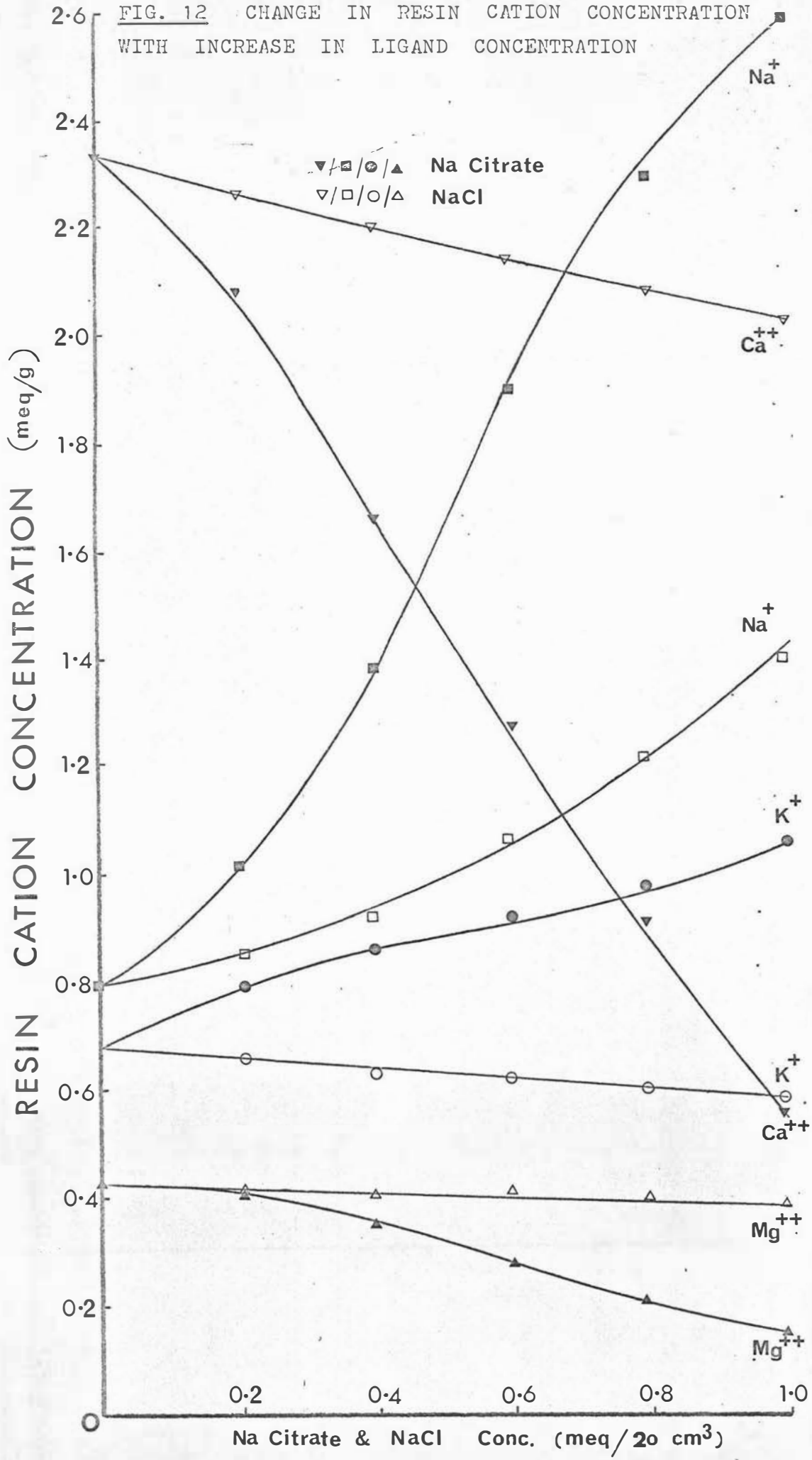
solution organic acids were added as the sodium salt to a salt solution lacking NaCl and the solution then made up to the standard salt solution concentration by the addition of the appropriate amount of NaCl. After ten 15 minute equilibrations, the solutions were decanted from the resin which was then quickly transferred with two washings of deionised water (10 cm^3) to a sintered-glass filter and eluates prepared as outlined in section 3.2.3.

3.3. RESULTS

3.3.1. Effect of ligand concentration

In order to determine the optimum quantity of plant fraction complexant to use, it was initially necessary to gauge the effect of a known complexing ligand upon the resin - standard salt solution equilibrium. The citrate ion is one such suitable ligand and was added to the standard solution as sodium citrate in concentrations ranging from 0.2 - 1.0 meq/20 cm^3 (1 meq Na citrate = $\frac{\text{molecular weight}}{3}$). However, the addition of sodium citrate at the upper limit of 1 meq per 20 cm^3 of standard salt solution, increased the $[Na^+]$ in the solution from 1.6 to 2.6 meq/20 cm^3. Consequently, a control series of solutions containing equivalent amounts of additional NaCl instead of sodium citrate were also equilibrated with the standard resin. The effect of ligand concentration in the solution upon the load of all cations (except NH_4^+ on the resin, is given in Fig. 12. It is clear that the addition of sodium citrate brings about a marked decrease in resin $[Ca^{++}]$ and $[Mg^{++}]$ and that this is compensated for by increases in resin $[K^+]$ and particularly $[Na^+]$. That this considerable increase in resin $[Na^+]$ is not caused solely by the increase in solution $[Na^+]$ is evident from the small increase in resin $[Na^+]$ produced by the addition of NaCl. It may be concluded, therefore, that the citrate ligand has decreased the solution $[Ca^{++}]$ and $[Mg^{++}]$.

FIG. 12 CHANGE IN RESIN CATION CONCENTRATION WITH INCREASE IN LIGAND CONCENTRATION



It should also be noted that the sum of all resin cation concentrations for either Na citrate or NaCl solutions at any point along the x-axis is constant, with a value of approximately 4.2 meq/g.

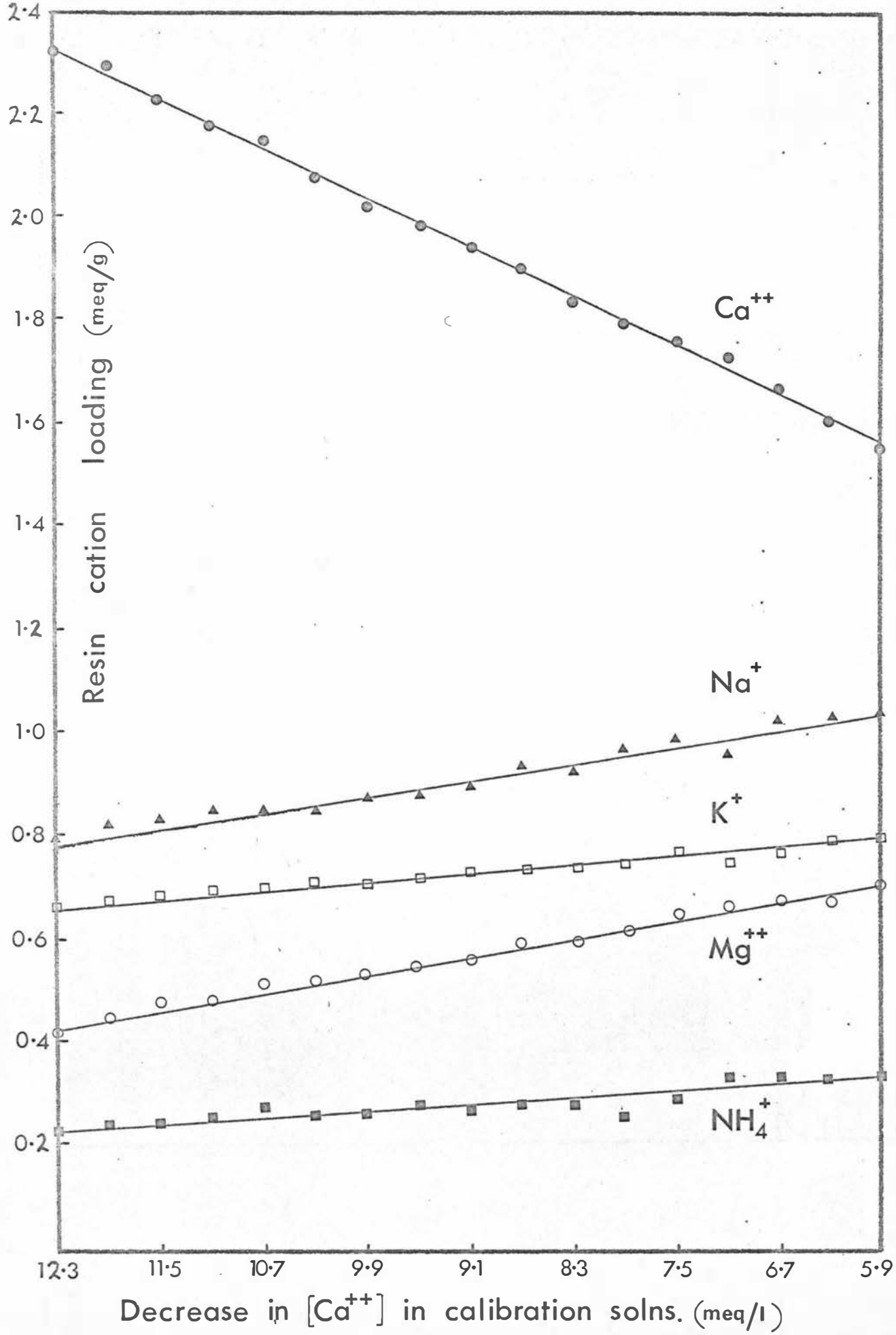
The midpoint of the citrate concentration range, 0.5 meq/20 cm^3 (corresponding to approximately 50 mg/20 cm^3 on a weight basis), seemed a suitable concentration maximum for complexing ligands. Since the plant fractions from Yorkshire fog were polymers of unknown molecular weight it was meaningless to compare their binding affinity with that of organic acids, such as citric acid, on a molar or 'equivalent' basis, so a potential complexant concentration of 50 mg/20 cm^3 standard salt solution was adopted for all future equilibrations.

3.3.2. Resin calibration results

It was now necessary to calibrate the relationship between resin cation loads and their equilibrium solution cation concentrations. The results for 17 calibration solutions decreasing in $[Ca^{++}]$ by steps of 0.4 meq/l from 12.3 meq/l to 5.9 meq/l (section 3.2.4.) are shown in Fig. 13, where each point is the mean of six values (two dilutions of triplicate resin eluates). Over this Ca^{++} concentration range, the resin shows remarkably constant behaviour, with a linear decrease in $[Ca^{++}]$ being compensated for by uptake of the other four cations, particularly magnesium and sodium. The total resin load was 4.419 $\pm$ 0.004 meq/g (mean $\pm$ standard error of the mean). Table 15 gives the simple linear regressions relating changes in resin load of all five cations for changes in Ca^{++} activity in these calibration solutions. The size of the correlation coefficients indicate the very symmetrical behaviour of the resin with changing solution composition.

The mean total resin load of 4.419 $\pm$ 0.004 meq/g for these calibration solutions was a more reliable value (mean of 102 individual values) than that for the standard resin given in Table

FIG. 13 VARIATION IN RESIN INDIVIDUAL CATION LOAD WITH CHANGE IN Ca^{++} IN CALIBRATION SOLUTIONS



12 (4.435 ± 0.011 meq/g, mean of six individual values). However, the small standard errors for both values indicate the reproducibility of the whole equilibration, elution and photometric procedure (% coefficient of variation for the means in Table 12: Ca^{++}, 0.86; Mg^{++}, 3.95; Na^{+}, 3.04; K^{+}, 1.81). The standard error for NH_4^{+} in Table 12 (giving a larger coefficient of variation of 11.1%) reflects the errors in the Nessler analyses which have already been discussed in section 3.2.3.

TABLE 15

REGRESSIONS FOR VARIATION OF RESIN INDIVIDUAL CATION LOAD $[M^{n+}]_R$ WITH SOLUTION CALCIUM CONCENTRATION $[Ca^{++}]_S$

Regression	r
$[Ca^{++}]_R = 0.889 = 0.114\ [Ca^{++}]_S$	0.998***
$[Mg^{++}]_R = 0.952 - 0.042\ [Ca^{++}]_S$	1.000***
$[Na^{+}]_R = 1.246 - 0.037\ [Ca^{++}]_S$	0.984***
$[K^{+}]_R = 0.875 - 0.016\ [Ca^{++}]_S$	0.995***
$[NH_4^{+}]_R = 0.455 - 0.019\ [Ca^{++}]_S$	0.981***
	(p 0.001***)

3.3.3. Plant fraction complexing

The variation in resin cation load, shown in Fig. 13, gave a simplified model for the evaluation of the complexing effect of plant fractions on the cations of the standard salt solution. A resin load conforming with the curves in Fig. 13 would indicate a situation where only Ca^{++} was bound in a non-ionic form.

Table 16 shows the individual and total mean resin cation loads obtained on equilibration of the two groups of plant fractions, organic acids and cell wall substances, with the standard salt solution. The same data for a variety of plant metabolites, including a commercial pectin isolated from apples and pure samples of glucuronic and galacturonic acids, is given in Table 18. Where

possible, each fraction was evaluated at both a weakly acidic and a weakly alkaline pH. The final column in both tables gives the total resin cation load, $\sum M^{n+}_R$ which has a mean of 4.415 ± 0.005 meq/g for the 21 plant fractions from Yorkshire fog (Table 16) and 4.425 ± 0.007 meq/g for the 10 metabolites in Table 18.

To test whether these values lay on the curves of Fig. 13, (which applies to the case where only Ca^{++} is bound by the ligand) the experimental values of $[Mg^{++}]_R$, $[Na^+]_R$, $[K^+]_R$ and $[NH_4^+]_R$ of tables 16 and 18 were subtracted from the respective values read from Fig. 13 for <u>identical</u> values of $[Ca^{++}]_R$. These differences, and their statistical significance, are given in Table 17 and the bottom half of Table 18. The first column in each of these tables gives the mean change in resin Ca^{++} load from that of the standard resin Ca^{++} load (2.330 meq/g) and is, therefore, an index of the extent of Ca^{++} binding. The following columns give the corresponding differences ($\bar{d}$) in resin Mg^{++}, Na^+, K^+ and NH_4^+ from the values predicted for each ion in Fig. 13. Since the total resin load must remain constant (c. 4.419 ± 0.004 meq/g), the sum of the mean differences $\bar{d}_{Mg^{++}}$, $\bar{d}_{Na^+}$, $\bar{d}_{K^+}$ and $\bar{d}_{NH_4^+}$ in each case should be zero. The final summation column in Tables 17 and 18 shows that, within the limits of experimental errors this condition is met.

The significance of these mean differences, $\bar{d}_{M^+}$, were determined by testing the hypothesis $\bar{d}_{M^+} = 0$ by using the relationship $t = \frac{\bar{d}_{M^+}}{SE_{mean}}$ where $\bar{d}_{M^+}$ = the mean difference of any particular cation, M^+, in the resin load from that value predicted in Fig. 13 at any resin Ca^{++} value and SE_{mean} = the standard error of the mean of the six individual differences, $d_1 - d_6$.

