STUDIES ON SUGAR-METAL INTERACTIONS IN AGRICULTURE

THESIS

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Preface

The work described in this thesis was carried out in the Agricultural Section of the Chemistry Department, University of Glasgow, from October, 1964, to September, 1967.

During this time I was in receipt of a Research Studentship from the Department of Agriculture and Fisheries for Scotland, which I gratefully acknowledge.

I should like to thank Professor J. M. Robertson for the use of laboratory facilities and specialised equipment.

I also wish to record my thanks to Dr. W. R. Rees, who initiated this work, for his constant help and encouragement throughout all its stages.

The typing services of my mother are also gratefully acknowledged.

CONTENTS

NOTE

UNLESS OTHERWISE STATED, ALL SUGARS USED IN THIS STUDY BELONG TO THE D - SERIES.

ABBREVIATIONS

^{o}C	Degrees Centigrade
cm. cms.	Centimetres
EDTA	Ethylenediamine tetraacetic acid
Exp. No.	Experiment Number
G.M.	General Methods
gm. gms.	grammes
M	Molar
mg. mgs.	milligrammes
ml. mls.	millilitres
N	Normal
O.D.	Optical Density Units
ppt.	precipitate
pptd.	precipitated
rediss.	redissolving stage
UMP	Uridine mono phosphate

General Introduction

1. Considerations on which this study is based.

The value of an extensive investigation into the effects exerted by sugars on metallic compounds was pointed out by Rees (1), and attention was drawn to the probable importance of such effects in biological environments.

Although the nineteenth century literature contains many references to metal containing derivatives of sugars, some of which were isolated and to some extent characterised, modern carbohydrate textbooks devote little or no space to them, and relatively few workers have mentioned the phenomenon in recent years. A review article appearing in 1966 (2) concerning complexes of alkaline-earth and alkali metals with carbohydrates, may indicate that interest is now reviving.

Research into this somewhat unexplored, and to some extent unexpected, property of carbohydrates was initiated by a consideration of the architecture, or stereochemistry, of carbohydrate molecules. The numerous possible configurations of sugar hydroxyl groups, may endow some sugars with the ability to complex certain metals, although sugars do not contain the active groupings generally associated with the complexing agents in common use. It is not intended to give a fully detailed summary of typical chelating agents, or of their

mode of action. A brief account will, however, be given, in order to demonstrate the principles involved in complex formation. More detailed information may be obtained in several textbooks on the subject (4, 5, 6).

When a metal combines with an electron donating compound, or ligand, the product is said to be a complex, or coordination compound. This group of substances may be further subdivided into simple complexes, and chelate compounds. A simple complex is formed when a donor molecule becomes attached to a metal by only one donor group. If the electron donating substance is to function as a chelating agent it must possess two or more appropriate functional groups, each capable of combining with a metal by donating a pair of electrons. By this mechanism one or more ring structures, known as chelate rings, may be formed. This generally imparts a greater stability to the metal chelate than that possessed by a simple metal complex.

The electrons donated by the functional groups of a chelating agent may be contributed either by basic coordination groups, or by acidic groups which have lost a proton. Under the first category come such groups as :-

=O ; $-NH_2$; -NH ; -N= ; -O-R ; =NOH ;

-OH (alcoholic) ; -S- (thioether)

Under the latter category come :-

-COOH ; $-SO_3H$; -OH (enolic and phenolic) ; -SH :

The mere possession of two or more such functional groups does not necessarily endow a molecule with chelating ability. These functional groups must be so situated in the molecule that they permit the formation of a chelate ring structure, with the metal atom as the closing member. Thus the stereochemistry of the molecule is of great importance.

In addition to the stereochemistry of the donor molecule determining whether or not a complex may form, the properties of the metal have an effect. It is generally agreed that the transition metals have the strongest tendency to form complexes with electron donors, but strong complexes have also been found with alkaline-earth and alkali metals. This latter has only been accomplished with chelating compounds, as chelating agents tend to show a greater affinity for metals than simple complexing agents.

The binding of the alkaline-earth and alkali metals appears to be predominantly ionic, the stability of the complex increasing with increasing charge on, and decreasing radius of, the central metal atom. Thus magnesium and calcium complexes possess a high stability, magnesium being more stable than calcium ; whereas sodium and lithium complexes possess a low stability, the lithium complexes being less stable than those of sodium.

Transition metals, on the other hand, usually form covalent bonds, the stability of the complex being determined by the basicity of the metal. Mellor and Maley (7, 8.) found that the stability of bivalent metallic complexes was in the order :-

Pd > Cu > Ni > Pb > Co > Zn > Cd > Fe > Mn > Mg

where the least basic metals form the strongest complexes.

Further steric factors, such as steric hindrance, may influence the formation of chelate rings. Where the various groupings on a chelating molecule interfere with, or are repulsed by, the groups of a further molecule, attachment of more than one molecule of the ligand may be prevented, or chelate ring formation may be completely inhibited. Where the interference does not completely prevent chelation, it may reveal its influence by lowering the stability of the resultant chelate. The radius of the central metal atom may determine whether or not such interference will take place, as this effect is often demonstrated only with atoms below a certain radius.

In addition, the normal order of ionic binding strengths may be disrupted because of the special requirements of a chelating agent. For example, the chelating agent ethylenediamine tetraacetic acid does not chelate magnesium

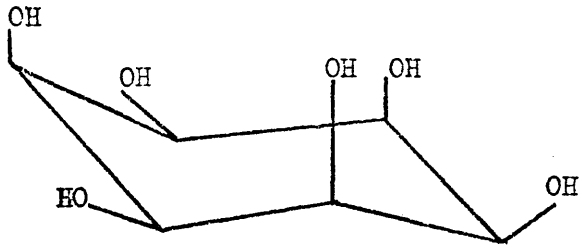
effectively, since the magnesium atom is apparently too small for the most effective binding of the donor groups. The larger calcium ion, however, seems to possess the necessary properties and forms a more stable chelate with this ligand.

A further complicating factor which may disrupt the normal order of stabilities, is that metals of low atomic weight tend to possess a more tightly bound sphere of solvation than metals of higher atomic weight. This inhibits the close approach of donor groups and results in a complex of low stability. This is the explanation for the previously mentioned lower stability of lithium complexes, as compared to those of sodium.

Since the work of Ley (9, 10) the alteration of the 'normal' chemical reactions of a metal in solution has been used as one of the principal methods for determining the presence of chelates, and is of particular importance when a ligand combines with a metal to form a water soluble chelate. This phenomenon is not restricted to chelates, but is applicable to complex formation in general. (11)

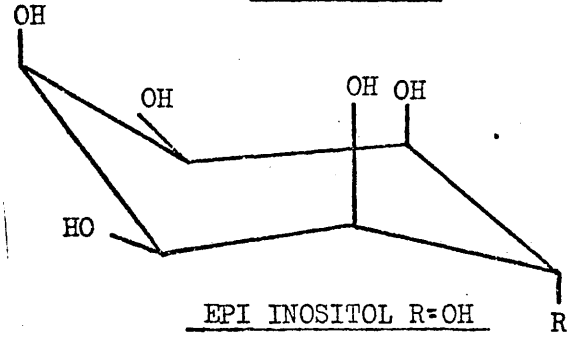
The foregoing gives a brief and simplified summary of the principles on which the phenomenon of complex formation is based. As this study is concerned with examining the complexing ability of sugars with metals, the most important functional group to be considered is the hydroxyl group. It

a)



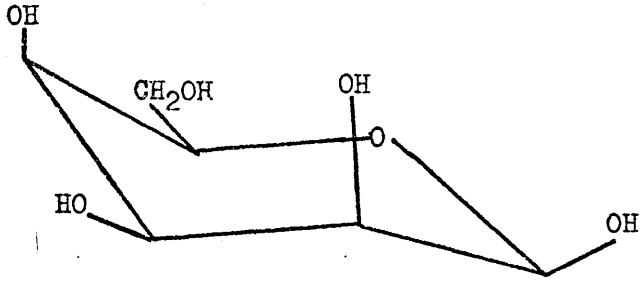
CIS INOSITOL

b)



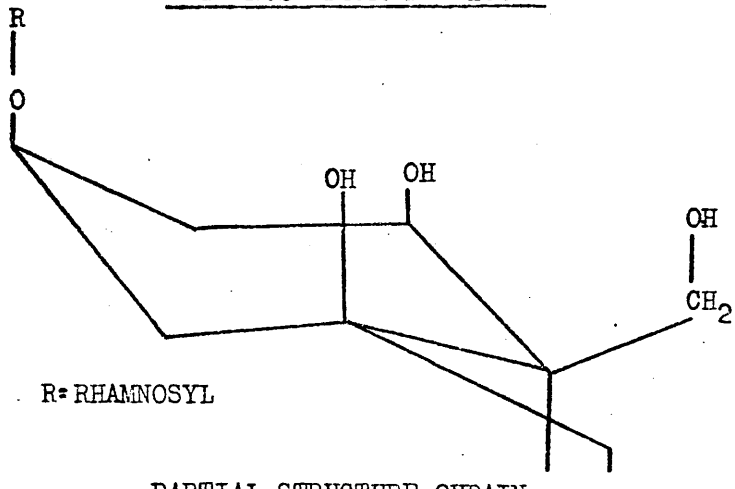
EPI INOSITOL R=OH
CIS QUERCITOL R=H

c)



PYRANOSE FORM D-TALOSE

d)



R= RHAMNOSYL

PARTIAL STRUCTURE OUBAIN

FIGURE 1

has been ascertained that polyhydroxy compounds such as diols and polyols exhibit complexing powers. That the stereochemistry of such molecules is of importance in determining the relative strengths of the bonds formed, was demonstrated by Mills in 1961. (3) He examined the association of polyhydroxy compounds with metals in solution and found that cis inositol (Fig. 1a) forms a very strong complex, whereas epi inositol (Fig. 1b) forms a complex which does not possess the stability of its isomer. A comparison of the stereochemistry of these molecules shows very little difference, but sufficient to allow closer approach of the hydroxyl groups in the former.

Sugar molecules bear a great deal of resemblance to these polyhydric alcohols, and it does not require much extrapolation to see that some sugars may form complexes with metals, their ability to do this being once again dependent on the spatial arrangement of their hydroxyl groups. Mills has established that the sugar D - talose (Fig. 1c) possesses some complexing powers. The position of the hydroxyl groups in the β-pyranose form of this sugar bear a close resemblance to those of cis inositol.

Although this sugar has not a very wide occurrence in nature, very many different sugars do exist in considerable quantities in biological environments as monosaccharides,

disaccharides and polysaccharides. With the possible configurations of different oligosaccharides and polysaccharides approaching infinity, it is evident that sugars cannot be discounted as valuable complexing agents under natural conditions, being such a rich source of hydroxyl groups.

Thus it was a consideration of this feature of sugars, together with the knowledge that they are found in appreciable concentration in soils, plants, and the diet of many animals, that prompted this examination of the role such complexes could possibly play under physiological conditions; if, indeed, they do occur under such conditions.

Indicative of the role which hydroxy complexes may play is the action of oubain (Fig. 1d). This physiologically active compound selectively influences sodium and potassium transport in the body, exerting an apparently equivalent effect in both cases (218). It possesses a configuration in which three hydroxyl groups, at positions 1, 5 and 19, have the same spatial arrangement as the axial hydroxyls of cis inositol.

The primary aim of the present study was to establish the conditions under which some selected sugar-metal interactions could occur, and furthermore, if such conditions were biologically meaningful, to study and consider the possible role of such compounds. Thus, it was in no way the prime purpose

of this work to become involved in studies on the mechanism of these interactions, or to decide precisely whether they led to the formation of 'chelates' in the sense that the term is usually used. The term'complex' will be used throughout this study as being an entity which fulfils Rossotti and Rossotti's definition of a complex (12) as being a : 'species formed by the association of two or more simpler species, each capable of individual existence.' Thus, any complexing which results in the formation of a readily available, or transportable form of a metal, by its interaction with a sugar, will be of interest, if such complexing occurs under physiological conditions.

2. Early investigation of the phenomenon of sugar-metal complexing.

A fairly comprehensive review of any previous research applicable to this problem has been attempted. From the point of view of convenience, however, this sub-section will deal only with information directly related to the formation of complexes between sugars and metals.

Since the last century, the occurrence of some form of interaction between sugar compounds and a variety of metallic cations, has been noted by numerous chemists. Generally these observations were made while investigating another specific problem, so that their importance has never been fully elucidated.

This is ably demonstrated by a study of the research undertaken to improve the refining processes of raw sugar. Calcium oxide has been used as a purifying agent in this industry for many years, and many of the early references are associated with this phenomenon.