From Tables 17 and 18 it is clear that the simple case of Ca^{++} alone being bound by the complexing ligand is true only in a few cases. The lower levels of magnesium on the equilibrated

TABLE 16. INDIVIDUAL AND TOTAL MEAN* RESIN CATION LOADS (meq/g) AFTER EQUILIBRATION WITH COMPLEXANTS

Complexant	pH	Mean individual resin cation loads (meq/g) Ca_R^{++}	Mg_R^{++}	Na_R^{+}	K_R^{+}	$NH_4{}_R^{+}$	$\sum M_R^{n+}$
Organic Acids							
Malic acid	5.04	1.871	0.489	1.002	0.742	0.302	4.406
	6.66	1.839	0.464	1.048	0.758	0.304	4.413
Citric acid	4.84	1.683	0.350	1.235	0.836	0.336	4.440
	7.00	1.159	0.304	1.574	0.973	0.413	4.423
Oxalic acid	4.88	1.918	0.411	1.071	0.765	0.271	4.436
	7.40	1.945	0.397	1.064	0.770	0.265	4.441
Malonic acid	6.16	1.955	0.434	0.969	0.744	0.280	4.382
trans-Aconitic acid	5.42	2.058	0.499	0.897	0.730	0.250	4.434
	7.42	1.884	0.424	1.044	0.794	0.269	4.415
Quinic acid	4.94	2.240	0.467	0.813	0.696	0.220	4.436
	8.44	2.248	0.454	0.781	0.708	0.230	4.421
Cell Wall Fractions							
Pectin	6.30	2.026	0.536	0.898	0.723	0.248	4.431
	7.24	1.983	0.556	0.892	0.734	0.264	4.429
Lignin	6.2	2.104	0.469	0.892	0.683	0.252	4.400
Hemicellulose B (unfractionated)	5.00	2.300	0.439	0.785	0.621	0.234	4.379
	8.20	2.287	0.426	0.829	0.028	0.234	4.404
Hemi. B (branched)	7.90	2.217	0.472	0.812	0.666	0.244	4.411
Hemi. B (linear)	7.86	2.287	0.438	0.808	0.643	0.234	4.410
Hemi. B (I)	5.90	2.331	0.435	0.780	0.650	0.230	4.427
Cellulose	4.70	2.273	0.443	0.798	0.651	0.230	4.395
	8.12	2.282	0.437	0.798	0.645	0.237	4.399

* Mean of six individual determinations (2 duplicate dilutions of 3 resin eluates)

Mean total load (± S.E.) = 4.415 ± 0.005 meq/g

TABLE 17

MEAN[+] DEVIATION (in meq/g) OF RESIN Mg^{++}, Na^{+}, K^{+} AND NH_4^{+} LOADS FROM VALUE PREDICTED IN FIG. 13 FOR RESIN Ca^{++} LOADS (in meq/g) OBTAINED ON EQUILIBRATION WITH PLANT FRACTIONS

	pH	Change in $[Ca^{++}]$ (meq/g) from standard resin Ca^{++} loading ‡	Mean difference (in meq/g) of each cation from predicted value (Fig. 13) for each new Ca^{++} level: $\bar{d}_{Mg^{++}}$	$\bar{d}_{Na^{+}}$	$\bar{d}_{K^{+}}$	$\bar{d}_{NH_4^{+}}$	$\sum \bar{d}$
Organic Acids							
Malic acid	5.04	-0.459***	-0.106***	+0.075***	+0.022***	+0.012*	+0.003
	6.66	-0.491***	-0.146***	+0.098***	+0.032***	+0.010*	-0.006
Citric acid	4.84	-0.677***	-0.318***	+0.248***	+0.076***	+0.016*	+0.022
	7.00	-1.171***	-0.564***	+0.414***	+0.133***	+0.027*	+0.010
Oxalic acid	4.88	-0.412***	-0.169***	+0.159***	+0.037***	-0.012^{NS}	+0.015
	7.40	-0.385***	-0.172***	+0.160***	+0.048***	-0.015*	+0.021
Malonic acid	6.16	-0.375***	-0.129***	+0.069***	+0.029***	+0.010^{NS}	-0.021
trans-Aconitic acid	5.42	-0.272***	-0.029*	+0.027^{NS}	+0.028*	-0.012^{NS}	+0.014
	7.42	-0.446***	-0.166**	+0.121*	+0.071*	-0.021*	+0.005
Quinic acid	4.94	-0.090*	+0.007^{NS}	-0.007^{NS}	+0.014^{NS}	-0.018^{NS}	-0.004
	8.44	-0.082*	-0.002^{NS}	-0.027^{NS}	+0.027^{NS}	-0.006^{NS}	-0.008
Cell wall fractions							
Pectin	6.30	-0.304***	-0.002^{NS}	+0.020^{NS}	+0.021^{NS}	-0.014^{NS}	+0.025
	7.24	-0.347***	+0.003^{NS}	0.000^{NS}	+0.024*	-0.012^{NS}	+0.015
Lignin	6.20	-0.226***	-0.039***	+0.038**	-0.015*	+0.002^{NS}	-0.014
Hemicellulose B	5.00	-0.030^{NS}	+0.005^{NS}	-0.003^{NS}	-0.024*	+0.004^{NS}	-0.022
	8.20	-0.043*	-0.008^{NS}	+0.019*	-0.017^{NS}	0.000^{NS}	-0.026
Hemi. B (branched)	7.90	-0.113***	+0.002^{NS}	-0.008^{NS}	-0.012^{NS}	+0.004^{NS}	-0.014
Hemi. B (linear)	7.86	-0.043*	-0.002^{NS}	+0.006^{NS}	-0.003^{NS}	-0.008^{NS}	-0.007
Hemi. B (I)	5.90	+0.001^{NS}	-0.001^{NS}	+0.021^{NS}	-0.013^{NS}	-0.006^{NS}	+0.001
Cellulose	4.70	-0.057*	-0.007^{NS}	-0.006^{NS}	-0.009^{NS}	0.000^{NS}	-0.022
	8.12	-0.048*	-0.009^{NS}	-0.001^{NS}	-0.005^{NS}	+0.010^{NS}	-0.025

[+] Mean of 6 individual determinations (2 duplicate dilutions of 3 resin eluates)

‡ Standard resin Ca^{++} load = 2.330 ± 0.008 m.eq./g

NS Not significant ($P > 0.05$), *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$)

TABLE 18. MEAN+ RESIN CATION LOADS (meq/g) PLUS CORRESPONDING MEAN DEVIATIONS ($\bar{d}$, DESCRIBED IN TABLE 17) FROM Ca_R^{++} LOADS PREDICTED FROM FIG. 13 AFTER EQUILIBRATION WITH COMPLEXANTS

Complexant	pH	Ca_R^{++}	Mg_R^{++}	Na_R^{+}	K_R^{+}	$NH_4{}_R^{+}$	$\sum M_R^{n+}$
Inositol (meso)	4.78	2.323	0.434	0.777	0.673	0.227	4.434
	7.96	2.331	0.431	0.762	0.677	0.230	4.431
Inositol hexaphosphate	5.70	2.134	0.473	0.851	0.732	0.236	4.426
	7.48	1.423	0.525	1.299	0.871	0.309	4.427
Glucuronic acid	5.68	2.234	0.463	0.823	0.684	0.230	4.434
	7.86	2.210	0.453	0.838	0.692	0.230	4.423
Galacturonic acid	4.20	2.222	0.461	0.830	0.678	0.240	4.431
	7.68	2.184	0.462	0.842	0.696	0.246	4.430
Pectin (apple)	4.40	2.034	0.487	0.898	0.726	0.262	4.407
	8.56	2.002	0.511	0.898	0.724	0.278	4.413
		$\overline{\lfloor Ca^{++} \rfloor}_{standard}$ $(2.330)-\overline{\lfloor Ca^{++} \rfloor}_R$	$\bar{d}_{Mg^{++}}$	$\bar{d}_{Na^{+}}$	$\bar{d}_{K^{+}}$	$\bar{d}_{NH_4{}^{+}}$	$\sum \bar{d}$
Inositol (meso)	4.78	-0.007^{NS}	-0.001^{NS}	-0.003^{NS}	-0.003^{NS}	$+0.005^{NS}$	+0.001
	7.96	$+0.001^{NS}$	-0.004^{NS}	-0.018^{NS}	$+0.001^{NS}$	$+0.008^{NS}$	-0.012
Inositol hexaphosphate	5.70	-0.196***	-0.031^{NS}	$+0.007^{NS}$	$+0.027^{NS}$	-0.010^{NS}	-0.007
	7.48	-0.907***	-0.245***	+0.201***	+0.069**	-0.011^{NS}	+0.014
Glucuronic acid	5.68	-0.096*	-0.005^{NS}	$+0.003^{NS}$	$+0.002^{NS}$	-0.008^{NS}	-0.008
	7.86	-0.120**	-0.025^{NS}	$+0.010^{NS}$	$+0.006^{NS}$	-0.012^{NS}	-0.021
Galacturonic acid	4.20	-0.108*	-0.011^{NS}	$+0.008^{NS}$	-0.006^{NS}	-0.002^{NS}	-0.011
	7.68	-0.146**	-0.026^{NS}	$+0.008^{NS}$	$+0.006^{NS}$	-0.004^{NS}	-0.016
Pectin (apple)	4.40	-0.296***	-0.050*	$+0.020^{NS}$	$+0.022^{NS}$	-0.010^{NS}	-0.018
	8.56	-0.328***	-0.036*	$+0.012^{NS}$	$+0.016^{NS}$	$+0.002^{NS}$	-0.006

+ Mean of 4 individual determinations (2 duplicate dilutions of 2 resin eluates)

NS, Not significant ($P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$)

resins (ie. negative $\bar{d}_{Mg^{++}}$), especially for the organic acids and lignin, indicated that magnesium had also been complexed. To fulfil the constant total resin load requirement, greater amounts of the monovalent cations had been taken up by the resin. Overall, the new resin loads reflected completely different cation activities and the simple case outlined in Fig. 13 did not hold true as a prediction model for the amounts of cations bound by a complexant.

3.3.4. Resin calibration with solutions lower in calcium and magnesium concentration

To interpret the more complex situation where both calcium and magnesium in the standard salt solution are bound, similar procedures to the earlier calibration were followed with 16 solutions decreasing in $[Ca^{++}]_S$ from 12.3 meq/l to 5.0 meq/l and in $[Mg^{++}]_S$ from 7.2 meq/l to 4.0 meq/l. The concentrations of Na^+, K^+ and NH_4^+ were the same as that of the standard salt solution. Multiple regression analyses of the resin cation loads (mean cation load = 4.412 $\pm$ 0.010) were undertaken and equations of the following type derived:

$$[Ca^{++}]_R = \alpha_1 + \beta_1[Ca^{++}]_S + \beta_2[Mg^{++}]_S \quad .....(3.3.1.)$$

$$[Mg^{++}]_R = \alpha_2 = \beta_3[Ca^{++}]_S + \beta_4[Mg^{++}]_S \quad .....(3.3.2.)$$

Equation (1) reduced to a linear regression (r^2 = 0.962)*

$$[Ca^{++}]_R = 1.232 + 0.089[Ca^{++}]_S \quad .....(3.3.3.)$$

since an analysis of variance (see Appendix 1 for details) showed a negligible contribution of the $\beta_2[Mg^{++}]_S$ term in the multiple regression (r^2 = 0.963 cf. 0.962). Consequently, within the concentration range investigated, the magnesium level in the solution had no significant effect upon the resin calcium load. The multiple regression for the resin magnesium load could not be simplified, however, since $[Mg^{++}]_R$ was found to be dependent upon both the calcium and magnesium concentrations in the solution.

This multiple regression ($r^2 = 0.789$)

$$[Mg^{++}]_R = 0.448 - 0.037[Ca^{++}]_S + 0.061[Mg^{++}]_S \quad ...(3.3.4.)$$

did not give as good an explanation of the behaviour of the resin as did the linear regression for $[Ca^{++}]_R$. Solution calcium concentration is the dominant variable since the linear regression on $[Ca^{++}]_S$ comprised 94% of the sums of squares for the multiple regression on both $[Ca^{++}]_S$ and $[Mg^{++}]_S$ (see Appendix 1). Attempts were made to introduce a third variable, especially one covering changes in ionic strength, eg. $\beta 5 \cdot \frac{1}{[Ca^{++}]_S + [Mg^{++}]_S}$, but no improvement in the regression was obtained.

3.3.5. Calculation of Ca^{++} and Mg^{++} bound by plant fractions

The solution calcium concentration, $[Ca^{++}]_S$, after equilibration with a complexant, was calculated from equation (3.3.3.). The corresponding $[Mg^{++}]_S$ was obtained by solving equation (3.3.4.) using the estimated value of $[Ca^{++}]_S$. The original $[Ca^{++}]_S$ and $[Mg^{++}]_S$ of the standard salt solution were known (12.3 and 7.2 meq/l respectively) so the concentration of Ca^{++} and Mg^{++} bound by the complexing ligand was calculated by subtraction. The percentage of calcium and magnesium in the standard salt solution (20 cm^3) bound by the different plant fractions (50 mg) is given in Table 19. This weight of 50 mg is also expressed in Table 19 as milli-moles (m mol) for each fraction; for the cell wall polymers the weight of 50 mg was expressed as the number of milli-moles of 'structural repeating unit' calculated from the analyses in Part 2. For cellulose this unit was glucose (M.W. = 180); for pectin, galacturonic acid (M.W. = 194); for lignin, coniferaldehyde (M.W. = 178); and for the hemicelluloses, the total weight of 100 monose units calculated from the known % of xylose, arabinose, glucose, galactose and glucuronic acid divided by 100 (M.W. = 154 - 159).

Errors The values presented in Table 19 are inverse estimates

TABLE 19

PERCENTAGE OF Ca^{++} AND Mg^{++} IN STANDARD SALT SOLUTION (20 cm^3) BOUND BY PLANT FRACTIONS (50 mg)

	pH	Weight of* complexant	Percentage Ca^{++} bound	Percentage Mg^{++} bound
Organic acids				
Malic	5.04	0.373	41.9	29.9
	6.66	0.373	44.7	38.9
Citric	4.84	0.260	56.5	73.6
	7.00	0.260	97.6	100
Oxalic	4.88	0.556	37.8	43.1
	7.40	0.556	35.4	43.8
Malonic	6.16	0.481	34.1	34.7
trans-Aconitic	5.42	0.287	24.8	9.7
	7.42	0.287	40.7	43.8
Quinic	4.94	0.260	8.5	0
	8.44	0.260	7.7	2.1
Cell wall fractions				
Pectin	6.30	0.259	28.0	3.5
	7.24	0.259	31.7	4.2
Lignin	6.20	0.281	20.7	12.5
Hemicellulose B	5.00	0.250	2.8	0.7
(unfractionated)	8.20	0.250	4.5	4.9
Hemi. B (branched)	7.90	0.253	10.6	1.4
Hemi. B (linear)	7.86	0.246	4.5	1.4
Hemi. B (I)	5.90	0.234	0	0
Cellulose	4.70	0.278	6.5	3.5
	8.12	0.278	4.5	4.5
Plant metabolites				
Inositol (meso)	4.78	0.278	0	0
	7.96	0.278	0	0
Inositol	5.70	0.078	17.9	8.3
hexaphosphate	7.48	0.078	82.5	64.6
Glucuronic acid	5.68	0.258	8.95	4.79
	7.86	0.258	11.13	5.93
Galacturonic acid	4.20	0.258	10.04	2.97
	7.68	0.258	13.50	6.38
Pectin (apple)	4.40	0.159	27.12	16.34
	8.56	0.259	30.03	12.51

* 50 mg complexant added per 20 cm^3 standard salt solution, expressed as milli-moles

from the regressions described by equations (3.3.3.) and (3.3.4.). Estimates of $\left[Ca^{++}\right]_S$, using equation (3.3.3.), had a confidence interval of approximately $\pm$ 10% (see Appendix 2 for details). Estimates of $\left[Mg^{++}\right]_S$ are subject to error from two sources - the error initially introduced in the estimation of $\left[Ca^{++}\right]_S$ and the error associated with inverse estimation after substitution of $\left[Ca^{++}\right]_S$ into equation (3.3.4.). There is no precise formulation for confidence intervals on inverse estimates of one independent variable in multiple regression where the second independent variable has known errors (Williams, 1959). In view of the comparatively poor (r^2 = 0.789) determination by equation (3.3.4.), a conservative limit of $\pm$25% is placed on estimates of $\left[Mg^{++}\right]_S$.

PART 4 DISCUSSION

4.1. GENERAL DISCUSSION OF METHODOLOGY AND RESULTS

The constant ionic medium provided by the bulk electrolytes NaCl, KCl, and NH_4Cl allowed the assumption of constant activity coefficients for the ionic species present (p 70). The equilibration with plant fractions reduced the ionic strength very slightly in most cases and the maximum change experienced (citric acid at pH 7.0) reduced the ionic strength by only 13% from 0.146 to 0.127. Consequently, it was considered that changes in ionic activities could be adequately described by changes in ionic concentration - both being an index of the degree to which Ca^{++} and Mg^{++} are bound in a non-ionic form. Since there may be a multitude of equilibria between the different cations of such a mixed electrolyte and the complexing ligand, it was not possible to determine the actual chemical nature of the metal-ligand complex; therefore, Table 19 expresses only the percentage of Ca^{++} and Mg^{++} in the standard salt solution bound in some non-ionic form by the plant ligands under these standard conditions. This does, however, enable the two different categories of plant fraction ligands - organic acids and cell wall polymers - to be compared.

The ion exchange resin behaved very symmetrically on calibration with widely differing pure salt solutions. The mean total cation load for 17 salt solutions varying in $[Ca^{++}]$ was 4.419 ± 0.004 meq/g; for 16 salt solutions varying in both $[Ca^{++}]$ and $[Mg^{++}]$, 4.412 ± 0.010 meq/g; for 21 solutions containing plant ligands with unknown $[Ca^{++}]$ and $[Mg^{++}]$, 4.415 ± 0.005 meq/g; and for the 10 metabolites of Table 18, 4.425 ± 0.007 meq/g. These values are in excellent agreement and indicate the constant pattern in resin behaviour with

changes in cation activities in the solutions.

The wide limits placed on estimates of $[Ca^{++}]_S$ and $[Mg^{++}]_S$ (± 10% and 25% respectively) do not detract from the validity of the estimation procedure. Calibration lines such as equation (3.3.3.) are often fitted and interpolated visually with complete disregard for the confidence which can be placed on inverse estimates. The more rigorous treatment using least squares analysis indicates the fallibility of assuming, as in the case of visual calibration, that inverse estimates have zero error. This error is emplasized in estimates of $[Ca^{++}]$ using equation (3.3.3.) in spite of the excellent determination ($r^2 = 0.962$) of $[Ca^{++}]_R$. The multiple regression technique used to estimate $[Mg^{++}]_S$ is the only rigorous method available to interpret the complex behaviour of those complexants binding both Ca^{++} and Mg^{++}. In any case, this limit of ± 25% on $[Mg^{++}]_S$ does little to mask the clear cut differences in Mg^{++} binding by the organic acids and lignin on the one hand and the remaining plant fractions on the other (Table 19).