Scheibler, in 1883 (13) developed a strontium process for the separation of sugar from molasses, strontium saccharate being shed as a precipitate. Meanwhile Juneman, (14) while working on the purification of beet juice, observed that a solution of magnesium hydroxide added to hot syrup, produced a granular precipitate of magnesium saccharate. In addition, ferric chloride was used to purify these crude syrups (15), while Klein and Berg in 1886 (16) showed that invert sugar malt infusion and cane sugar dissolved considerable quantities of iron, resulting in severe corrosion of the boilers used during the purification process.

In the following years some work was carried out on the periphery of this essentially industrial application.

Stromeyer in 1887 (17) added freshly ignited calcium oxide to an excess of a sugar solution and precipitated the product with alcohol. This product analysed to correspond with the formula $C_{12}H_{22}O_{11}CaO$, a similar result being obtained with barium. Petit (18) in 1893, investigated the dissolving action of sugars on calcium compounds. In the following

year, Evers (19) obtained a crystalline, reddish brown powder of iron sucrate by pouring a solution of cane sugar and ferric chloride into a slight excess of sodium hydroxide. This powder contained 48.5 per cent of iron, whereas a similar powder obtained by use of maltose as sugar, contained 32 per cent of iron, corresponding to the formula :-

$$2Fe_2O_3 . C_{12}H_{22}O_{11}$$

The above observations represented the first spate of references on this phenomenon, although as early as 1831, Philips (20) had recorded that the presence of sugar in solution hindered the hydrolysis of ferric chloride. This work does not appear to have been followed up until 1874, when Riffard (21) claimed that invert sugar was seven times more effective than sucrose in preventing the precipitation of ferric hydroxide when ferric salt solutions were treated with alkali.

Thus it can be seen that this curious property of sugars was not closely investigated in those early years, the literature containing merely a series of isolated and disjointed references, rather than a systematic examination. In all cases the conditions used were so extreme, that any attempt to relate these findings to natural conditions would be impossible.

Around the 1920s further results began to appear in the literature, much of it in regard to the effect of short chain

organic acids and sugars on the precipitation of various metallic hydroxides from solutions of their salts, on addition of sodium hydroxide or other alkalis.

Fischer (22) observed that the presence of glycerol gave a stable solution of ferric hydroxide under alkaline conditions, and equated the effect with that of tartrate in Fehlings solution, as being due to the hydroxyl groups of the glycerol. Chatterji and Dhar (23) reported that the hydroxides of iron, nickel, thorium, mercury, and cobalt, did not precipitate from solutions of their respective salts upon addition of alkali, in the presence of glycerol or sugars. No effect was, however, noted when glycerol or sugar were added subsequent to precipitation.

Britton (24) recorded an effect between zirconium and dextrose, as did Sen, (25, 26) who included in his study the metals lanthanum, yttrium, uranium, chromium and iron, along with the sugars lactose, dextrose, and laevulose. Mehrotra and Sen (27) examined the effect of these same sugars, plus sucrose, on the metals copper, mercury, iron and cerium. In every case, inhibition of precipitation of the hydroxide was observed, the different sugars showing some degree of selectivity for the metal which it would complex most effectively. Sen, however, concludes that the phenomenon is only observed if :-

'an excess of alkali is present in the solution'.

Dumanski and his co-workers published extensively on the phenomenon in 1930. (28, 29, 30, 31, 32) They examined a wide range of sugars, and found that the efficacy of each sugar to inhibit the precipitation of ferric hydroxide was, in order of decreasing effectiveness :-

lactose, laevulose, maltose, galactose, glucose, sucrose, and raffinose.

They concluded that the property was dependent on the structure of the sugar. In addition, several polymeric carbohydrates were examined and found to exhibit these same properties.

The foregoing workers attributed the effects they observed to 'peptisation' of the metallic hydroxides by the sugars. This is a term originally used by Graham, and is sometimes employed in a general way to imply the opposite of coagulation, that is, dispersion, especially when the process results in the formation of a colloidal sol. It is, however, generally restricted to a chemical means of dispersion, in which the colloidal particles are stabilised by the adsorption of charged ions.

Bachmann et al (33) investigated by several methods the effect of addition of glycol, glycerol, mannitol, dextrose, sucrose, inulin, dextrin and starch on the behaviour of copper sulphate and ferric chloride solutions when sodium hydroxide

was added. They concluded that the character of the product ranged from crystalloid, through colloid, to suspension, depending on the proportions of alkali and organic additive present.

The question as to whether this phenomenon of precipitate inhibition is due to formation of a colloidal sol or of a true molecular complex, has not been pursued in this initial study, since the phenomenon itself, and the conditions under which it becomes manifest, are of greater preliminary importance. If sugars are capable of holding metals in a form suitable for translocation under physiological conditions, be it chelate, simple complex, or protected colloid, then they can be of great biological significance as complexing agents. All of the forms described above fulfil Rossotti and Rossotti's definition of a complex.

In addition to the research referred to above, a considerable amount of work has been carried out by various workers to investigate complex formation in alcoholic media. The work of Percival (34, 35, 36) and Rendelman (37) is notable in this field. The results of experiments carried out in this solvent demonstrated the formation of complexes with definite stoichiometric relationships between metal and sugar. The greater stability of complexes formed in alcohol has been attributed to the lower polarity of the solvent (38), just as

the relatively low stability in water has been explained by the tendency for metal ions to associate with water molecules, rather than the sugar (39).

This work is mentioned, since it cannot be ignored in any summary of research previously carried out on the formation of sugar-metal complexes. It is, however, of no direct applicability to this study as a whole, since no correlation with natural conditions is possible.

Thus it can be seen from the above survey of the early literature that, in general, the conditions used to demonstrate a complexing effect were far removed from conditions found in nature. No early workers, therefore, attempted to correlate the effects which were found, with any physiological metal translocation process.

It should be pointed out here that the effects described above represent complex formation between sugars and metal bases or hydroxides, as distinct from the conventional complexing effects described earlier (p.2 - 5) where complexing between the ligand and the metal ion is general. This point will be further discussed in subsection 4 of this introduction and in Section I.

Around 1950 came the first example of this property of metals and sugars being applied to a physiological role. A number of workers reported the use of saccharated iron oxide as an injectable preparation for the treatment of anaemia.(40,41,42)

Many different sugars were used to produce these preparations. These included, apart from sucrose: various polysaccharides (43) dextran (44, 45) polyglucose and polygalactose (46). All were used with success, although some patients developed undesirable side effects. Latterly, however, preparations were found which did not produce these side effects, and similar compounds are still in general use for the treatment of anaemia.