The multiple regression (eqn. 3.3.4.) relating $[Mg^{++}]_R$ to both $[Ca^{++}]_S$ and $[Mg^{++}]_S$ is of particular interest in view of the findings of van't Klooster (1967) for a similar cation exchange resin, Dowex 50, and similar mixed electrolyte solutions. He calculated the concentration of Ca^{++} and Mg^{++} in the intestinal ultrafiltrates of ruminants from the following simple linear regression formulae:

$$Ca_1 = \overline{Ca}_1 + 0.218\ (Ca - \overline{Ca}) \qquad \ldots(4.1.)$$

$$Mg_1 = \overline{Mg}_1 + 0.214\ (Mg - \overline{Mg}) \qquad \ldots(4.2.)$$

where Ca_1, Mg_1 = the % concentration of the Ca^{++} and Mg^{++} in the unknown solution,

Ca, Mg = the % concentrations of these ions in their corresponding resin eluate,

$\overline{Ca}_1$, $\overline{Mg}_1$ = the average % concentration of 3 test solutions corresponding to the unknown in $[Na^+]$, $[K^+]$ and $[NH_4^+]$ (all assumed to be ionic and determined by normal chemical analysis) but varying in $[Ca^{++}]$ and $[Mg^{++}]$,

$\overline{Ca}$, $\overline{Mg}$ = the average % concentration of these ions in the 3 test solution resin eluates.

The simple linear regression for Ca^{++} expressed by eqn. 4.1. is similar to that of eqn. (3.3.3.) derived from the test solutions employed in the present investigation; however, his simple linear relationship between $[Mg^{++}]_S$ and $[Mg^{++}]_R$ expressed in eqn. (4.2.) is quite at variance with the multiple regression (eqn. 3.3.4.) derived in the present work. An analysis of variance of eqn. (3.3.4.) showed that variation in $[Ca^{++}]_S$ comprised 94% of the variation in $[Mg^{++}]_R$ (p. 89) and, consequently, resin Mg^{++} loadings were only partly dependent upon $[Mg^{++}]$ in the calibration solutions. Unfortunately, van't Klooster gives scant detail on the calculation of his linear regressions except that they were based upon 64 test solution observations.

No doubt the equilibrium resin loads for the five cations of any salt solution could be related to their solution concentrations by a sufficiently large polynomial but this would be a mammoth task involving many test solutions, thousands of analyses and a computer analysis of the results. By considering only one electrolyte solution (the standard salt solution) and, furthermore, by assuming that Na^+, K^+ and NH_4^+ would not be bound by the investigated complexants (as was later found to be the case) it was possible to derive eqns. (3.3.3) and (3.3.4) which were then used to determine the amounts of bound and ionic Ca^{++} and Mg^{++} in solutions in equilibrium with complexing ligands.

Plant fraction complexing

Of the plant fractions studied, the organic acids, particularly the tribasic citric and trans-aconitic acids, are most active in complexing the divalent cations while the cell wall fractions, with the notable exceptions of pectin and lignin, have little binding effect (Table 19). Pectin binds Ca^{++} to the same extent as some of the organic acids but appears to give no significant binding of Mg^{++}. Lignin on the other hand, while complexing Ca^{++} to a lesser extent than pectin, does have a significant affinity for Mg^{++} (Table 17). The hemicelluloses (except branchedhemicellulose B at the slightly alkaline pH) and cellulose had little affinity for complex formation with either Ca^{++} or Mg^{++}.

The complexants listed in Table 18 were not fracticnated from Yorkshire fog but are a series of miscellaneous plant metabolites included for the purpose of comparison. The inositols, of which 'meso-' is one of nine possible isomers, are a widespread group of plant cyclitols which are generally found as polyphosphate esters, especially inositol hexaphosphate (phytic acid), the Ca salt of which has a low solubility and is known as phytin. The ability of inositol hexaphosphate to bind Ca^{++} and Mg^{++} (at the same slightly alkaline pH) was even superior to that of citric acid on a molar basis (Table 19). The relationship of the structure of the above-mentioned plant fractions to their ability to complex Ca^{++} and Mg^{++} is discussed in more detail in section 4.2.

For most fractions examined, pH had an effect on the extent of complexing since the 'bound' percentage of both Ca^{++} and Mg^{++} is larger at the weakly alkaline pH (cf. ileal region) than at the slightly acidic pH (cf. duodenal region) (van't Klooster,

1967). Since most of the plant fractions examined can be considered as weak acids, an increase in pH would tend to bring about ionization of carboxyl groups as well as increasing the polarisation of hydroxyl groups. Furthermore, a tendency towards polynuclear complexes would be expected to increase with higher pH and the stability constants of the complexes have been shown to increase correspondingly (Schnitzer and Hansen, 1970).

4.2. RELATIONSHIP OF POLYMER STRUCTURE TO CATION AFFINITY

4.2.1. Pectic substances and polyuronides

It is now well established that plant pectic substances are a heterogeneous group of polysaccharides (Worth, 1967) comprising the following three polymers:

(a) a linear α-(1→4) linked poly-D-galacturonic acid chain as a major constituent. Sometimes L-rhamnose and other neutral sugars are present as side chain substituents.

(b) a minor β-(1 → 4) linked D-galactan

(c) a minor L-araban

These pectic substances are generally found in the primary cell wall and middle lamella where they are associated with cellulose, hemicelluloses and lignin. The exact nature of this association is not clear, as yet, but it does seem that the hydrophilic and adhesive properties of the pectic substances are utilised in holding the cellulose microfibrils together in a rigid network. The extensive literature on the role of pectins in plant growth and the physiological role of Ca^{++} and Mg^{++} in binding the polygalacturonan chains together has been reviewed by Wilson (1964) and Roelofsen (1965).

The carboxyl groups of the polygalacturonan chain of cell wall pectins *in situ* have been shown to be part free acid, part

esterified (methyl) and partly associated with Ca^{++} and Mg^{++}; consequently the well known gelatinisation of pectic substances with traces of divalent or trivalent cations has been attributed to the formation of a huge molecule by a 'salt bridge' cross-linkage through the cations and the carboxyl groups of different chains (Whistler, 1959).

The 'pectin' extracted from Yorkshire fog is almost a pure polygalacturonan (Table 4) with a low degree of methyl esterification (1.9% -OMe or one in ten galacturonic acid monomers esterified). This polymer exhibited a binding of Ca^{++} of the same order as many of the dicarboxylic acids yet it had no significant affinity for Mg^{++}. Despite the longstanding knowledge of this gelation phenomenon, and its economic importance, the nature of these cation/polygalacturonide complexes has received scant investigation and the limited amount of recent work with plant pectins has stemmed from the more popular field of cation complexes of alginic acids extracted from seaweeds.

In a long term study of the properties of these alginates, Norwegian workers (Smidsrod and Haug, 1965; Haug and Smidsrod, 1965) have found that these polymers can function as cation exchangers with calcium and other divalent metals. The concentration of cation required to bring about gel-formation and precipitation increased in the order:

$$Ba < Pb < Cu < Sr < Cd < Ca < Zn < Ni < Co < Mn, Fe < Mg$$

but this order was not the same as the affinity series in which the ions are preferentially bound by the alginate. These alginic acids consist of non-uniformly distributed residues of D-mannuronic acid and L-guluronic acid (Haug, Myklestad, Larsen and Smidsrod, 1967) which can be degraded to polymeric fragments consisting almost entirely of $\beta(1 \rightarrow 4)$-poly-D-mannuronic acid

and $\alpha(1 \rightarrow 4)$-poly-L-guluronic acid. Furthermore, the ion exchange properties of the alginates depend upon their uronic acid composition, with the selectivity for the exchange reaction Ca^{++}/Mg^{++}, Ca^{++}/Sr^{++}, Sr^{++}/Mg^{++} and Ca^{++}/Ca^{++} almost entirely due to the guluronic acid residues in the polymers (Smidsrod and Haug, 1968).

The Ca^{++}/K^{+} ion-exchange equilibrium selectivity coefficients were determined for pectin and four alginate samples of differing uronic acid composition (Kohn, Furda, Haug and Smidsrod, 1968); an extrapolation of their results showed that polygalacturonate and polyguluronate have a much higher selectivity for Ca^{++} than has polymannuronate. This investigation employed the complexiometric determination of Ca^{++} activity using tetramethylmurexide as an auxiliary ligand (Kohn and Furda, 1967a and b) and, as such, seems an improvement on the earlier viscosity and equilibrium dialysis techniques of Haug and Smidsrod.

The degree of methyl esterification of the galacturonic acid unit of pectin seems to be of importance in cell wall extension (see review in Wilson, 1964) as well as having a marked effect upon the bond strength of Ca^{++} with pectin (Kohn and Furda, 1967a). These latter workers found that with a decreasing degree of esterification (ie., with a rising charge density) there was a corresponding decrease in the activity coefficient of Ca^{++}; they were later able to express the stability constant for Ca pectinate, and the selectivity coefficient for the Ca^{++}/K^{+} ion exchange in pectin, not only as a function of the degree of esterification of the polymer but also of the ionic strength and mean distance of free carboxyl groups in the molecule.

Nature of the cation-uronide linkage

The findings on the binding of cations by polyuronides, as discussed above, can be summarised as follows:

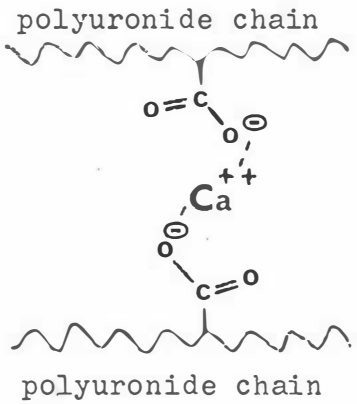
Cation binding seems to involve both,

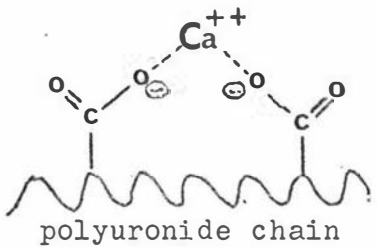
(a) a simple charge neutralisation of the free $-COO^-$ groups, as indicated by the influence of the degree of esterification

(b) other effects possibly involving the -OH groups of the polymer, eg.,the stereochemistry of uronide configuration as shown by Haug and Smidsrod for alginic acids.

Case (a) is probably a simple ionic association, providing a bridge between $-COO^-$ groups of different polyuronide chains (I) or a chelate type of structure with $-COO^-$ groups on neighbouring uronic acid groups in the same polymer (II).

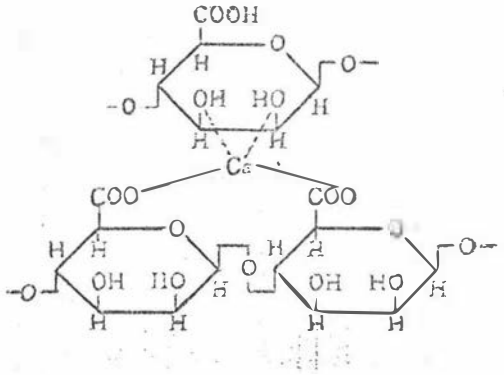
I





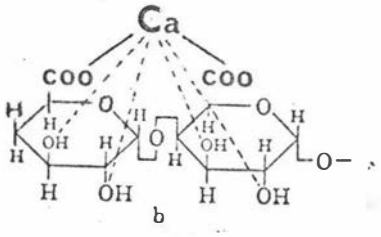
II

There is much evidence, however, for the existence of case (b) types of association. In a study of the binding affinity of acetyl alginates of varying degrees of acetylation, Schweiger (1962b) concluded that the Ca complex was a chelate structure involving two carboxyl groups from neighbouring units and two hydroxyl groups in a unit of probably another chain (III), thus;



III

where the bonds to the two vicinal -OH groups were of the co-ordinate type. Again, he was able to obtain only a low gelation with magnesium ion, the gelation strength order being Ba> Ca> Zn≫ Mn/Mg. Schweiger (1964) found the same type of behaviour for pectic acids of similar degrees of acetylation and postulated a similar type of complex to that above for alginic acids except that the ion formed an intramolecular complex with the carboxyl and hydroxyl groups on adjacent residues of the same chain (IV)



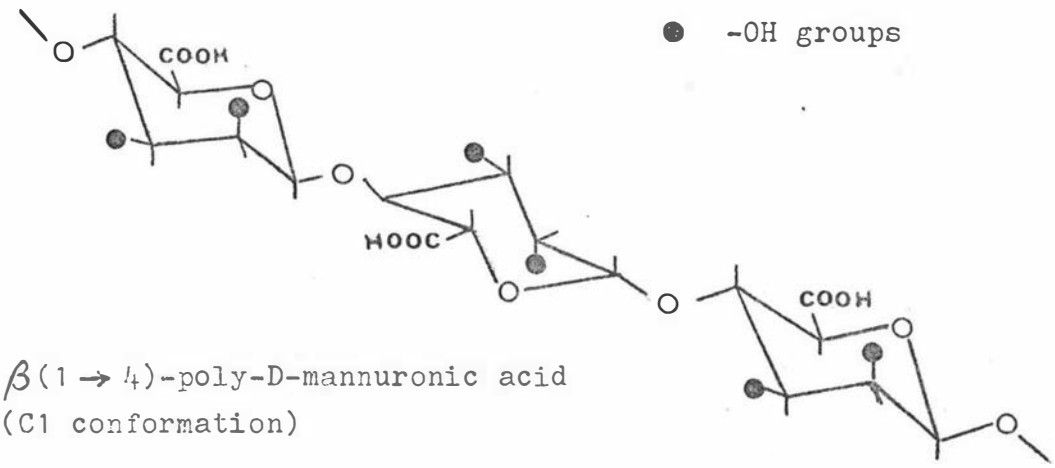
IV

In this case also, Mg^{++} gave the lowest degree of gelation of the 14 divalent metals investigated.

Configuration of polyuronides

To obtain an accurate 3-dimensional picture of the structure of these polyuronides it is necessary to discuss them in terms of their 'conformations', ie., arrangements in space of a single chemical structure (configuration), rather than the above mis-leading 2-dimensional Haworth diagrams used by Schweiger.

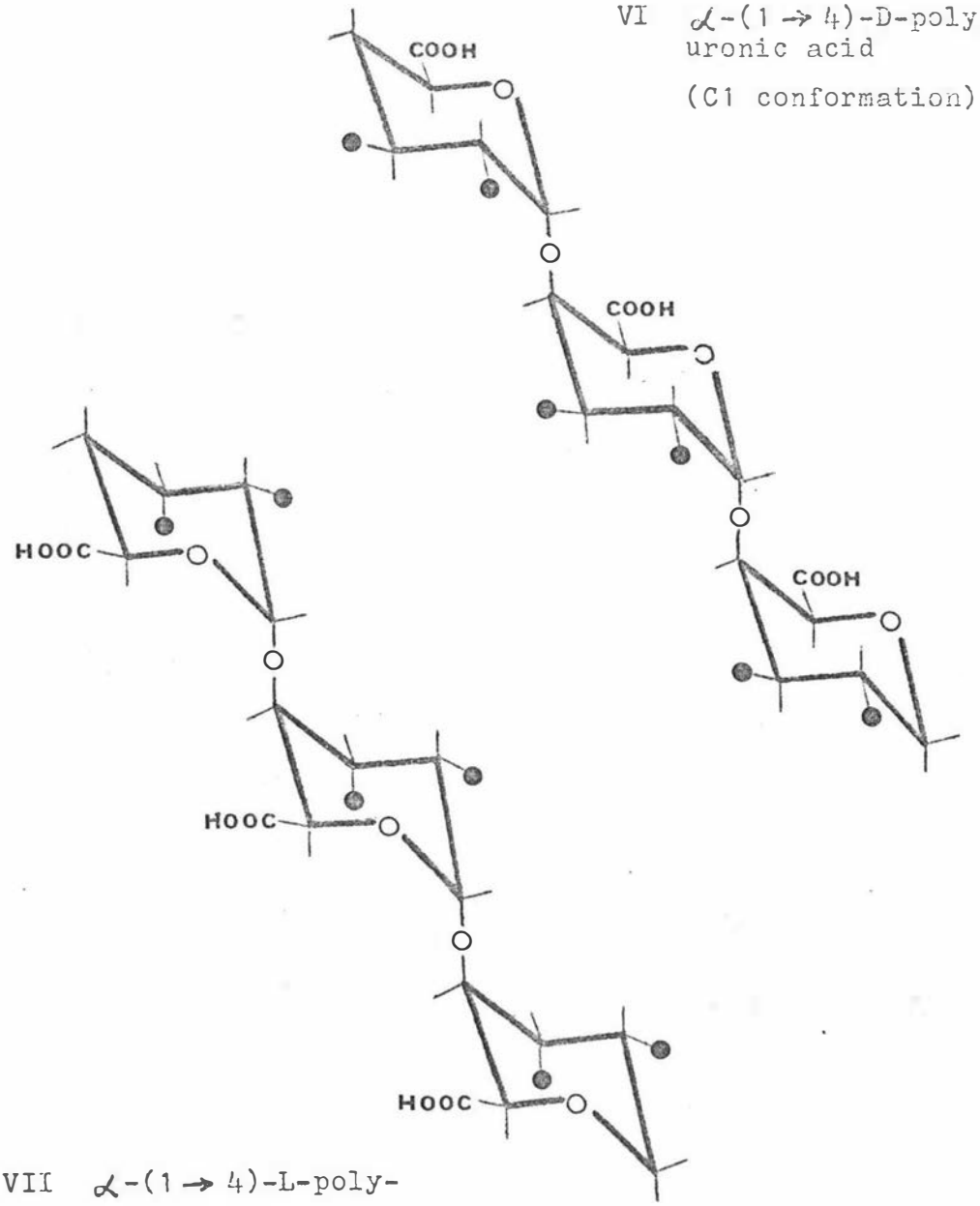
In the C1 conformation the free hydroxyls at C_2 and C_3 would be axial/equatorial in mannuronic acid and equatorial/equatorial in galacturonic acid; however, Schweiger ignores the fact that the binding properties of alginic acids are associated with the L-guluronic acid residues (Smidsrod and Haug, 1968) which have been shown by X-ray fibre diffraction to be in the 1C conformation with three axial hydroxyl groups (Atkins, Mackie and Smolko, 1970) as opposed to the single axial hydroxyl group in mannuronic acid. The fibre repeating distance for poly-mannuronic acid obtained by these workers is identical to those observed for $\beta(1 \rightarrow 4)$ linked hexosans such as cellulose and mannan (Marchessault and Sarko, 1967), thereby confirming the $\beta(1 \rightarrow 4)$ linkage of D-mannuronic acid units postulated from chemical evidence. To obtain this fibre repeating distance the monosaccharide units are in the energetically favourable C1 chair conformation with a diequatorial linkage, thus:



V $\beta(1 \rightarrow 4)$-poly-D-mannuronic acid (C1 conformation)

Similar recent X-ray fibre work has shown a shorter repeating distance for poly-L-guluronic acid and pectic acid, both of which can be explained by an α-configuration of the glycosidic linkage

(di-axial) and a 1C conformation for L-guluronic acid and a C1 conformation for D-galacturonic acid, thus:

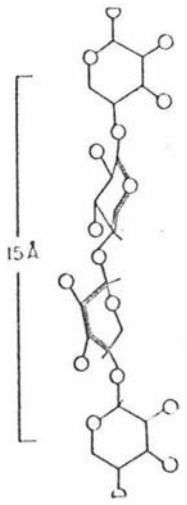


VI α-(1 → 4)-D-polygalacturonic acid
(C1 conformation)

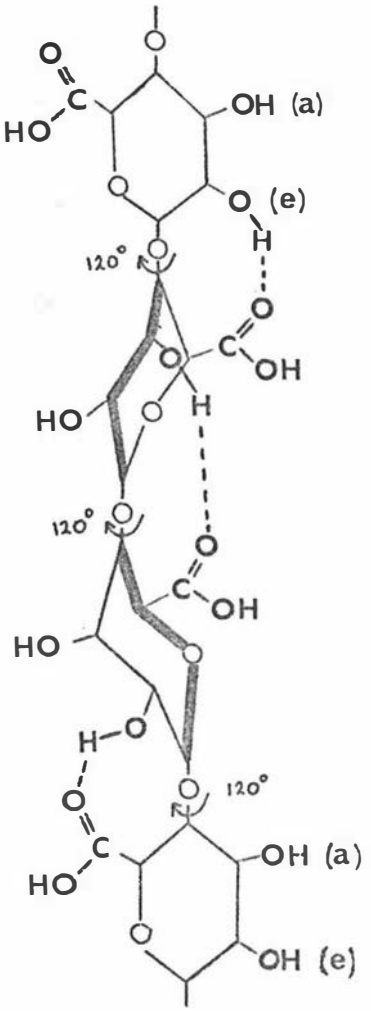
VII α-(1 → 4)-L-polyguluronic acid
(1C conformation)

It must be stressed that the polymeric diagrams drawn above are only attempts to represent the 3-dimensional situation where rotation about the glycosidic bond can occur, thereby enabling the polyuronide to adopt a helical structure. Two- or three-fold screw axes have been proposed for cellulose, amylose,

poly-D-galacturonic acid and D-xylan (Marchessault and Sarko, 1967), the latter (VIII), for instance, containing three xylose residues progressively rotated 120° in repeating units of 15A° in length (Marchessault and Liang, 1962). The energetic considerations involved in the steric configuration of these polymers are very complex but most workers seem to agree (Atkins, Mackie and Smolko, 1970; Marchessault and Sarko, 1967) that the most stable configuration is that involving the maximum number of intra-polymer (ie., inter-monomer) hydrogen bonds - usually between the hydroxyl substituents of C_2/C_3 and either oxygen atom of the carboxyl group of the adjacent sugar unit in the chain. If poly-L-guluronic acid is represented with a three-fold screw axis (diagram IX, as shown below) it is clear that the formation of such intrapolymer hydrogen bonds is possible.



VIII Repeating unit and conformation of a (4-O-methyl-D-glucurono)-D-xylan. Marchessault and Liang, (1962).

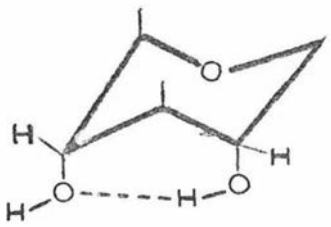


IX

Correspondingly, poly-D-mannuronic acid (V) and poly-D-galacturonic acid (VI) could be represented by similar three-dimensional diagrams showing their intrapolymer hydrogen bonds. The extent of this hydrogen-bonding would be expected to be of prime importance in determining the degree of cation binding by the polymer and is discussed in the following section.

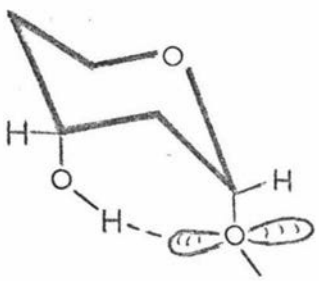
Hydrogen-bonding and steric factors involved in cation binding

The polyhydroxylic nature of the polyuronides gives rise to a variety of situations for hydrogen-bonding in the polymer. These may be between two hydroxyl groups (X)

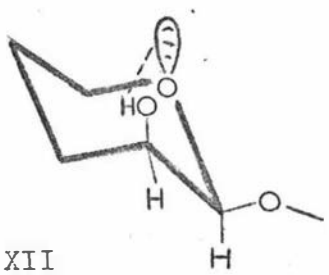


X

or between axial hydroxyl- groups and the p-orbital of the oxygen atom of the glycosidic bridge (XI) (Lemieux and Levine, 1964) or the ring (XII) (Lemieux and Stevens, 1965).

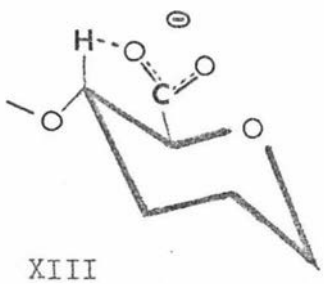


XI

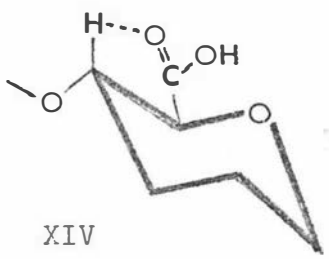


XII

In uronides, similar bonds may occur between carboxyl and hydroxyl groups when ionised (XIII) or unionised (XIV)

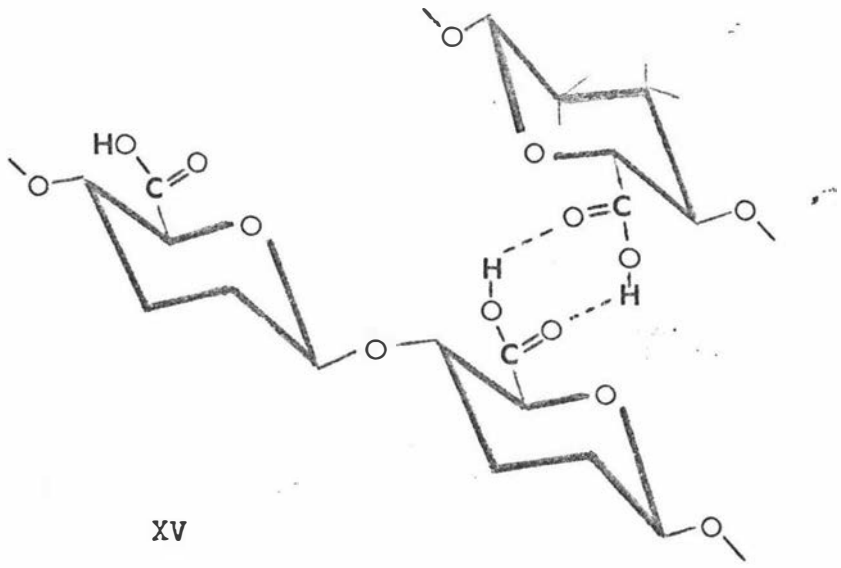


XIII



XIV

and, in acid solution, even between two non-ionised carboxyl groups on different polymers (XV).



XV

Hydrogen-bonding is generally favoured by non-hydroxylic solvents since water solvates the dipole associated with the ring oxygen atom and other substituents containing a lone pair of electrons, thereby reducing electrostatic interactions. Along with hydrogen bonding, solvation of lone electron pairs, is of considerable importance in determining monomer conformation but in this context, as yet, this is a poorly understood phenomenon. It is accepted, however, that the polymer conformation adopted will depend upon the minimisation of angular strain and energetically unfavourable interactions (non-bonded interactions) between substituents; consequently, the most stable conformations of the monomers in these polyuronides will have the greatest number of hydroxyl- and carboxyl substituents in the equatorial position, thereby avoiding the unfavourable interaction between axial substituents on the same side of the ring ('Hassel-Ottar effect').

As mentioned above, solvation will markedly reduce and

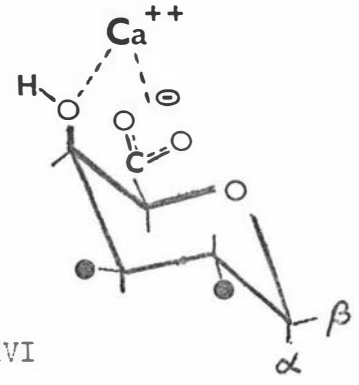
modify these non-bonded interactions in the polysaccharide and since the present investigation was carried out in aqueous media, solvation of both the cell wall fractions and the cations in the standard salt solution would be expected to occur. Consequently, hydroxylic solvents such as water will probably favour the carbohydrate conformation without intra-molecular (-polymer) hydrogen bonds because of the 'swamping effect' of the solvent in forming inter-molecular hydrogen bonds (Lemieux and Levine, 1964). A greater knowledge of the mode of incorporation of water molecules (both free and associated with the 'hydration sphere' of a cation) into the expanding lattices of these polyuronides seems one of the limitations to a better understanding of cation-uronide complexing. The difficulty encountered by Schweiger and other workers in acetylating alginates and pectins by normal methods is possibly due to a degree of hydrogen-bonding in the dried polymers; chemical reactivity of such polyuronides seems to require a pre-wetting of the material (Schweiger, 1962a).

In conclusion, it is difficult to explain the difference in cation affinity between poly-D-galacturonic and poly-L-guluronic acid on the one hand and poly-D-mannuronic on the other, on the basis of the steric distribution of hydroxyl groups alone. Gould and Rankin, (1970) suggest structure-function relationships for α-linked polyuronides on the premise that axial hydroxyl groups allow for greater interaction with a cation. Yet Rendleman (1966), in a review of alkali- and alkaline-earth metal complexes of carbohydrates, maintains that there is no indication that the oxygen atom of a glycosidic linkage can serve as an electron donor and, therefore, bind a cation. If this is the case, then poly-D-galacturonic acid has no free

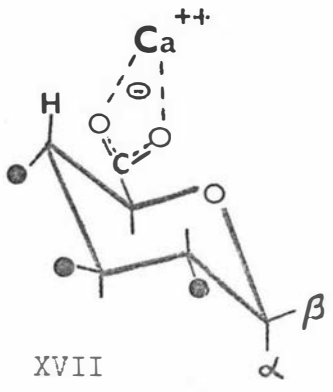
axial hydroxyl groups while poly-D-mannuronic and poly-L-guluronic acid each have one such axial hydroxyl group - a situation which offers no correlation between the Gould and Rankin (loc. cit.) hypothesis and the present, and literature, results on cation binding. One explanation for the non-reactivity of poly-D-mannuronic acid is the possibility of a strong hydrogen-bond between the axial hydroxyl group on C_2 and the p-orbitals of the ring or carboxyl group oxygen. Another possibility is an impedence of the flexibility of this polymer in aqueous solution; since all evidence points to a favourable juxtaposition of carboxyl- and hydroxyl- ring substituents for cation bonding to occur, it is possible that the diaxially-linked α-polymers are endowed with greater flexibility.

Structure-function relationships in monouronides

A definite structure-function relationship is suggested in the difference in Ca^{++} affinity in the monomers, D-galacturonic acid and D-glucuronic acid (Table 18). The greater strength of the galacturonate complex may be related to the hydroxyl group on C_4 which is axial in galacturonate (XVI) and equatorial in glucuronate (XVII).



XVI



XVII

This difference is in agreement with the results of Gould and Rankin (1970) yet at variance with other findings of non-

significant differences in Ca^{++} binding between monomers of D-galacturonate, L-guluronate, D-mannuronate and D-glucuronate (Kohn, Furda, Haug and Smidsrod, 1968; Buddecke and Drzeniek, 1962; Triffitt, 1968).

It is of interest that neither the polygalacturonic acid (pectic substance) from Yorkshire fog, or the two monomer uronic acids investigated, bound Mg^{++} ion - again, in agreement with the investigations of Haug, Smidsrod and Schweiger (see p. 97-100) This difference in cation affinity can partly be explained by the differences in the e^2/r ratio discussed for synthetic ionic exchangers on p. 74-75, but it is likely that the size and degree of hydration of the cation is of considerable importance in determining the extent to which it is bound by these selective polyuronides. The large hydrated radius of Mg^{++} has already been discussed in this context for ion exchange on synthetic resins in Section 3.2.2, p. 75 and for chelation in general, Section 1.2, p. 5.

4.2.2. Hemicelluloses and other polysaccharides

The limited and undistinguished literature on carbohydrate/alkali- and alkaline-earth metal complexes has been reviewed by Rendleman (1966). While the existence of carbohydrate/salt complexes has been assumed for over 60 years, the weak nature of these complexes and the analytical difficulties involved, have to date precluded the accumulation of quantitative data such as stability constants. Formation of such complexes is facilitated by alcoholic media and high concentration of salt, and discussion of complex formation in these non-physiological solutions is outside the scope of this discussion. So too, is the formation of soluble chelates of monosaccharides in aqueous alkaline solutions of Ca^{++}, Mg^{++}, Ba^{++} and Sr^{++} (Charley, Sarkar,

Stitt and Saltman, 1963).

Most investigations have been on aqueous complexes with common mono- and disaccharides and the principles involved seem to be those of chelation between metal ion and a multidonor molecule - similar to that discussed for uronides in the previous section. A group of two, or more, properly oriented hydroxyl groups, or a combination of a carbonyl group with one, or more, properly oriented hydroxyl groups on the carbohydrate molecule is necessary for the formation of these complexes.

There is very little information on the formation of polysaccharide/cation complexes. A conductivity study of locust-bean gum (a neutral polymer of β (1 → 4) linked mannose units with short branches of single α (1 → 6) linked galactose units) in aqueous solutions of the cations H^+, Na^+, K^+, Ag^+ and Ba^{++} led Barry and Halsey (1963) to conclude that no complex formation occurred. The electrolyte concentrations were in the physiological range of 0.0005 to 0.1N and the maximum polysaccharide concentration was 0.0277M (on a monosaccharide residue basis), equivalent to 0.5% concentration by weight; these polysaccharide concentrations are of the same order as those in the present investigation where concentrations were approximately 0.0125M (Table 19). The only known polysaccharide complexes of a definite stoichiometric type are the addition compounds of amylose (Senti and Witnauer, 1952) but these were formed from alkali metal hydroxide adducts in aqueous ethanolic medium.

In contrast to the pectic acid fraction, the hemicelluloses and cellulose from Yorkshire fog showed little ability to bind Ca^{++} and Mg^{++} in the standard salt solution. If it is assumed that, like polyuronides, the complexing of cations depends primarily upon the presence of carboxyl groups in the polymers,

then there are two possible reasons for the lack of any appreciable binding:

(a) the glucuronic acid substituents are randomly distributed as short side chains along a xylan backbone which may differ in its degree of flexibility as compared with the polyuronide chains discussed in the previous section.

(b) the degree of cation binding is a function of the distance apart of the glucuronic acid groups on the xylan chain.