This was the first evidence produced which indicated that iron-sugar complexes had a role to play in physiological processes. Since they were artificially synthesised compounds, however, no evidence for the occurrence of complexing under natural conditions had yet been provided.

Gaisford and Jennison (47) reported in 1955 that orally administered ferrous gluconate was absorbed from the gastro-intestinal tract to a far greater extent than ferrous sulphate. The figures obtained were 28 per cent and 14 per cent utilisation respectively.

Around the same time, Kusakawa (48) reported on experiments where he compared animals fed a supplement of lactose, with controls which were fed only ferric sulphate. He found that iron absorption was far more complete, and more rapid, in the former. An indication was thereby obtained that sugars under natural conditions, and not in the form of an

artificially synthesised complex, could affect the transport of iron in the body.

Several other workers have since reported a similar enhancement of absorption with sorbitol fed as a supplement (49, 50, 51). Rapid absorption of iron when administered orally in the form of a fructose complex, has also been reported by Saltman et al (52) and Manzini (53).

Saltman and his co-workers are almost an isolated case of research workers who have examined the possible dietary role of sugars, and their papers of 1963 and 1965 make interesting reading (54, 55).

They have shown that sugars facilitate the diffusion of iron across the membrane of the intestine mucosal wall, and have advanced the theory that dietary sugars can play an important role in iron metabolism. A suggested mechanism for the occurrence of 'Bantu siderosis' has been put forward. This is a disease characterised by excessive iron storage, and Saltman et al suggest that this occurs due to the fact that the diet of the Bantu is largely carbohydrate in nature, being cooked in rusty iron pots. Large quantities of iron are therefore present in the diet. Under the acid conditions of the stomach the iron is hydrolysed, as is much of the carbohydrate. On passage to the alkaline conditions of the small intestine, these contents are neutralised, thereby

forming low molecular weight, soluble, iron-carbohydrate complexes. Thus, instead of iron precipitating and becoming unavailable to the organism, it is now in a form readily taken up by the intestinal mucosa. This is followed by transportation into the blood and ultimate deposition in the body tissues.

In addition to the suggested mechanism for iron transport, a similar mechanism has been proposed for calcium transport, by the same workers (56).

This summary shows that the early work done on the phenomenon of sugar-metal complexing, has provided little information as to the precise conditions under which such complexing will occur. In addition, it is only fairly recently that any possible role for sugar-metal complexes under natural conditions has been visualised, so that the full range of possible applications has yet to be investigated.

3. The possible role of sugar-metal complexes in agriculture.

Since the prime purpose of this study is to investigate the possible role which could be played by complexes between sugars and metals under biological conditions, discussion of this topic must thus necessarily range over the three main subdivisions of agricultural interest, namely plants, soils and animals. A fully detailed discussion of any one of these subjects is obviously outwith the scope of this study, but a

summary of the most pertinent points will be made.

Plants can be regarded as extensive stores of carbohydrate material, with over 50 per cent of their dry weight being composed of this class of substances. Frequently, much of this is in a readily water-soluble form, for example, the fructosans of grasses. These can account for up to 25 per cent of the dry weight of herbage at certain times of the year (57). As such, plants supply a high quantity of carbohydrate material both to soils and animals.

When grasses are ploughed into the soil, or when vegetation is left to decompose on the surface, an appreciable concentration of sugars, both simple and polymeric, must be leached down into the lower horizons of the soil profile (58). Much of this material will be subjected to attack by bacteria, but the bacteria themselves produce quantities of polysaccharides, such as levans (59) and other more complex polysaccharides (60). As stated by Forsyth (61) the source of soil carbohydrates is not readily identifiable, being perhaps plant or perhaps bacterial in origin. The carbohydrate content will, however, be maintained, the actual level varying from soil to soil. In general, a content of from 5 to 20 per cent is accepted for the carbohydrate content of soil organic matter (62), although higher values have been reported (63). The higher the soil organic matter content, then the higher the proportion of carbohydrates present (62).

Two reviews have been written summarising the numerous investigations into this field of research (62, 64), and a more detailed discussion of the carbohydrate level in soils will be presented in the introductory sub-section to Section II. This will be accompanied by a more complete summary of the features of soil development where, it is felt, sugars can play an important role. Inclusion in this relevant section, which is devoted to the dissolving action of various sugars, will give a greater continuity to the text.

Up to the present the most important role which has been attributed to soil carbohydrates is their influence on soil structure. It has been shown (65) that soil polysaccharides are capable of binding imorganic soil particles into stable aggregates. It has also been suggested that the presence of carbohydrates in the soil may inhibit the precipitation of iron and aluminium by phosphate (66).

However, it is felt that a far more important role may be played by soil carbohydrates, namely, that of mobilising iron and aluminium, and transporting them to lower horizons. The upper horizons of the soil would thereby become depleted of these minerals.

Many workers have shown that leachates from various plants are capable of mobilising insoluble iron and aluminium salts in the soil, but the entity responsible for this has never been determined. These leachates, mainly from grasses and

leaves, will contain an appreciable concentration of carbohydrates, but sugars have never been used on their own in any experiment investigating iron mobilisation. It is here that carbohydrates in the soil may play a hitherto unappreciated role.

In addition, the carbohydrate material in the soil may affect the nutrition of the plant itself, by keeping nutrients such as phosphate, iron, aluminium, magnesium etcetera in a soluble form which will facilitate their uptake. Plants are known to be able to absorb and utilise monomeric sugars (62), and this may indicate a mechanism whereby mineral uptake by the plant could be enhanced.

Since herbage provides a large proportion of the diet of farm animals, it is obvious that they are subjected to very high levels of carbohydrate intake. The implications of this have, however, never been fully investigated. Several reports have indicated that feeding high levels of sugar to animals increases the absorption of various ions (48, 49, 67), but the full significance, or even the quantitative importance of varying levels of sugar, and differing conditions, have not been at all thoroughly investigated. Thus the level of sugars in the herbage could affect the 'availability' of a number of ions to the animal in a way not yet fully appreciated. This subject will be further discussed in Section III.

These, then, are the main areas where complexes between sugars and metals could have a considerably important role to play. In essence, the role which these complexes may play can be summarised by the term 'translocation', whether it be the movement of metals in soils, plants, or animals.