In view of the shortcomings in present knowledge of the fine structure of polyuronides and xylans, and the effect of the spatial distribution of hydroxyl and carboxyl groups upon their flexibility, it is difficult to answer possibility (a). The ability of a polymer to adopt a variety of coiled orientations with variation in solution cation concentration, and thereby achieve maximum interaction of cations with the anionic sites, would be expected to be a criterion of prime importance in complex formation; it is possible that $\alpha(1 - 4)$ linked polymers like pectic acid are relatively inflexible because of the axial nature of the linkage, but, unfortunately, there seems to be no information on such flexibility. Regardless of polymer flexibility it is clear from the work of Kohn and Furda (1967a) discussed on p.98 that the bond strength for complexes with Ca^{++} and other alkaline-earth cations depends upon the distribution pattern of free carboxyl groups in the polymer, ie., case (b). Molecules with segments richer in free carboxyl groups or with a blockwise arrangement will, due to a higher charge density in these segments, bind cations more firmly.

The present investigation did not include detailed information on the statistical distribution of glucuronic acid units

along the xylan chains of hemicellulose B fractions isolated from Yorkshire fog. Furthermore, the non-linear nature of hemicellulose B (branched) is an additional factor complicating the determination of a mean 'distribution distance' of carboxyl groups. Nevertheless, if it is assumed that carboxyl groups are distributed regularly along these xylans, then Table 20 shows the dependence of hemicelluloses, like pectins, upon their uronide content for the degree of cation binding exhibited.

TABLE 20

RELATIONSHIP BETWEEN URONIDE CONTENT OF PLANT FRACTIONS AND DEGREE OF Ca AND Mg BINDING

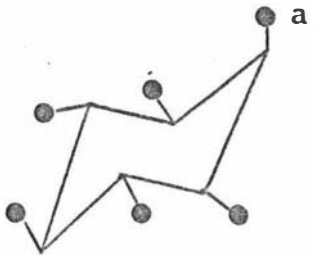
Polymer*	% uronide	% Ca bound	% Mg bound
Pectin	87.4	31.7	4.2
Hemicellulose B (branched)	12.9	10.6	1.4
Hemicellulose B (linear)	3.2	4.5	1.4

* pH range 7.2 - 7.9

Cellulose, like the hemicellulose B (linear) fraction, gave a low degree of complexing with Ca^{++} but, as with all the hemicellulose B fractions investigated, there was no significant binding of Mg ion (Table 17). It is not possible to offer any explanation, except that of experimental error at this low level of significance, for the slightly higher level of Ca^{++} binding at the acidic pH.

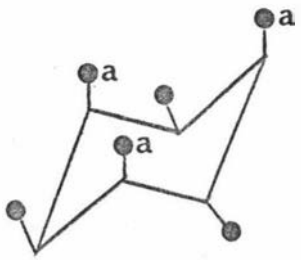
The alicyclic polyhydric alcohol (cyclitol), meso-(myo-) inositol is widely distributed in plants where it is usually found as the hexaphosphate (phytic acid); for this reason, and the fact that the nine stereoisomers of inositol offer an

interesting variety of hydroxyl group arrangements for associations with cations, these two synthetically-prepared metabolites were investigated (Table 18). The inositols exist as strainless non-planar rings and show a conformational isomerism in the same manner discussed for hexose sugars; in particular, meso-inositol (XVIII) is thought to exist in a chair form with five hydroxyl groups disposed equatorially and one axially:



XVIII meso-inositol

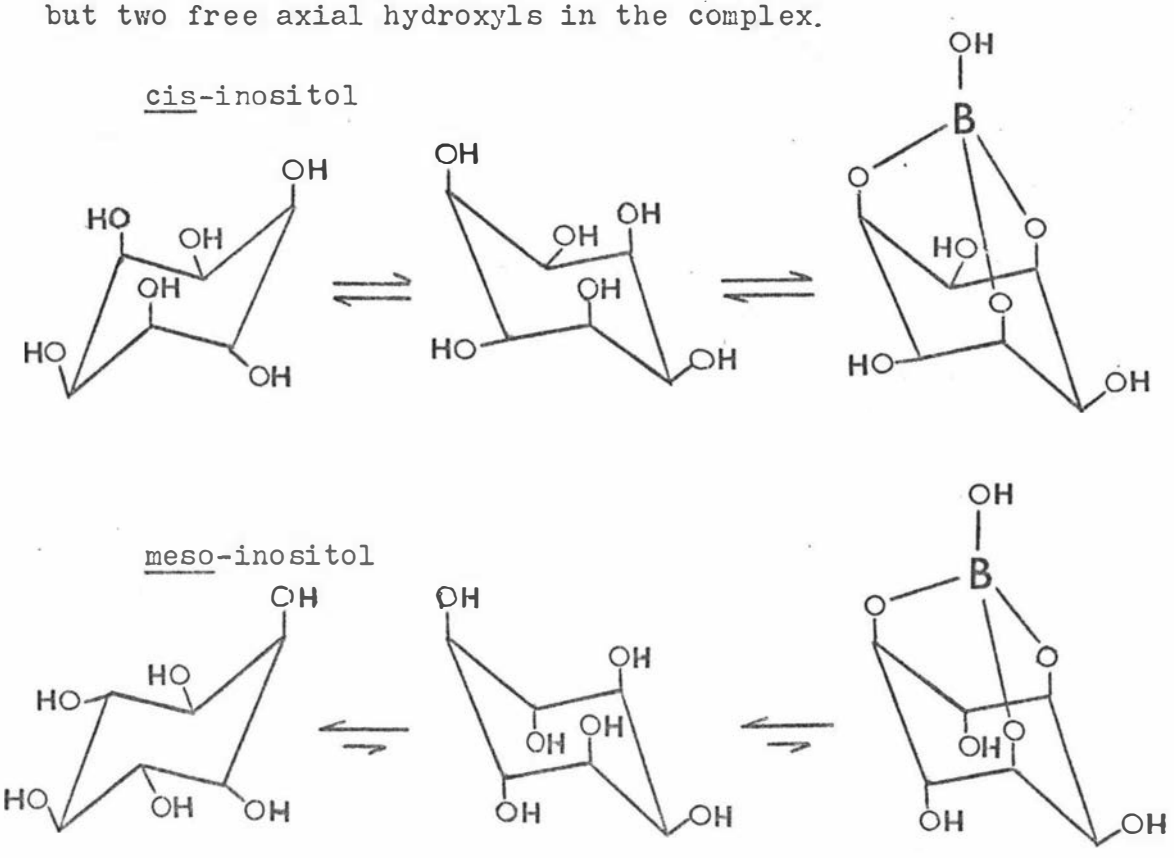
Mills (1961/62) used cellulose paper electrophoresis to provide evidence for the existence of complexes of inositols and other polyhydroxy compounds with cations of alkali and alkaline-earth metals in dilute aqueous solutions. He attributed the outstanding complexing power of cis-inositol to the three axial hydroxyl groups in the chair conformation (see below) which were considered to be suitably oriented for the close approach of a cation.



cis-inositol

This conclusion follows from the energies of interaction of axial hydroxyl groups on cyclitols, as determined by the formation of borate complexes (Angyal and McHugh, 1957). Cyclitols with axial hydroxyls at C_1, C_3 and C_5 (such as cis-inositol) react reversibly with borate to give a tridentate complex, in

contrast to the usual bidentate complex formed between borate and cis-1,2-diols. The non-bonded interactions between three such axial hydroxyl groups would be considerable and is analagous to such interactions between an axial hydroxyl group at C_3 in the 1C conformation of hexoses such as glucose, galactose and mannose, and the anhydro bridge. However, conversion to such unfavourable conformations involving axial hydroxyl groups has been shown to be necessary prior to the formation of 1,6-anhydrides and other sugar tridentate anionic complexes such as those with periodate (Barker and Shaw, 1959). The extent to which such tridentate complexes are formed, therefore, is related to the non-bonded interactions in the parent inositol and in the complex. Thus cis-inositol (as illustrated below) has three axial hydroxyls in either conformation of the parent cyclitol but no free axial hydroxyls in the complex; therefore, it undergoes more extensive complex formation than meso-(myo-) inositol which has only one free axial group in the parent form but two free axial hydroxyls in the complex.



Consequently, the lack of Ca^{++} and Mg^{++} complexing shown by meso-inositol in the present investigation is easily explained. The marked binding of these cations by meso-inositol hexaphosphate is due to the presence of the six esterified phosphate groups. In fact, 'phytin', which is found in many plant seeds, is the calcium magnesium salt of 'phytic acid' (the hexaphosphate ester of meso-inositol) and, because of its affinity for these elements, has long been known for its antinutritional effects in humans. The marked affinity of the hexaphosphate for Ca^{++} and Mg^{++} (Table 18) at a slightly alkaline pH (7.48) is comparable only with that of citric acid at pH 7.00 and is probably due to the high density of donor groups (in this case phosphate) on the molecule compared with the other plant fractions investigated. The sharp increase in binding by inositol hexaphosphate with an increase in pH from 5.70 to 7.48 can be attributed to a degree of dissociation by the acidic phosphate groups.

4.2.3. Lignin

The limited information on the chemical composition of Yorkshire fog lignin and the inadequate data on other grass lignins was discussed in Section 2.5.2; consequently, it is only possible to speculate on the mode of cation complexing by this polymer. Although any repeating unit formula is inadequate in denoting the structure of lignin, it is still clear that oxygen atoms in the polymer are present in several different groupings - such as primary hydroxyl, phenolic hydroxyl, carbonyl and ether linkages. As such, the polymer would be expected to offer a variety of situations for associations with cations. A variety of acid, alkali and dioxane/acid extracted lignins from oat straw, rye straw and bromegrass have exhibited cation exchange capacities between 50 and 130 milliequivalents per 100g for Ca^{++}

at pH 7.0 (Thompson, Chesters and Engelbert, 1964). The percentage of Ca^{++} plus Mg^{++} complexed by the Yorkshire fog lignin (Table 19) is equivalent to a high cation exchange capacity of approximately 125 meq/100g, (Ca^{++}, 95 meq/100g; Mg^{++}, 30 meq/100g), compared with a figure of 80 meq/100g obtained by the above authors for and oat straw lignin, also extracted with 5% aqueous alkali at 25°C. As previously mentioned, the significant binding of Mg^{++} by Yorkshire fog lignin, in contrast to the polysaccharides, is of interest but, unfortunately, no other investigations of lignin affinity for Mg^{++} are known to this author.

Thompson, Chesters and Engelbert (1964) also found a considerable decrease in cation exchange capacity for all lignins with a decrease in solution pH from 7.0 to 5.7. Unfortunately, inadequate quantities of isolated Yorkshire fog lignin allowed the use of only one pH - the intermediate value of 6.2. Such an increase in cation exchange capacity with pH would be expected in this lignin also from the ionisation of the moderate concentration of phenolic hydroxyl groups (Table 6) in the polymer.

4.2.4. Organic acids

The ability of carboxylic acids, particularly those with an α-hydroxyl group, to form ring complexes of exceptional stability (chelates) is well documented (Martell and Calvin, 1952) and has already been discussed in the context of intestinal absorption in Section 1.2. Lehman (1963) has given a more recent review of aspects of chelation chemistry, especially considerations of the favourable entropy increase in the formation of alkaline-earth cation - organic ligand ring complexes. Stability constants for a wide variety of alkaline-earth cation/carboxylic acid chelates are now well documented (Martell and Calvin, 1952; Bjerrum, Schwarzenbach and Sillen, 1958).

The aliphatic carboxylic acids present in Yorkshire fog were predictable in their ability to bind both Ca^{++} and Mg^{++}; because of the complexity of the standard salt solution and the inability of the resin method to give information on thermodynamic activities of the different ionic species in such a solution, it was not possible to derive stability constants for these different acids. Nevertheless, it is clear in a qualitative way that the extent to which these acids bind Ca^{++} and Mg^{++} under these experimental conditions is related to their stability constants as documented in the literature. In all cases more Ca^{++} was bound than Mg^{++} although, somewhat inexplicably, oxalic acid had a greater preference for Mg^{++} than the other acids. There was no evidence at these concentrations of complex formation with Na^{+}, K^{+} or NH_4^{+}; evidence of such weak complexes in concentrated solutions (up to 3.0M) has been shown by NMR studies of malate and other α- and β-hydroxy carboxylic acid anions (Jardetzky and Wertz, 1956; Erickson and Alberty, 1962).

In most cases, pH had an appreciable effect upon chelation of Ca^{++} and Mg^{++} (Table 17). Only for oxalic acid was there no significant increase in complexed cations with increase in pH; in fact, the reverse was true for this acid. Since the chelating ligand is effective as a Lewis base, the hydrogen ion can act as a successful competitor to the metal ion and, consequently, chelate formation is very dependent upon pH. Generally, Ca^{++} is not strongly chelated below pH 5 and at moderate alkaline pH (9 - 10) there is a likelihood that the solubility product of $Ca(OH)_2$ may be reached. As discussed earlier, the physiological pH range throughout the ruminant intestine is about pH 4.5 to 8.5 and generally the two pH levels chosen for this investigation were near each of these extreme values. All the aliphatic

carboxylic acids gave a highly significant degree of chelation at the lower pH while the tricarboxylic acids, citric and trans-aconitic, gave marked increases at the higher pH value.

The alicyclic acid, quinic acid (1,3,4,5,tetrahydroxycyclohexane carboxylic acid) showed a low affinity for Ca^{++} and none whatever for Mg^{++}. Shikimic acid (3,4,5,trihydroxy-1-cyclohexene carboxylic acid) could not be obtained in sufficient quantities to be investigated. However, the non-benzenoid ring structure of both probably precludes the formation of stable anions which normally occurs in aromatic carboxylic acids by interaction of the negative charge with the delocalised π-orbitals of the aromatic nucleus. This inability to form resonance-stabilised anions is probably the prime reason for the observed low degree of complexing by quinic acid, although both acids also have structures which, like meso-inositol, are not conducive to chelate formation.

4.3. POSSIBLE IN VIVO IMPLICATIONS OF COMPLEXING RESULTS

One of the major obstacles to a better understanding of the mechanisms of intestinal absorption in ruminants is the complexity of the organic and inorganic 'milieu' in the digesta. The standard salt solution used in this investigation is a simplified version of the complex solution of ions that make up the intestinal fluid of a ruminant. The only anion used in this solution was chloride whereas both inorganic (bicarbonate, sulphate and phosphate) and organic (organic acids, proteins, bile and pancreatic secretions) anions would be present to some extent in vivo. The intestinal fluid also contains water-soluble organic non-electrolytes which are largely responsible for the osmotic pressure of the small intestine of a ruminant being considerably greater than the osmotic pressure of its blood

(van Weerden, 1961). Furthermore, the gross fractions studied would be expected to undergo extensive chemical modification in their passage through the gut and their binding effect, *in toto*, may be considerably different from that observed with the individual fractions. The present results then, while indicating the relative binding abilities of the extracted plant fractions *in vitro*, do not necessarily reflect the *in vivo* situation. As already pointed out in Section 1.4., this investigation was not undertaken for the express purpose of extrapolating the complexing findings to the *in vivo* situation of absorption in ruminants. However, these results do raise the following implications which are worth discussing in the general context of ruminant nutrition and alkaline-earth metal absorption discussed in the introduction, Part 1.

Although the major organic acids of Yorkshire fog, citric, malic and *trans*-aconitic, chelate Ca^{++} and Mg^{++} strongly their survival in the rumen, and consequent passage into the small intestine, is very doubtful. For example, citric acid is reported to be rapidly utilised by rumen micro-organisms (Packett and Fordham, 1965) and *trans*-aconitic acid is rapidly absorbed through the rumen epithelium (Kennedy, 1968).

The pectic substances bound Ca^{++} to almost the same extent as some of the organic acids but polygalacturonides are only present in grasses in small amounts (2 - 5% dry weight) and artificial rumen studies (Dehority, Johnson and Conrad, 1962) have shown that they are fermented at a faster rate than hemicellulose or cellulose. Pectic substances are lost from the reticulo-rumen of cattle, by either fermentation or passage out of the rumen as undigested particles, at a rate between that of the rapidly utilised soluble sugars and the slowly-fermented hemicellulose and cellulose (Bailey, 1967). In sheep, however,

Waite, Johnson and Armstrong (1964) found that the apparent digestibility of pectin was considerably lower than that of either hemicellulose or cellulose at all levels of pasture maturity. At the flower emergence stage only lignin had a lower digestibility than pectin. Furthermore, these same authors found a corresponding appreciable decrease in the apparent digestibility of the xylan-uronic fraction of the hemicellulose. There is a distinct possibility, therefore, that polyuronides may be present in the intestinal fluids of ruminants but there is no specific evidence of this as yet.

In view of the strong correlation between an increasing degree of uronide esterification and decreasing cation affinity (Kohn and Furda, 1967a; p. 98), the presence of pectin esterases in the ruminant intestinal tract will be of considerable significance in the possible binding of Ca^{++} and Mg^{++} by these polyuronides.

Hemicellulose and cellulose degradation seems to be restricted to the reticulo-rumen (Gailliard and van't Klooster, 1969) with little further digestion of these cell wall constituents during their passage through the intestines. In view of the resistance of these polysaccharides to further degradation their slight binding of Ca^{++}, especially that by branched hemicellulose B at the higher pH, may assume some significance in the intestine. Phytic acid is completely hydrolysed in the rumen of sheep and, in general, inositol polyphosphates are not considered a problem in ruminant Ca and Mg nutrition since they are effectively absorbed by rumen micro-organisms (Reid, Franklin and Hallsworth, 1947).

Lignified plant tissues are degraded only slowly in the reticulo-rumen (Bailey, 1967) to a size which permits their

passage on to the intestines where further digestion of the lignin is minimal (Gailliard and van't Klooster, 1969). Because of this persistence, the results indicating an appreciable binding of Ca^{++} by lignin and a small, but significant, binding of Mg^{++} are of interest. Furthermore, the absence of binding by the partly lignified hemicellulose B(I) indicates that the cleavage of the lignin-hemicellulose bond, be it a glycosan uronide ester (Mercwether, 1960), benzyl ether (Freudenberg and Neish, 1968) or acetal (Eolker and Terashima, 1966), may be of significance.

The apparent digestibility in sheep of organic acids, pectin, hemicellulose, cellulose, lignin and protein decreases markedly with the advancing maturity of grasses (Waite, Johnson and Armstrong, 1964). The higher lignin and aldobiuronic acid content of more mature grasses is probably responsible for rendering their structural polysaccharides more resistant to attack by rumen micro-organisms. Since the present investigation has indicated that these cell wall polymers can individually bind Ca^{++} and Mg^{++} to varying extents <u>in vitro</u>, it is reasonable to assume that the degree of lignification of the feed, and the rate of digestion of the cell wall polysaccharides in the rumen, will considerably influence the form and concentration of the modified digestion products which may bind these cations in the intestine.

APPENDIX I

DERIVATIONS AND ANALYSIS OF VARIANCE OF MULTIPLE REGRESSIONS FOR THE RELATIONSHIP BETWEEN RESIN Ca^{++} AND Mg^{++} AND SOLUTION Ca^{++} AND Mg^{++}

Let the required expressions be of the type

$$Y_1 = \alpha_1 + \beta_1 X_1 + \beta_2 X_2 \qquad \text{and}$$

$$Y_2 = \alpha_2 + \beta_3 X_1 + \beta_4 X_2$$

where

Y_1 = Resin Ca^{++} (Ca^{++}_R); X_1 = Solution Ca^{++} (Ca^{++}_S)

Y_2 = Resin Mg^{++} (Mg^{++}_R); X_2 = Solution Mg^{++} (Mg^{++}_S)

16 sets of data (n = 16)

Ca^{++}_S (meq/l)	Ca^{++}_R (meq/g)	Mg^{++}_S (meq/l)	Mg^{++}_R (meq/g)
5.0	1.673	6.0	0.687
5.0	1.670	5.0	0.606
5.0	1.681	4.0	0.492
6.0	1.710	6.0	0.636
6.0	1.711	5.0	0.544
6.0	1.782	4.0	0.477
7.0	1.907	5.0	0.424
7.0	1.816	6.0	0.466
8.0	2.030	5.0	0.409
8.0	1.953	6.0	0.553
9.0	2.079	5.0	0.424
9.0	2.058	6.0	0.434
10.0	2.182	6.0	0.411
11.0	2.228	6.0	0.399
12.3	2.269	5.0	0.356
12.3	2.291	6.0	0.374

In the following computations, a shorthand notation is used:

eg., $$C\sum y_1^2 = \sum y_1^2 - \frac{(\sum y_1)^2}{n}$$

and $$C\sum y_1 x_1 = \sum y_1 x_1 - \frac{\sum y_1 \sum x_1}{n}$$

$\sum y_1^2 = 60.990104$ $\sum y_1 = 31.04$ $C\sum y_1^2 = 0.772504$

$\sum y_2^2 = 3.842118$ $\sum y_2 = 7.692$ $C\sum y_2^2 = 0.144189$

$\sum x_1^2 = 1094.58$ $\sum x_1 = 126.6$ $C\sum x_1^2 = 92.8575$

$\sum x_2^2 = 470$ $\sum x_2 = 86$ $C\sum x_2^2 = 7.75$

$\sum x_1x_2 = 690.3$

$\sum y_1x_1 = 253.912$ $C\sum y_1x_1 = 8.3080$

$\sum y_1x_2 = 167.648$ $C\sum y_1x_2 = 0.8080$

$\sum y_2x_1 = 57.993$ $C\sum y_2x_1 = -2.86995$

$\sum y_2x_2 = 41.451$ $C\sum y_2x_2 = 0.1065$

Consider the first case (eqn. 3.3.1., p. 88),

$$y_1 = \alpha_1 + \beta_1 x_1 + \beta_2 x_2$$

by using the following relationships,

(1) $$(n)\alpha + (\sum x_1)\beta_1 + (\sum x_2)\beta_2 = (\sum y_1)y_1$$

(2) $$(\sum x_1)\alpha + (\sum x_1^2)\beta_1 + (\sum x_1x_2)\beta_2 = (\sum x_1y_1)y_1$$

(3) $$(\sum x_2)\alpha + (\sum x_1x_2)\beta_1 + (\sum x_2^2)\beta_2 = (\sum y_1x_2)y_1$$

estimates of β_1 and β_2 can be obtained:

$$\beta_1 = 0.090591$$

$$\beta_2 = -0.010588$$

The sum of squares due to fitting β_1 and β_2

$$= \beta_1(C\sum y_1x_1) + \beta_2(C\sum y_1x_2)$$

$= 0.744075$ for two degrees of freedom (df)

An Analysis of Variance for this multiple regression is as follows.

Source	Sum of Squares (SS)	Degrees of freedom (df)	Mean square (MS)	Variance ratio (F)	Probability (P)
Total	0.772504	15			
R*($\beta_1\beta_2$)	0.744075	2	0.372038	170.1133	P < 0.001
Residual	0.028429	13	0.002187		

R* Reduction due to the fitting of β_1 and β_2

∴ Residual sum of squares

as % of Total sum of squares = 96.32%

(= r^2 or coefficient of multiple determination x 100%)

Now to test the hypothesis that $\beta_2 = 0$, ie., that the multiple regression is effectively a simple linear regression, a solution of β' in the equation

$$y_1 = \alpha' + \beta' x_1$$ is needed.

Solving using the relationships outlined above,

$$\beta' = 0.08947$$

and the sum of squares due to $\beta' = \beta'(C\sum y_1 x_1)$

$= 0.743317$ for 1df

Analysis of Variance for the Multiple Regression involving the test of $\beta_2 = 0$

Source	SS	df	MS	F	P
Total	0.772504	15			
R($\beta_1\beta_2$)	0.744075	2			
R(β_1 alone)	0.743317	1			
R(β_2 after adjusted for β_1)	0.000758	1	0.000758	0.34	N.S.
Residual	0.028429	13	0.002187		

Hence it is possible to conclude that β_2 makes no significant

contribution to an explanation of the variance in y_1 and, consequently, the alternate model $y_1 = \alpha' + \beta_1 x_1$ is adequate. This can be verified by a further Analysis of Variance.

Source	SS	df	MS	F	P
Total	0.772504	15			
Regression	0.743317	1	0.743317	356	p 0.001
Residual	0.029187	14	0.002085		

and in this case the regression sum of squares as a % of the total sum of squares = 96.22%, a value negligibly different from that obtained with the full multiple regression containing β_2. The constant, α', can now be computed from any normal equation and the simple linear equation

$$y_1 = 1.232069 + 0.08947\ X_1 \qquad \text{(eqn. 3.3.3, p. 88)}$$

can be used as a very useful estimation equation for solution calcium.

The second case (eqn. 3.3.2, p. 88) involves the multiple regression for resin magnesium,

$$y_2 = \alpha_2 + \beta_3 X_1 + \beta_4 X_2$$

A solution similar to that outlined above for $[Ca^{++}]_R$ gives the multiple regression

$$Mg^{++}_R = 0.447936 - 0.037374\ Ca^{++}_S + 0.061123\ Mg^{++}_S$$

(eqn. 3.3.4, p. 88)

The sum of squares due to fitting β_3 and β_4

$$= \beta_3(c\{y_2x_1) + \beta_4(c\{y_2x_2)$$

$$= 0.107262 + 0.006510$$

$$= 0.113772 \text{ for 2 degrees of freedom,}$$

and it is clear that the β_3 term provides most (94%) of the total sum of squares.

An analysis of variance for this multiple regression gives:

Source	SS	df	MS	F	P
Total ($c\sum y_2^2$)	0.144189	15			
R ($\beta_3 \beta_4$)	0.173722	2	0.056886	24.310	<0.001
Residual	0.030417	13	0.002340		

Regression sum of squares as % of

total sum of squares = 78.9%

Consequently the β_3 and β_4 terms are significant in this multiple regression which does not give as good an explanation for the variance in $[Mg^{++}]_R$ as does the linear regression for $[Ca^{++}]_R$.

APPENDIX 2

CONFIDENCE LIMITS FOR ESTIMATES OF SOLUTION CALCIUM

The simple linear regression (eqn. 3.3.3, p. 88) derived in Appendix I is used to estimate Ca^{++}_S which is then substituted in eqn. 3.3.4. for the estimation of Mg^{++}_S (section 3.3.5, p. 89). To examine the confidence limits of this equation it is necessary to calculate the Standard Deviation of the coefficient β' (Appendix 1), thus:

$$\sigma_{\beta'} = \frac{\sqrt{\text{Residual Mean Square}}}{\sqrt{c \sum x^2}} = \sqrt{\frac{0.002085}{92.8575}} = 0.00473814$$

Now, for (n-2)df (ie, 14 df),

the 95% confidence limits of $\beta' = \beta' \pm t_{0.05} \cdot \sigma_{\beta'}$

For 14df, $t_{0.05} = 2.145$

$\therefore t_{0.05} \cdot \sigma_{\beta'} = 0.01016331$

thus $\hat{\beta}' = 0.08947 \pm 0.010163$

$= 0.079307 - 0.099633$

ie., approximately $\pm$ 10% (p. 91).

Since eqn. 3.3.3. is to be used for calibration, (i.e., for a measured value of Ca^{++}_R it is intended to estimate the corresponding Ca^{++}_S, and, in general, any X_i from a particular Y_i), there are errors of estimation associated with it, as follows:

$$X = \frac{\hat{x} \pm t_{0.05} \cdot \sqrt{RMS} \cdot \sqrt{\frac{n+1}{n}(1 - c^2) + \frac{\hat{x}^2}{c \sum x^2}}}{1 - c^2}$$

x = the limit values of the predicted solution concentration

$\hat{x}$ = is the value calculated from the prediction equation, ie., $= \frac{Y - \alpha}{\beta'}$

$$c^2 = \frac{1}{c\sum x^2} \cdot \left(\frac{t.\sqrt{RMS}}{\beta'}\right)^2$$

RMS = Residual mean square (Appendix 1)

Since x, the predicted value of x for a given y will be different each time, these limits need to be computed for each estimate undertaken. However, some portions of the expression, such as $\frac{t_{0.05}\cdot\sqrt{RMS}}{\beta'}$ and c^2, are constant each time and, consequently can be inserted for each limit calculation.

Individual ranges for the values of % Ca^{++} bound and % Mg^{++} bound (Table 19, p. 90) were not computed but were set at approximately $\pm$ 10% and $\pm$ 25% respectively (p. 91). However, the calculation of one of these 10% confidence limits in x (solution Ca^{++}) is given below to illustrate the point. Consider a y value of 2.085 (ie, $[Ca^{++}]_R$ = 2.085 meq/g).

Then $\hat{x} = \frac{y - \alpha}{\beta'} = 9.53315$

$\hat{x}^2 = 90.880949$

Since $\frac{t_{0.05}\cdot\sqrt{RMS}}{\beta'}$ is constant for this regression,

$$= \frac{2.145 \times 0.002085}{0.08947}$$

$$= 1.09471957$$

and squared = 1.19841094

Now $c^2 = \frac{1}{c\sum x^2} \times 1.19841094$

$$= 0.012906$$

$\therefore 1 - c^2 = 0.987095$

Since n = 16, $\frac{n+1}{n} = 1.0625$

$$\therefore x_{range} = \frac{9.53315 \pm \left[1.09471957\sqrt{(1.0625)(0.987095) + \frac{90.880949}{92.8575}}\right]}{0.987095}$$

$$= 8.078631 - 11.112304$$

ie., a $[Ca^{++}]_R$ of 2.085 meq/g arose through equilibrium with a solution of $[Ca^{++}]_S$ range, 8.079 to 11.112 meq/l (ie., $\pm$ 10%)

Now, original $[Ca^{++}]_S$ = 12.3 meq/l

So mean $[Ca^{++}]_{bound}$ in 20 cm^3 of solution = (12.3 - 9.595)meq/50mg complexant

= 2.705 meq $\pm$ 10%

ie., this is the value expressed as a percentage in Table 19 ($[Mg^{++}]_{bound}$ limits in Table 19 = $\pm$ 25%)

ADDENDUM

Insert after 'hexa-acetate', line 5, p.29:

..... These two hexose derivations could have been resolved if it had been possible to use the medium polarity liquid phase, ECNSS-M, which has proven satisfactory for the separation of monoses as their alditol acetates (Sawardeker, Sloneker and Jeanes, 1965; Oades, 1967) or acetylated nitrite derivatives (Blake and Richards, 1970).

Insert after 'galactose', third to bottom line, p.35:

..... It is likely that the pH employed in the overnight dialysis of this pectin may have been sufficiently low to hydrolyse off any L-arabinofuranose residues in the polymer. These units may be present as highly branched L-arabinofuranose sidechains or completely unattached 'pectic arabans' (Rees, 1967).

Insert after 'seaweeds', line 17, p.97:

.....In the following pages the relationship of polymer structure to cation affinity is discussed in terms of the configurations of polyuronides, however, many of the principles involved are applicable to other polysaccharides. A general, but extensive, coverage of the structure, shape and function of some of these plant polysaccharides is given by Rees (1967).

REFERENCES

Blake, J. D. and Richards, G. N., 'Polysaccharides of tropical pasture herbage'. Aust. J. Chem., 1970, 23, 2361-8.

Oades, J. M., 'Gas-liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates'. J. Chromatogr., 1967, 28, 246-52.

Rees, D. A., "The shapes of molecules; Carbohydrate polymers". 1967, Oliver and Boyd, Edinburgh and London.

Sawardeker, J. A., Sloneker, J.H. and Jeanes, A., 'Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography'. Anal. Chem., 1965, 37, 1602-4.

ADDENDUM

Summary of resin equilibration with plant complexant (p.75 and pp.79-81)

Fifty milligrams of plant complexant and 0.7 cm^3 of standard resin (equilibrated with the standard salt solution) were gently shaken in 20 cm^3 of the standard salt solution for 15 minutes. If any Ca^{++} or Mg^{++} in the solution is bound by the plant complexant, then the resin adjusts its cation load to reflect this new equilibrium. This resin adjustment further changes the activities of all the cations in solution, a situation illustrated graphically in Fig.11. It was found that 10 separate 15 minute shakings with fresh portions of standard salt solution and 50 mg of plant complexant were necessary to bring the resin cation loads to their 'true' equilibrium level (Fig.11).

To shorten this procedure a large solution of plant complexant and standard salt solution was made up (bottom p.80). Portions of this solution (20 cm^3) were shaken with the standard resin, decanted, and replaced by a further 20 cm^3 portion until the procedure had been repeated ten times.

After the final decantation the resin was quickly washed onto a sintered glass filter under suction with deionised water (2 x 10 cm^3). The washings were discarded and the resin spooned back into the shaking flask; the few beads remaining on the filter were washed into the shaking flask with 20 cm^3 2 m HCl. The cations were eluted from the resin by shaking this mixture for 15 minutes and then transferred to a weighed sintered glass filter as described in section 3.2.3 (bottom p.75). Resin eluates were prepared and the resin weighed exactly as outlined in this section (3.2.3.).

REFERENCES

Adams, G. A., 'Structure of an arabinogalactan from Tamarack'. Can. J. Chem., 1960, 38, 280-93.

Allcroft, R., and Burns, K. N., 'Hypomagnesaemia in cattle'. N.Z. Vet. Jl., 1963, 16, 109-128.

Allcroft, W. M., and Green, H. H., 'Seasonal hypomagnesaemia of the bovine without clinical symptoms.' J. comp. Path., 1938, 51, 176 91.

Ames, B. N., and Mitchell, H. K., 'The paper chromatography of imidazoles.' J. Am. Chem. Soc.; 1952, 74, 252-4.

Angyal, S. J., and McHugh, D. J., 'Cyclitols. V. Paper ionophoresis, complex formation with borate and the rate of periodic acid oxidations.' J. Chem. Soc., 1957, 1423-31.

Aspinall, G. O., 'Structural chemistry of the hemicelluloses.' Adv. Carbohyd. Chem., 1959, 14, 429-68.

Aspinall, G. O., Cairncross, I. M., and Ross, K. M., 'A xylan from the roots of perennial ryegrass (Lolium perenne).' J. Chem. Soc., 1963, 1721-7.

Aspinall, G. O., and McGrath, D., 'The hemicelluloses of Lucerne.' J. Chem. Soc., 1966, C. Pt. 2, 2133-39.