4. Scope of the present work.

It is pertinent to discuss at this point what chemical form of the metal is envisaged as forming complexes with sugars. The possibilities of complex formation with the ionic form of the metal, or with a metallic compound such as hydroxide, carbonate, or phosphate, both exist. Indeed Rendelman (2) makes the distinction between complex formation with alkaline-earth and alkali metal salts, and the corresponding bases. All of the early workers (subsection II of this introduction) refer to the alkaline conditions necessary for complex formation, under which conditions the metals would be in the form of their hydroxides. Thus the majority of information implies that the form in which the metal is bound to the sugar is the hydroxide. This may indicate that the nature of the bonding between metals and sugars is an interaction between hydroxyls, and may have led the early workers to assume that an excess of alkali was necessary. Although the precise nature of the form of bonding is not of great concern in this study, it is of importance to determine the chemical form of

the metal which undergoes bonding. The investigations on this point are described and discussed in Section I.

It was originally intended to examine a wide range of sugars and biologically important metal compounds. However, most of the work in this study has, in fact, been confined to ferrous and ferric iron and, to a lesser extent, magnesium. The analytical problems presented by the necessity of estimating iron in the presence of high sugar concentrations, and of estimating sugars in the presence of variable quantities of iron, were themselves time-consuming; in any case, the results obtained with iron suggested so many other avenues for investigation, that a decision to confine the work in this way was clearly advisable.

Initially it was intended to conduct much of the work using the polysaccharide fructosan. The use of this polysaccharide was planned, since it is readily soluble in water, is found in high quantities in herbage at certain times of the year, and is virtually identical in structure to the levans synthesised by soil bacteria. Since it is the most common soluble polysaccharide found in British feed grasses, it is ingested in large amounts by livestock, and leached in quantity into the soil. Having such a widespread occurrence in nature, it was obviously the most useful polysaccharide which could be used in an investigation of this type. However, difficulty was experienced in extracting

sufficient quantities of this carbohydrate to conduct the numerous necessary experiments, so that its monomer fructose was used as the main sugar in this study. After conducting much of the preliminary work with this sugar, the more important experiments were then repeated using fructosan. Other monosaccharides and disaccharides were also tested for comparison, but in every case fructose proved to be the most effective simple sugar used; as will be shown, even more enhanced effects were obtained with its polymer.

The preliminary studies with a large scale fructosan preparation obtained from ryegrass, gave results which led to some analytical work upon it, and necessitated the isolation of further fructosan samples for confirmatory studies. Section III of this thesis is devoted to these findings. For the sake of continuity, all the analytical results are presented together in this section, reference being made elsewhere in the text to certain results where this is necessary for clarification.

The layout of this thesis is as follows:-

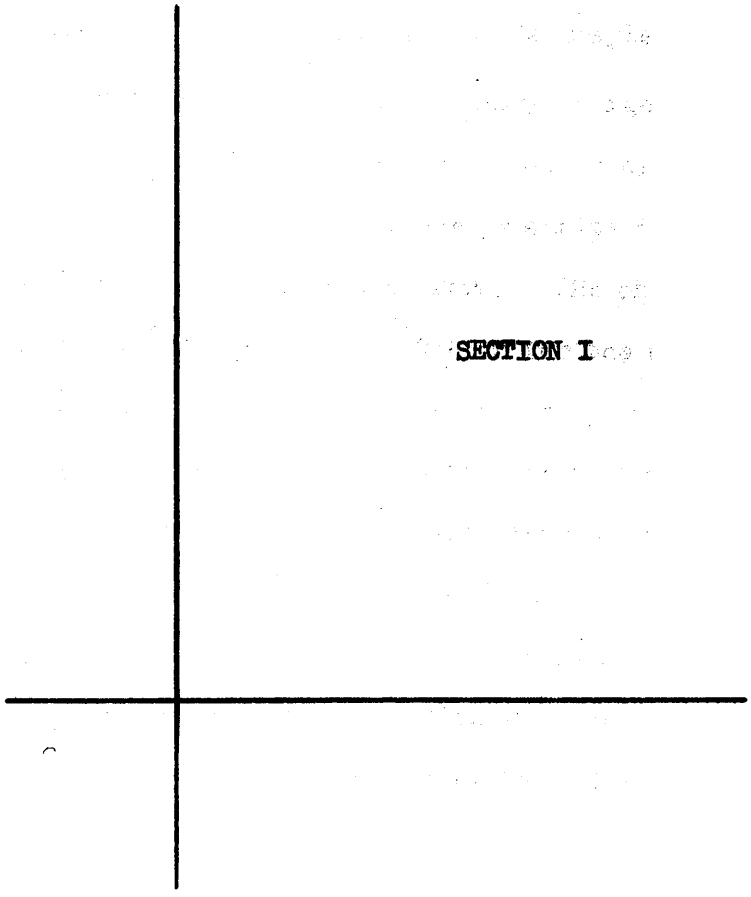
Section I deals with the initial investigation to determine the conditions under which sugars may exhibit complexing powers with various metals. All the methods used were based on the premise that, when a metal forms a complex with any complexing agent, the 'normal' chemical properties of the metal become altered. The complication introduced by the

possibility that metal hydroxides were the entities bound by the sugars was, of course, borne in mind in these experiments.

Section II is a description of the work directed towards an examination of the practical applications of the preceding results, with respect to the capacity of sugars to dissolve insoluble materials. These substances were : magnesium oxide; freshly precipitated samples of ferric and ferrous hydroxides; and the naturally occurring ferrous minerals biotite, tremolite, and siderite. In conducting this work full use was made of the results obtained in the preceding section.

Section III gives an account of the analytical work carried out on the original fructosan sample, and of the subsequent examination of further samples of the polysaccharide.

Each section is prefaced by a short introductory sub-section discussing the previous research work relevant to the particular topic. A summary and discussion follows each section.



SECTION I

Section I. Investigation of the conditions under which complexing will occur.

1. Introduction.

This introduction will provide a brief summary of the methods frequently used in the investigation of complex formation. Many of these methods are based on the fact that the 'normal' properties of the metal become altered on formation of a complex with an organic complexing agent.