Atkins, E. D. T., Mackie, W., and Smolko, E. E., 'Crystalline structures of alginic acids.' Nature, 1970, 225, 626-8.

Aulin-Erdtman, G., 'Studies on ultra-violet absorption changes caused by modifications of the chromophores, with special reference to lignin chemistry.' Svensk. Kemisk. Tidskrift., 1958, 70, 145-156.

Aulin-Erdtman, G., and Hegborn, L., 'Spectrographic contributions to lignin chemistry. 8. $\Delta\varepsilon$-studies on Braun's 'native lignins' from coniferous woods.' Svensk. Pappers-Tidning,

1958, 61, 187-210.

Bailey, R. W. and Gailliard, B. D. E., 'Carbohydrases of the rumen ciliate Epidinium ecaudatum (Crawley).' Biochem. J., 1965, 95, 758-766.

Bailey, R. W., 'Quantitative studies of ruminant digestion. II. Loss of ingested plant carbohydrates from the reticulo-rumen.' N.Z. Jl. agric. Res., 1967, 10, 15-32.

Barker, G. R.,and Shaw, D. F., 'Ribose and its derivatives. VIII. Ring structure and periodate oxidation of ribose and related polyols.' J. Chem. Soc., 1959, 584-93

Barry, J. A. and Malsey, G. D., 'The interaction and noninteraction of ions with a natural polysaccharide.' J. Phys. Chem., 1963, 67, 1698-1701.

Bath, I. H., 'The determination of sugars and uronic acids.' Analyst, 1958, 83, 451-5.

Bathurst, N. O. and Allison, R. M., 'The preparation of plant tissue for analyses.' N.Z. Jl. Sci. Tech., 1949, 31(B), 1-14.

Becker, E. S., Hamilton, J. K. and Lucke, W. E., 'Cellulose oligosaccharides as model compounds in chlorine dioxide bleaching.' Tappi, 1965, 48, 60-64.

Bjerrum, J., Schwarzenbach, G. and Sillen, L. G., ed. 'Stability constants': I. Organic ligands. The Chemical Society, London. 1958.

Björndal, H., Lindberg, B. and Svensson, S., 'Gas liquid chromatography of partially methylated alditols as their acetates.' Acta. Chem. Scand., 1967, 21, 1801-4.

Blaxter, K. L., Rook, J. A. F. and MacDonald, A. M., 'Experimental magnesium deficiency in calves. I. Clinical and pathological observations.' J. comp. Path., 1954, 64, 157-75.

Bohman, V. R., Lesperance, A. L., Harding, G. D. and Grunes, D. L., 'Induction of experimental tetany in cattle.' J. Anim Sci., 1969, 29, 99-102.

Bolker, H. I., 'Lignin-carbohydrate bond as revealed by infrared spectroscopy.' Nature, Lond., 1963, 197, 489-90.

Bolker, H. I. and Terashima, N., 'Lignin structure and reactions.' 1966, pp. 110-24, (Washington D.C., American Chemical Society Symposium).

Bondi, A. and Meyer, H., 'Lignin in young plants.' Biochem. J., 1948, 43, 248-256.

Bonner, O. D., Argersinger, W. J. and Davidson, A. W., 'Factors involved in cation exchange equilibria.' J. Amer. Chem. Soc., 1952, 74, 1044-47, 1047-50.

Boyd, G. E., Schubert, J. and Adamson, A. W., 'The exchange adsorption of ions from aqueous solutions by organic zeolites. I. Ion exchange equilibria.' J. Amer. Chem. Soc., 1947, 69, 2818-29.

Briggs, D. R., Garner, E. F. and Smith, F., 'Separation of carbohydrates by electrophoresis on glass filter paper.' Nature, 1956, 178, 154-5.

Brouwer, E., Dijkstra, N. D. and Frens, A. M., 'Over de bijvoedering van het melkvee in de weide met copra, voederbieten en aardappelen, in verband met de stevigheid van de geproduceerde boter.' Versl. Landbouwk. Ouderz., 1943, 49(10)C, 347-406.

Brouwer, E., 'Sur les modifications de la composition des acides gras de l'herbe pendant la maturation et la conservation. Rec. Trav. Chim., 1944, 63, 35-8.

Buch, M. L., Montgomery, R. and Porter, W. L., 'Identification of organic acids on paper chromatograms.' Anal. Chem., 1952, 24, 489-91.

Buch, M. L., 'A bibliography of organic acids in higher plants.' Agr. Handbook, No. 164 A.R.S., U.S.D.A. (1960).

Buddecke, E.,and Drzeniek, R., 'Stability constants of the calcium complexes of acid mucopolysaccharides.' Z. Physiol. Chem., 1962, 327, 49-64.

Burau, R. G. and Stout, P. R., 'Trans-Aconitic acid in range grasses in early spring.' Science, 1965, 150, 766-7.

Burt, A. W. A. and Thomas, D. C., 'Dietary citrate and hypomagnesaemia in the ruminant.' Nature, Lond., 1961, 192, 1193.

Busch, H., Hurlbert, R. B. and Potter, V. R., 'Anion-exchange chromatography of the acids of the citric acid cycle.' J. biol. Chem., 1952, 196, 717-27.

Care, A. D., 'Factors which affect the availability of magnesium.' Proc. Nutr. Soc., 1965, 24, 99-105.

Care, A. D., 'Magnesium absorption and hypomagnesaemia.' Feed Forum, 1967, 2(1), 6-9.

Care, A. D., van't Klooster, A. Th., 'In vivo transport of magnesium and other cations across the wall of the gastro-intestinal tract of sheep.' J. Physiol., 1965, 177, 174-91.

Cartwright, R. A. and Roberts, E. A. H., 'Theogallin as a galloyl ester of quinic acid.' Chem. & Ind., 1955, p. 230-1.

Chanda, S. K., Hirst, E. L., Jones, J. K. N. and Percival, E. G.V., 'The constitution of xylan from Esparto grass (Stipa tenacissima, L.).' J. Chem. Soc., 1950, 1289-97.

Charley, P. J., Sarkar, B., Stitt, C. F. and Saltman, P. D., 'Chelation of Fe by sugars.' Biochim. Biophys. Acta., 1963, 69, 313-7.

Creighton, R. H. J. and Hibbert, H., 'Studies on lignin and related compounds. 76. Alkaline nitrobenzene oxidation of

corn stalks. Isolation of p-hydroxybenzaldehyde.' J. Am. Chem. Soc., 1944, 66, 37-8.

Curran, P. F., 'The biophysical nature of biological membranes.' In 'The transfer of Ca and Sr across biological membranes.' 1963, ed. R. H. Wasserman, pp. 3-23. Academic press, New York and London.

Czerkawski, J. W., 'Effect of storage on the fatty acids of dried ryegrass.' Br. J. Nutr., 1967, 21, 599-608.

Davies, A. W., Evans, R. A. and Evans, W. C., 'Studies on the biochemistry of pasture plants. I. A new technique for the preparation and preservation of herbage samples.' J. Br. Grassl. Soc., 1948, 3, 153-158.

Davies, C. W., 'The extent of dissociation of salts in water. VIII.' J. Chem. Soc., 1938, 2093-98.

Davies, D. D., 'Organic acid metabolism in plants.' Biol. Rev., 1959, 34, 407-44.

Dehority, B. A., Johnson, R. R. and Conrad, H. R., 'Digestibility of forage hemicellulose and pectin by rumen bacteria in vitro and the effects of lignification thereon.' J. Dairy Sci., 1962, 45, 508-12.

Deshusses, J. and Desbaumes, P., 'Recherche par chromatographie sur papier de la banilline et de l'ethylvanilline dans les denrees alimentaires.' Mitt. Lebensm. U. Hyg., 1957, 48, 49-51.

DeWit, C. T., Dijkshoorn, W. and Noggle, J. C., 'Ionic balance and growth of plants.' Versl. Landbouwk. Onderz., 1963, No. 69.15, Wageningen.

Dische, Z., 'A new specific color reaction of hexuronic acids.' J. Biol. Chem., 1947, 167, 189-198.

Dische, Z., in 'Microdosage des Glucides.' J. Montreuil and E. Spik. Faculte de Sciences de Lille Universite, Lille, France. Vol. 1, p. 60, 1963.

Dishington, I. W., 'Changes in serum magnesium levels of ruminants, as influenced by abrupt changes in the composition of the diet.' Acta. vet. Scand., 1965, 6, 150-77.

Dishington, I. W. and Tollersrud, S., 'Hypomagnesaemia and hypomagnesaemic tetany induced in lactating cows by changing the diet.' Acta. vet. Scand., 1967, 8, 14-25.

Doub, L. and Vandenbelt, J. M., 'The ultraviolet absorption spectra of simple unsaturated compounds. II. m- and o-disubstituted benzene derivatives.' J. Am. Chem. Soc., 1949, 71, 2414-20.

Ehrenthal, I., Montgomery, R. and Smith, F., 'The carbohydrates of gramineae. II. The constitution of the hemicelluloses of wheat straw and corn cobs.' J. Am. Chem. Soc., 1954, 76, 5509-14.

Erickson, L. E. and Alberty, R. A., 'Evidence from nuclear magnetic resonance for malate complexes of alkali metal cations.' J. Phys. Chem., 1962, 66, 1702-5.

Farmer, V. C., 'Effects of grinding during the preparation of alkali halide disks on the infra-red spectra of hydroxylic compounds.' Spectrochim. Acta., 1957, 8, 374-89.

Fowler, H. D., 'Grass tetany and histamine.' Nature. Lond., 1963, 197, 619.

Frahn, J. L. and Mills, J. A., 'Paper ionophoresis of carbohydrates. I. Procedures and results for four electrolytes.' Aust. J. Chem., 1959, 12, 65-89.

Frank, H. S. and Evans, M. W., 'Free volume and entrophy in condensed systems. III.' J. chem. Phys., 1945, 13, 507-32.

Fraser, C. G. and Wilkie, K. C. B., in press, Phytochem., 1970.

Freudenberg, F. and Neish, A. C., 'Constitution and biosynthesis of lignin.' 1968, pp.92-3, (Berlin, Springer-Verlag).

Gailliard, B. D. E., 'A detailed summative analysis of the crude fibre and nitrogen-free extraction fractions of roughages. I. Proposed scheme of analysis.' J. Sci. Fd. Agric., 1958a, 9, 170-177.
II. The analysis of straw, hay, grass and mangold Ibid., 1958b, 346-53.

Gailliard, B. D. E., 'Comparison of the hemicelluloses from plants belonging to two different plant families.' Phytochem., 1965, 4, 631-4.

Gailliard, B. D. E. and Nijkamp, H. J., 'Calculation of the digestibility for ruminants of roughages from their contents of cell-wall constituents. Neth. J. Agric. Sci., 1968, 16, 21-24.

Gailliard, B. D. E. and van't Klooster, A. Th., 'The digestion of the cell wall constituents of roughages.' Meded. Landbouwhogeschool Wageningen, 1969, 69/11, 20-25.

Garton, G. A., 'The digestion and absorption of lipids in ruminant animals.' World Review of Nutrition and Dietetrics, 1967, Vol. 7, pp. 225-50. S. Karger, Basel/New York.

Goldschmid, O., 'The effect of alkali and strong acid on the ultraviolet spectrum of lignin and related compounds.' J. Am. Chem. Soc., 1953, 75, 3780-83.

Goldschmid, O., 'Determination of phenolic hydroxyl content of lignin preparations by ultraviolet spectrophotometry.' Anal. Chem., 1954, 26, 1421-3.

Gould, R. O. and Rankin, A. F., 'Calcium complexes of uronic acid monomers.' Chem. Comm. (J.C.S.), 1970, Sect. D. 8, 489-90.

Gramera, R. E. and Whistler, R. L., 'Isolation of three polysaccharides from the hemicellulose B fraction of corn stalk.' Arch. Biochem. Biophys., 1963, 101, 75-80.

Guggenheim, E. A., 'Thermodynamics, an advanced treatment for chemists and physicists.' North-Holland Publishing Co., Amsterdam, 1949.

Gunner, S. W., Jones, J. K. N. and Perry, M. B., 'The gas-liquid partition chromatography of carbohydrate derivatives. I. The separation of glycitol and glycose acetates.' Can. J. Chem., 1961, 39, 1892-99.

Hanes, C. S. and Isherwood, F. A., 'Separation of the phosphoric esters on the filter paper chromatogram.' Nature, 1949, 164, 1107-12.

t'Hart, M. L., 'The influence of meteorological conditions and fertiliser treatment on pasture in relation to hypomagnesaemia.' In 'Conference on hypomagnesaemia': 1960, 88-95, London. British Veterinary Association.

Hasegawa, S., Johnson, R. M. and Gould, W. A., 'Effect of cold storage on chlorogenic acid content of potatoes.' J. Agr. Fd. Chem., 1966, 14, 165-69.

Haug, A. and Smidsrod, O., 'The effect of divalent metals on the properties of alginate solutions. II. Comparison of different metal ions.' Acta. chem. Scand., 1965, 19, 341-51.

Haug, A., Myklestad, S., Larsen, B. and Smidsrod O., 'Correlation between chemical structure and physical properties of alginates.' Acta. chem. Scand., 1967, 21, 768-78.

Hawke, J. C., 'Properties of New Zealand butterfats. VII. Effect of the stage of maturity of ryegrass fed to cows on the characteristics of butterfat and its carotene and vitamin A content.' J. Dairy Res., 1963, 30, 67-75.

Head, M. J. and Rook, J. A. F., 'Hypomagnesaemia in dairy cattle and its possible relationship to ruminal ammonia production.' Nature. Lond., 1955, 176, 262.

Head, M. J. and Rook, J. A. F., 'Some effects of spring grass on rumen digestion and the metabolism of the dairy cow.' Proc. Nutr. Soc., 1957, 16, 25-30.

Hergert, H. L., 'Infrared spectra of lignin and related compounds. II. Conifer lignin and model compounds.' J. org. Chem., 1960, 25, 405-13.

Hirst, E. L. and Ramstead, S., 'Changes in organic acid content of perennial ryegrass during conservation.' J. Sci. Fd. Agric., 1957, 8, 727-32.

Hough, L., Jones, J. K. N. and Wadman, W. H., 'Quantitative analysis of mixtures of sugars by the method of paper chromatography.' J. Chem. Soc., 1950, 1702-6.

Hulme, A. C. and Richardson, A., 'The non-volatile organic acids of grass.' J. Sci. Fd. Agric., 1954, 5, 221-25.

Hulme, A. C. and Wooltorton, L. S. C., 'Determination and isolation of non-volatile acids of pome fruits and study of acid changes in apples during storage.' J. Sci. Fd. Agric., 1958, 9, 150-158.

Immink, H. J., Geurink, J. H. and Deijs, W. B., 'The determination of the higher fatty acids in grass and cow faeces.' Jaarb. Inst. biol. scheik. Onderz. LaudbGewass, 1965, 103-107.

Jacques, W. A., 'Yorkshire fog as a pasture grass.' Proc. N.Z. Grassl. Assn., 1962, 139-150.

Jardetzky, O. and Wertz, J. E., 'The complexing sodium ion with some common metabolites.' Arch. Biochem. Biophys., 1956, 65, 569-72.

Jeanes, A. and Isbell, H. S., 'Chemical reactions of the chlorites with carbohydrates.' J. Res. Nat. Bur. Stand., 1941, A 27, 125-42.

Jenny, H., 'Studies on the mechanism of ion exchange in colloidal aluminium silicates.' J. Phys. Chem., 1932, 36(2), 2217-58.

Jones, J. K. N. and Stoodley, R. J., 'Fractionation of polysaccharides using copper complexes.' In 'Methods in Carbohydrate Chemistry', 1965, ed. R. L. Whistler, Vol. 5, p. 36-38.

Kemp, A., 'Hypomagnesaemia in milking cows: the response of serum magnesium to alterations in herbage composition resulting from potash and nitrogen dressing on pasture.' Neth. J. Agric. Sci., 1960, 8, 281-304.

Kemp, A., Deijs, W. B., Hemkes, O. J. and Es, A. J. H. van., 'Hypomagnesaemia in milking cows: intake and utilisation of magnesium from herbage by lactating cows.' Neth. J. Agric. Sci., 1961, 9, 134-49.

Kemp, A., Deijs, W. B. and Kluvers, E., 'Influence of higher fatty acids on the availability of magnesium in milking cows.' Neth. J. Agric. Sci., 1966, 14, 290-5.

Kennedy, G. S., 'Trans-aconitate utilisation by sheep.' Aust. J. biol. Sci., 1968, 21, 529-38.

Klooster, A. Th. van't, 'The concentrations of Na, K, Ca and Mg in the dialysates of gut contents of conscious sheep and the distribution of these minerals in the contents of the colon and in the faeces of a sheep.' Tijdschr. Diergeneesk., 1964, 89, 1709-23.

Klooster, A. Th. van't, 'The state of calcium, magnesium and some other minerals in gut contents and faeces of ruminants in relation to their absorption.' Meded. Landbouwhogeschool Wageningen, 67/5, 1967, 1-136.

Kohn, R., and Furda, I., 'Calcium ion activity in solutions of calcium pectinate.' Collect. Czech. Chem. Commun., 1967a, 32, 1925-37.

Kohn, R. and Furda, I., 'Interaction of calcium and potassium ions with carboxyl groups of pectin.' Collect. Czech. Chem. Commun., 1967b, 32, 4471-84.

Kohn, R., Furda, I., Haug, A. and Smidsrod, O., 'Binding of calcium and potassium ions to some polyuronides and mono-uronates.' Acta. chem. Scand., 1968, 22, 3098-3102.

Kratzl, K., 'Lignin - its biochemistry and structure.' In 'Cellular ultrastructure of woody plants', pp. 157-180. Ed. Cote, W. A., Syracuse, N.Y.; Syracuse University Press, 1965.

Krebs, H. A. and Eggleston, L. V., 'Micro-determination of iso-Citric and cis-Aconitic acids in biological material.' Biochem. J., 1944, 38, 426-37.

Kreveld, A. van, and Minnen, G. van, 'Calcium and magnesium ion activity in raw milk and processed milk.' Neth. Milk Dairy J., 1955, 9, 1-29.

Kunin, R., 'Ion exchange resins', 1952, 2nd edn., John Wiley and sons, Inc., New York.

Laidlaw, R. A. and Reid, S. G., 'Filter paper chromatography: Extraction of sugars from the paper at room temperature.' Nature, 1950, 166, 476-7.

Larvar, P. and Guegen, L., 'Grass composition and grass tetany.' Ann. Zeotech., 1963, 12(1), 39-52 (Reprinted in Potash review, 19/15 (1964).

Lehman, D. S., 'Some principles of chelation chemistry.' Proc. Soil Sci. Soc. Amer., 1963, 27, 167-70.

Lemieux, R. U. and Levine, S., 'Synthesis of alkyl 2-deoxy-α-D-glycopyranosides and their 2-deutero derivatives.' Can. J. Chem., 1964, 42, 1473-80.

Lemieux, R. U. and Stevens, J. D., 'Substitutional and configurational effects on chemical shift in pyranoid carbohydrate derivatives.' Can. J. Chem., 1965, 42, 2059-70.

Lengeman, F. W., 'The metabolism of magnesium and calcium by the rat.' Arch. Biochem. Biophys., 1959, 84, 278-85.

Lomba, F., Chauvaux, G. and Bienfet, V., 'Organic acids and grass tetany, I.' Ann. Med. Vet., 1968, 112(4), 261-75. (Fr.)

Lomba, F., Paquay, R., Bienfet, V and Lousse, A., 'Statistical research on the fate of dietary mineral elements in dry and lactating cows. II. Magnesium.' J. agric. Sci. Camb., 1968, 71, 181-8.

Lomba, F., Fumiere, I., Chauvaux, G., Binot, H. and Bienfet, V., 'Organic acids and grass tetany. III. Effect of a mixture of citric acid, trans-aconitic acid, and KCl, introduced into the rumen of cows by gastric intubation.' Ann. Med. Vet., 1969, 113(1), 22-33. (Fr.)

McDonald, P., MacPherson, H. T. and Watt, J. A., 'The effect of histamine on silage dry-matter intake.' J. Br. Grassld. Soc., 1963, 18, 230-2.

MacLennan, D. H., Beevers, H. and Harley, J. L., 'Compartmentation of acids in plant tissues.' Biochem. J., 1963, 89, 316-27.

MacLennan, D. H. and Beevers, H., 'Trans-aconitate in plant tissues.' Phytochem., 1964, 3, 109-113.

Marchessault, R. H. and Liang, C. Y., 'The infrared spectra of crystalline polysaccharides. VIII. Xylans.' J. Polym. Sci., 1962, 59, 357-78.

Marchessault, R. H. and Sarko, A., 'X-ray structure of polysaccharides.' Adv. Carbohyd. Chem., 1967, 22, 421-82.

Martell, A. E. and Calvin, M., 'Chemistry of the metal chelate compounds.' 1952, (New Jersey, Prentice Hall, Inc.)

Martin, S. M., 'A spray reagent for identification of organic acids.' Chem. & Ind., 1955, p. 427-8.

Melvin, J. F. and Simpson, B., 'Chemical changes and respiratory drift during the air drying of ryegrass.' J. Sci. Fd. Agric., 1963, 14, 228-234.

Melvin, J. F., 'Changes in the non-volatile organic acids of rye-grass during airdrying.' J. Sci. Fd. Agric., 1965, 16, 612-14.

Merewether, J. W. T., in 'The chemistry of lignin', F. E. Brauns and D. A. Brauns, 1960, pp. 633-4. (New York and London, Academic Press).

Metson, A. J., Saunders, W. M. H., Collie, T. W. and Graham V. W., 'Chemical composition of pastures in relation to grass tetany in beef breeding cows.' N.Z. Jl. Agric. Res., 1966, 9, 410-136.

Mills, J. A., 'Association of polyhydroxy compounds with cations in solution.' Biochem. Biophys. Res. Comm., 1961/62, 6, 418-21.

Molloy, L. F., 'Determination of trans-Aconitic acid in forage grasses.' J. Sci. Fd. Agric., 1969, 20, 238-41.

Mullins, L. J., 'Macromolecular properties of excitable membranes.' Ann. N.Y. Acad. Sci., 1961, 94, 390-404.

Nachod, F. C. and Wood, W., 'The reaction velocity of exchange, II.' J. Am. chem. Soc., 1945, 67, 629-31.

Northcote, D. H., 'Electrophoresis of some neutral polysaccharides.' Biochem. J., 1954, 58, 353-58.

Northcote, D. H., 'Zone electrophoresis', in 'Methods in carbo-hydrate chemistry', 1965, ed. R. L. Whistler, Vol. 5, p. 49-53.

O'Sullivan, M., 'Grass tetany - effects of environment and fertilisers on pasture histamine levels.' J. Sci. Fd. Agric., 1968, 19, 12-15.

Packett, L. V. and Fordham, J. R., 'Utilization of citric acid by rumen microorganisms.' J. Anim. Sci., 1965, 24, 488-93.

Pearl, I. A., 'The Chemistry of Lignin.' Edward Arnold, London. Marcel Dekker Inc., N.Y. 1967.

Pepper, J. M., Manulopoulo, M., and Burton, R., 'Gas-liquid chromatographic analysis of lignin oxidation products.' Can. J. Chem., 1962, 40, 1976-80.

Perkins, H. J.,'Note on chemical changes occurring in freeze-dried and fresh-frozen wheat leaves during storage.' Can. J. Plant Sci., 1961, 41, 689-91.

Raguse, C. A. and Smith, D., 'Carbohydrate content in alfalfa herbage as influenced by methods of drying.' J. Agr. Fd. Chem., 1965, 13, 306-9.

Reid, J. S. G. and Wilkie, K. C. B., 'Polysaccharides of the oat plant in relationship to plant growth.' Phytochem., 1969a, 8, 2045-51.

Reid, J. S. G. and Wilkie, K. C. B., 'An acidic galactoarabino-xylan and other pure hemicelluloses in oat leaf.' Phytochem., 1969b, 8, 2053-2058.

Reid, R. L., Franklin, M. C. and Hallsworth, E. G., 'The utilisation of phytate phosphorus by sheep.' Aust. Vet. J., 1947, 23, 136-40.

Reitsema, R. H., 'Characterisation of essential oils by chromatography.' Anal. Chem., 1954, 26, 960-3.

Richardson, A. and Hulme, A. C., 'Shikimic acid in grass.' Nature, 1955, 175, 43-4.

Roelofsen, P. A., 'Ultrastructure of the wall in growing cells and its relation to the direction of growth.' Adv. Bot. Res., 1965, 2, 69-149.

Rogers, P. A. M. and van't Klooster, A. Th., 'The fate of Na, K, Ca, Mg and P in the digesta.' Meded. Landbouwhogeschool Wageningen, 1969, 69/11, 26-39.

Rook, J. A. F., 'Experimental magnesium deficiency in the cow.' J. comp. Path., 1963, 73, 93-7.

Rubin, M., 'The biological implications of alkaline earth chelation.' In 'The transfer of Ca and Sr across biological membranes', 1963, ed. R. H. Wasserman, pp. 25-46. Academic press, New York and London.

Saffran, N. and Prado, J. L., 'Inhibition of aconitase by trans-aconitate.' J. biol. Chem., 1949, 180, 1301-9.

Schachter, D., Dowdle, E. B. and Schenker, H., 'Active transport of calcium by the small intestine of the rat.' Amer. J. Physiol., 1960, 198, 263-268.

Schmidt, G. C., Fischer, C. and McOwen, J. M., 'New method for location of organic acids on paper chromatograms.' J. Pharm. Sci., 1963, 52, 468-72.

Schnitzer, M. and Hansen, E. H., 'Organometallic interactions in soils. 8. An evaluation of methods for the determination of stability constants of metal-fulvic acid complexes.' Soil Science, 1970, 109, 333-340.

Schweiger, R. G., 'Acetylation of alginic acid. I. Preparation and viscosities of algin acetates.' J. org. Chem., 1962a, 27, 1786-9.

Schweiger, R. G., 'Ibid. II. Reaction of algin acetates with calcium and other divalent ions.' J. org. Chem., 1962b, 27, 1789-91.

Schweiger, R. G., 'Acetyl pectates and their reactivity with polyvalent metal ions.' J. org. Chem., 1964, 29, 1973-5.

Scott, J. E., 'The reaction of long chain quarternary ammonium salts with acidic polysaccharides.' Chem. Ind. (London), 1955, 168-9.

Senti, F. R. and Witnauer, L. P., 'X-ray diffraction studies of addition compounds of amylose with inorganic salts.' J. Polymer Sci., 1952, 9, 115-

Simesen, M. G., Commun. 16th Int. Vet. Congr., Madrid, 1959, 2, 85.

Simesen, M. G., Proc. 17th Int. Vet. Congr., Hanover, 1963,

Sjollema, B., 'On the nature and therapy of grass staggers.' Vet. Rec., 1930, 10, 425-31; 450-4.

Smidsrod, O. and Haug, A., 'The effect of divalent metals on the properties of alginate solutions. I. Calcium ions.' Acta. chem. Scand., 1965, 19, 329-40.

Smidsrod, O. and Haug, A., 'Dependence upon uronic acid composition of some ion-exchange properties of alginates.' Acta. chem. Scand., 1968, 22, 1989-97.

Smith, R. H. and McAllan, A. B., 'Binding of magnesium and calcium in the contents of the small intestine of the calf.' Br. J. Nutr., 1966, 20, 703-18.

Steyermark, A., 'Quantitative Organic Analysis', 1961, 2nd edn. Chapt. 8, pp. 188-220. Academic Press, N.Y. and London.

Stokes, R. H. and Robinson, R. A., 'Ionic hydration and activity in electrolyte solutions.' J. Amer. chem. Soc., 1948, 70, 1870-8.

Storry, J. E., 'Studies on calcium and magnesium in the alimentary tract of sheep.' J. agric. Sci., Camb., 1961, 57, 103-9.

Stout, P. R., Brownell, J. and Burau, R. G., 'Occurrences of trans-Aconitate in range forage species.' Agron. J., 1967, 59, 21-24.

Swan, J. B. and Jamieson, N. D., 'Studies on metabolic disorders in dairy cows. III.' N.Z. Jl. Sci. Technol., 1956, A38, 363-82.

Sweeley, C.C., Bentley, R., Makita, M. and Wells, W. W., 'Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances.' J. Am. Chem. Soc., 1963, 85, 2497-2507.

Thimann, K. V. and Bonner, W. D., 'Organic acid metabolism.' Ann. Rev. Pl. Physiol., 1950, 1, 75-108.

Thompson, S. O., Chesters, G. and Engelbert, L. E., 'Comparative properties of plant lignins and humic materials of soils. I. Yields and cation exchange properties of plant lignins isolated by different techniques.' Proc. Soil Sci. Soc. Amer., 1964, 28, 65-68.

Trevelyan, W. E., Procter, D. P. and Harrison, J. S., 'Detection of sugars on paper chromatograms.' Nature, 1950, 166, 444-5.

Triffitt, J. T., 'Binding of calcium and strontium by alginates.' Nature, 1968, 217, 457.

Waite, R. and Gorrod, A. R. N., 'The structural carbohydrates of grasses.' J. Sci. Fd. Agric., 1959a, 10, 308-17.

Waite, R. and Gorrod, A. R. N., 'The comprehensive analysis of grasses.' J. Sci. Fd. Agric., 1959b, 10, 317-326.

Waite, R., Johnson, M. J. and Armstrong, D. G., 'The evaluation of artificially dried grass as a source of energy for sheep. I. The effect of stage of maturity on the apparent digestibility of ryegrass, cocksfoot and timothy.' J. agric. Sci., Camb., 1964, 62, 391-98.

Ward, G. M., 'Oral potassium chloride fatal to a cow.' J. Am. Vet. Med. Assoc., 1966, 148, 543-4.

Wardrop, A. B. and Bland, D. E., 'The process of lignification in woody plants', in 'Biochemistry of Wood.' Symposium II, 4th International Congress of Biochemistry, Vienna, 1958. Ed. Kratzl, K. and Billek, G., Pergamon Press, London. 1959, pp. 92-114.

Weerden, E. J., van, 'The osmotic pressure and the concentration of some solutes of the intestinal contents and the faeces of the cow, in relation to the absorption of the minerals.' J. agric. Sci., Camb., 1961, 56, 317-24.

Weihe, H. E. and Phillips, M., 'The quantitative estimation of hemicelluloses by direct isolation.' J. Ag. Res., 1947, 74, 77-85.

Wexler, A. S., 'Characterisation of lignosulfonates by ultraviolet spectroscopy.' Anal. Chem., 1964, 36, 213-21.

Whistler, R. L., Bachrach, J. and Bauman, D. R., 'Preparation and properties of corn cob holocellulose.' Arch. Biochem., 1948, 19, 25-33.

Whistler, R. L., 'Industrial Gums', 1959, p. 409, Academic Press, N.Y. and London.

Whistler, R. L. and Feather, M. S., 'Extraction of hemicelluloses from annual plants with alkaline solutions.' In 'Methods in Carbohydrate Chemistry', 1965, Vol. 5, 144-5.

Whistler, R. L. and Richards, E. L., 'Hemicelluloses', Chapt. 37. 'The Carbohydrates, chemistry and biochemistry.' 2nd. Edn., Vol. IIa. Ed. W. Pigman and D. Horton, 1970, Academic Press, New York and London, pp. 447-69.

Whistler, R. L. and Sanella, J. L., 'Fractional precipitation of hemicelluloses with ethanol.' In 'Methods in Carbohydrate chemistry', 1965, ed. R. L. Whistler, Vol. 5, p. 34-36.

Whistler, R. L. and Smart, C. L., 'Polysaccharide Chemistry', 1953, (New York; Academic Press).

White, E. P., Thompson, F. B. and Brice, N., 'Application of the Dumas micro method to pasture nitrogen analysis.' Analyst, Lond., 1948, 73, 146-8.

Wiklander, L., 'Studies on ion exchange (Wolfatits P. and M.)' Ann. Royal Agr. Coll. Sweden, 1946, 14, 1-171.

Wildung, R. E., Chesters, G. and Behmer, D. E., 'Alkaline nitrobenzene oxidation of plant lignins and soil humus colloids.' Pl. Soil, 1970, 32, 221-37.

Williams, E. J., 'Regression analysis', 1959, (New York, Wiley).

Willis, J. B., 'Determination of metals in blood serum by atomic absorption spectroscopy. I. Calcium.' Spectrochim. Acta, 1960a, 16, 259-72.
'II. Magnesium.' Ibid., 1960b, 16, 273-8.

Wilson, G. F., Reid, C. S. W., Molloy, L. F., Metson, A. J. and Butler, G. W., 'Grass tetany. I. Influence of starch and peanut oil supplements on plasma magnesium, calcium and phosphorus levels in grazing dairy cows.' N.Z. Jl. agric. Res., 1969, 12, 467-88.

Wilson, K., 'The growth of plant cell walls.' Int. Rev. Cytol., 1964, 17, 1-49.

Wind, J., Deijs, W. B. and Kemp, A., 'Hogere vetzuren in het voedsel en hun mogelijke rol bij het opheden van hypomagnesemie in de weide.' Jaarb. Inst. biol. Scheik. Onderz. LandbGewass., 1966, 91-100.

Wise, L. E., Murphy, M. and d'Addieco, A. A., 'Chlorite holocellulose, its fractionation and bearing on sumnative wood analysis and on studies on the hemicelluloses.' Paper Tr. J., 1946, 122, 35-43.

Worth, H. G. J., 'The chemistry and biochemistry of pectic substances.' Chem. Rev., 1967, 67, 465-73.

Wright, D. E. and Wolff, J. E., 'Trans-aconitic acid and the magnesium status of guinea pigs and sheep.' N.Z. Jl. agric. Res., 1969, 12, 287-92.

Yuen, S. H. and Pollard, A. G., 'The determination of nitrogen in agricultural materials by the Nessler reagent. I. Preparation of the reagent.' J. Sci. Fd. Agric., 1952, 3, 441-7.