One of the most common property changes is a change in the normal chemical reactions of the metal, and this may be manifest as the inhibition of the precipitation of an insoluble compound which would otherwise form. The phenomenon may be regarded, in essence, as competition for the metal between the complexing agent and the precipitating anion. For example, ferric iron forms a very stable complex with EDTA under acid and neutral conditions. Although this possesses a high stability constant of 10^{25}, ferric hydroxide is one of the most insoluble metal hydroxides and has a solubility product of 10^{-36}. The precipitation reaction therefore triumphs over the complexing by EDTA, and the complex is broken down to yield a precipitate at pH values above 7.0. Thus, the extent to which any complexing agent will inhibit the formation of a precipitate, will be a measure of the stability of that complex.

This technique was utilised by Dumanskii et al (29) in determining the relative stabilities of the iron complexes of

several sugars. These were found to be, in order of decreasing effectiveness:-

lactose ; laevulose ; maltose ; galactose ; glucose ; sucrose ; and raffinose.

The ability of sugars to complex with metals can be effectively tested by this method. In addition, the quantity of sugar required to prevent precipitation, and the pH values at which complex formation occurs, can be determined.

A widely used method for determining the presence of complexes, is the isolation, purification and analysis of the complex itself. Many of the early workers who investigated the phenomenon of sugar-metal complexing made use of this technique, (13, 17, 19.) which was also used more recently by Saltman et al (54). The recent review article by Rendleman (2) gives a complete summary of the alkaline-earth and alkali metal complexes of sugars which have been isolated to date, together with a summary of the usual isolation procedure.

By conducting this technique at different pH values the extent of complex formation can be determined, and the effect of the initial sugar to metal ratio on the ultimate complex formed can be investigated.

Electrophoretic data has been used to investigate

complex formation both in aqueous and alcoholic media, a comprehensive review of the electrophoretic behaviour of sugars being given by Weigel (68). The rate of electrophoretic migration is a function of many variables, such as the net charge on the molecule, the stoichiometry of the complex, the salt concentration, the cationic radius, the favoured coordination geometry of the cation, and the size, configuration and conformation of the polyhydroxy compound. A further contributing factor is the stability of the complex, which would be expected to be influenced by the pH of the system. An indication of the conditions under which complexes form can therefore be obtained by conducting electrophoretic experiments in buffers of different pH values.

On formation of a complex a pH shift can occur. This is due to the release of titratable hydrogen ions, since all agents which form metal complexes can be considered to do so by the displacement of one or more usually weak acidic protons by the metal. Thus a pH drop occurs, which can be followed by means of a titration technique, recording the pH attained during alkali addition. The net result is a depressed titration curve, the extent of pH depression being a measure of the stability of the complex. Since proton release, and therefore depression of the pH, will only occur when complexing takes place, the exact point at which complexing commences can

be determined. The technique was developed by Schwartzenbach (69), and has subsequently been used by Britton (70) investigating the complexing of acetic, oxalic, and tartaric acids; by Britton and Jarrett (71) investigating malonic acid complexes; by Pecsoc and Sandera (72) examining the ferric-gluconate system, and more recently by Saltman et al (54) investigating fructose complexes with iron. Use of this technique should allow close examination of the complexing behaviour of sugars and metals to be made.

Finally, the investigation of absorption spectra is a method frequently used for the detection of complexes. Ley (9, 10) recognised that formation of a characteristic colour is a feature of complex compounds, and utilised absorption spectra to distinguish between free metals and their complexes. Again, this technique has been used recently by Saltman et al (54).

The foregoing are the main methods applied to any investigation of complexing ability. Although they have been used previously for the examination of complexing phenomena between sugars and metals, no precise details of the type necessary in this study have been provided by the earlier workers. This can be said of the precipitate inhibition experiments, and the analysis of isolated complexes. Both demonstrated that complexes do exist, but did not detail the

conditions under which they occur, In addition, the information provided by the electrophoretic examinations and the titration experiments has been rather limited.

This section is an account of the investigation into the conditions of complex formation, together with a short discussion of the possible significance of the results obtained.

2. Chromatographic and electrophoretic investigation of complexing between sugars and metals.

(a) Chromatography.

Individual substances exhibit characteristic R_f values in a given solvent by virtue of their distribution coefficient between that solvent and the stationary phase. Any change in the characteristic value for either sugar or ferric chloride alone, effected by mixing the two entities, will reflect a change in the distribution coefficient, and will suggest the formation of a complex under the conditions used.

Exp. No.1. Paper chromatography (G.M.1)

Aliquots of 0.02 Molar ferric chloride with sugar additions to give molar ratios of sugar to iron from 1:1 to 50:1, were chromatographed in aqueous phenol (160 grams phenol: 40 mls water) and ethyl acetate : pyridine : water (120:50:40) solvents, along with samples of ferric chloride and sugar for comparison. In addition, a range of samples previously

adjusted to pH values from 3.5 to 10.0 were also examined. Sugars were then located with a silver reagent, whilst iron was detected with 5 per cent acid thiocyanate (G.M.1).

Although many chromatograms were run, no substantial change in movement of either sugar or ferric iron was observed. Typically, the sugars ran well down the paper, and ferric iron remained on the origin. The greatest effect observed with mixed ferric chloride and sugar was a slight tendency for iron to streak, possibly indicating some easily dissociable association between it and the sugar. Several sugars were tested in this way. Although fructose appeared to be more effective than glucose, galactose, mannose and xylose, similar affects were demonstrated in all cases.

The inconclusive results obtained by this technique were thought to be due to two factors. Firstly, no attempt was made to control the pH of the samples during elution, since buffering the solvent would have necessitated the introduction of inorganic salts , which themselves may have undergone complexing. It was desirable to avoid complications of this type. Secondly, there was the possible effect of the cellulose matrix. If complexing between sugars and metals can occur, then cellulose itself, an insoluble glucose polymer, could be expected to exhibit a complexing effect, thus hindering any movement of iron. The adsorption of ferric iron on

cellulose has been reported by Haerting (73), by Belford et al (74), and by Pickering (75).

Exp.No.2. Chromatography on a synthetic fibre sheet.

To remove this influence, similar solutions to those used in Exp.No.1 were chromatographed on a sheet of the synthetic fibre known generally as 'paper nylon'. Only ethyl acetate : pyridine : water, (120 : 50 : 40) of the several solvents tested, ran successfully. The location reagents were the same as those used previously. (Exp.No.1)

As before, ferric iron in the absence of sugar remained completely on the origin, but where sugar was present, extensive streaking of iron from the origin to the solvent front was observed. Although not running as a definite spot, a tendency for iron to run along with the sugar was demonstrated, indicating some form of complex formation. That this occurred on a synthetic fibre sheet, devoid of hydroxyl groups, indicated adsorption of iron on cellulose as one reason for the failure of paper chromatography.

These experiments, however, do not provide any detailed information as to the conditions under which complexing can occur, as attempts to control pH were not made.

b.) Paper electrophoresis.

By the use of buffer systems of different pH values, the variation of the stability of the sugar-metal complex with pH may be observed. In any one buffer system sugars will possess

typical mobility values, as will ferric chloride. Any change in these values effected by mixing the two entities will indicate some degree of complexing at the pH value of the buffer.

Sample solutions were made up as in Exp.No.1 using sugar to iron ratios of 20 : 1 to 50 : 1, together with samples of sugar and ferric chloride as standards. No previous pH adjustment was carried out, as the samples would rapidly attain the pH value of the buffer system used. The method used was an immersed strip technique, using Analar carbon tetrachloride as coolant. After spotting on the samples, each paper strip, 15 inches long, was subjected to a voltage of 500 volts for 1 hour, before drying, and location of the sugar and iron with the reagents already described (Exp.No.1). A full description of the method used is given in G.M.2.

All results showed evidence of cellulose interference, but it proved impossible to utilise the synthetic sheet as a supporting medium for the electrophoresis runs, due to the impossibility of adequately wetting the sheets.

Exp.No.3. The electrophoretic properties of sugar and iron in buffers of different pH.

a) Borate buffer pH 10.0

This buffer system was used since borates are known to form complexes with sugars (76, 77, 78). Thus fructose, the

sugar used in this initial investigation, should show a fairly high mobility. The features observed on location of fructose and iron are shown in Fig.2.

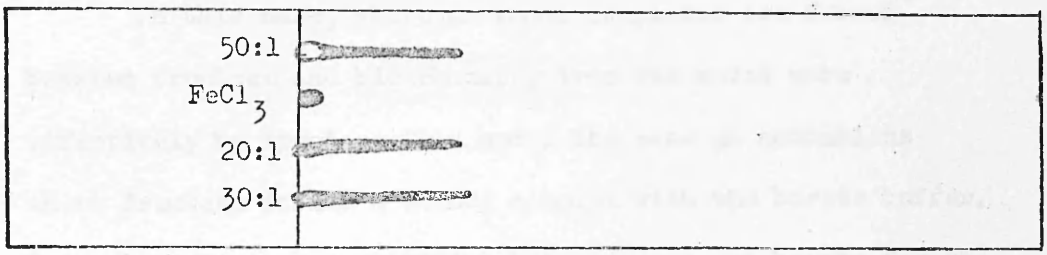
Fructose alone ran as a well defined spot, with ferric iron alone remaining on the origin. However, with mixed fructose and iron, a quantity of iron left the origin running as a streak, the amount of iron remaining at the origin decreasing as the ratio of fructose to iron increased. Moreover, fructose was held back with the iron and also ran as a streak. This is suggestive of the existence of complex formation between fructose and iron at this pH value. The fact that both appeared in the form of streaks, may indicate that the association between the two entities was not very strong, and was affected by the cellulose matrix. In addition, the complication exists that the interaction being studied here was that of fructose/borate and iron, not that of fructose and iron.

b) Electrophoresis at pH 9.0, again in a borate buffer, gave a similar effect, the only difference being that less iron was moved from the origin.

c) Bicarbonate buffer pH 10

The features exhibited in this buffer system are shown in Figs. 3a and 3b. Although still running as a streak, iron removal from the origin was more complete, and the distance run was far greater, when fructose was present, than with borate buffer. Again, when fructose was absent, no iron movement was

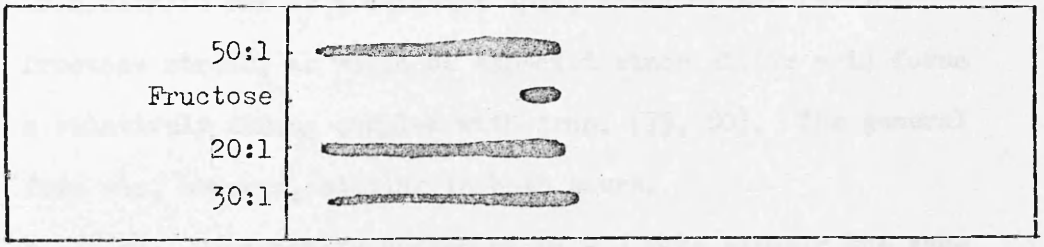
a)



Borate Buffer pH 10 Sprayed for iron

CATHODE ANODE

b)



Borate Buffer pH 10 Sprayed for Fructose

Diagrammatic representation of electrophoretograms

Figure 2

demonstrated. Fructose alone ran a very short distance, but when iron was present, a quantity was carried forward in the form of a streak.

In this case, where no known complexes are formed between fructose and bicarbonate, iron was moved more effectively by fructose than under the same pH conditions where fructose formed a strong complex with the borate buffer. This suggests that competition between iron and borate for the fructose, took place.

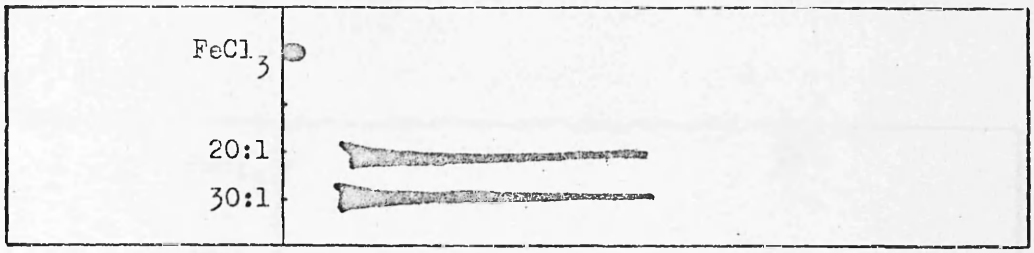
To compare the form of these runs with that of a known ferric-organic complex, a sample of a citric acid/ferric chloride mixture of ration 10 : 1 was tested.(Fig.3c). Iron:citrate ran as a definite spot, ahead of the ferric: fructose streak, as would be expected since citric acid forms a relatively strong complex with iron. (79, 80). The general form was, however, similar in both cases.

d. e. f) Bicarbonate buffer of pH 9.1 gave exactly the same result, while at pH 8.0 more iron was left at the origin. In pH 7.0 bicarbonate buffer, only very slight movement was observed, even when the voltage was doubled.

g. h) No movement was noted in phosphate buffers of pH 7.0 and 8.0.

i. j. k) At lower pH values, citrate buffers of pH values 4.0, 5.0 and 6.0 were used. The features observed (Fig.4) were the exact opposite of those noted when borate systems were

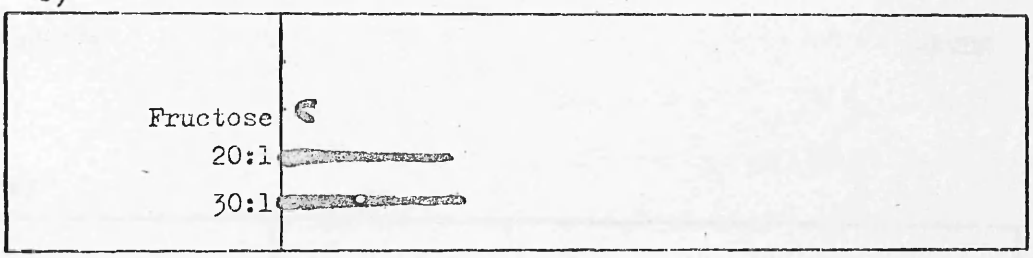
a)



Bicarbonate Buffer pH 10 Sprayed for iron

CATHODE ANODE

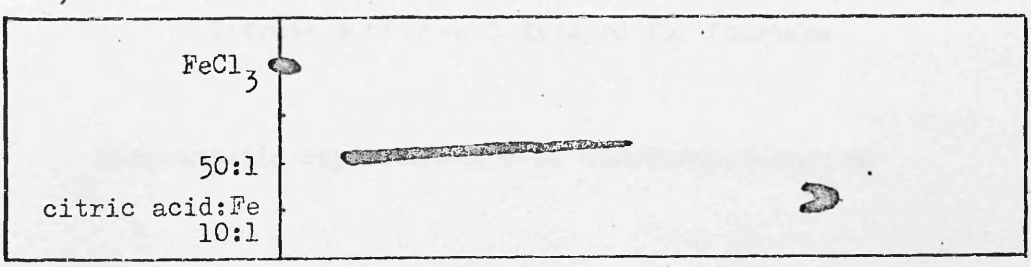
b)



Bicarbonate Buffer pH 10 Sprayed for fructose

CATHODE ANODE

c)

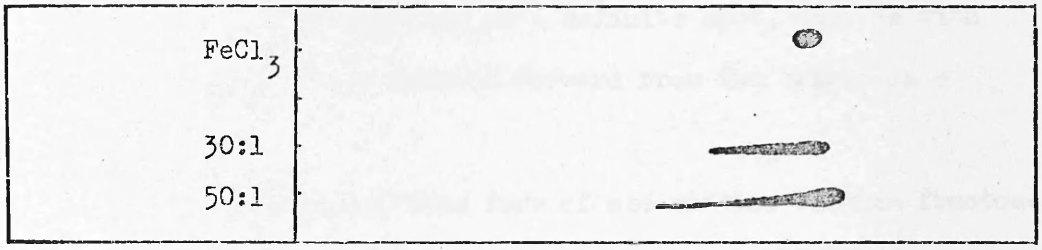


Bicarbonate Buffer pH 10 Sprayed for iron

Diagrammatic representation of electrophoretograms

Figure 3

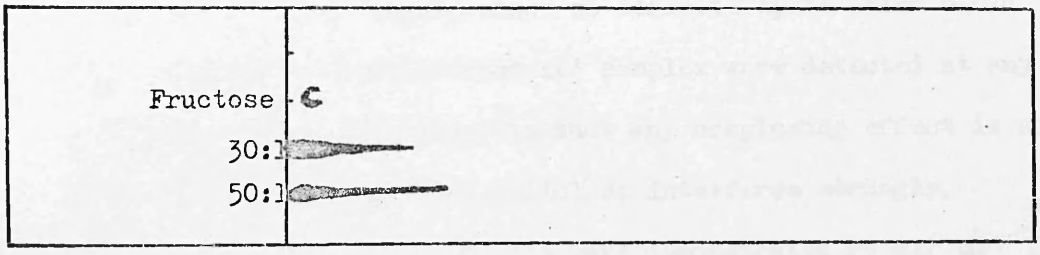
a)



Citrate Buffer pH 5 Sprayed for iron

CATHODE ANODE

b)



Citrate Buffer pH 5 Sprayed for fructose

Diagrammatic representation of electrophoretograms

Figure 4

used. In this case, citrate formed a complex with the iron, which ran fairly rapidly towards the anode. With fructose present, however, a tailing effect was observed. Fructose alone ran a short distance as a definite spot, whereas with iron present, it was carried forward from the origin as a streak.

This indicates some form of association between fructose and iron, even at these low pH values, since the presence of iron affects the characteristic mobility of fructose and vice versa. Although the effect is slight, fructose is in competition with a strong complexing agent.

The results obtained by this technique were not as conclusive as first hoped, since no definite spots which could be identified as 'ferric-fructose' complex were detected at any pH values used. This suggests that any complexing effect is not of high stability, and that cellulose interferes strongly. However, since streaking effects were demonstrated at all pH values, some association between iron and fructose is indicated. It is further suggested that this association is at a maximum at higher pH values, becoming reduced as the pH falls to neutrality, and followed by a further slight increase at acidic pH values.

These experiments were repeated, using sugars other than fructose. All gave effects similar to these noted for fructose, as judged by their ability to effect the movement of iron. In

order of decreasing effectiveness, these were:-

fructose > fructosan > glucose; galactose > xylose > sucrose; lactose

Since a wide range of carbohydrates exhibited this phenomenon of complexing with iron, it is not surprising that cellulose should also form a complex, and that this should contribute to the streaking effects noted.

3. Inhibition of hydroxide precipitation by sugars.

The procedure finally used was developed using fructose, as pilot experiments indicated that this was the most effective simple sugar in inhibiting precipitation. Quantities of sugar were weighed dry into beakers, to give the desired molar ratios on pipetting in 25 mls of 0.02 molar salt solution. All solutions were stirred at a constant speed by a magnetic stirrer. 1.0 Normal sodium hydroxide was added from the same burette at a fairly constant speed in all experiments. After addition of each drop of alkali, a short time was allowed for equilibrium to be reached. Fine adjustment of pH was effected by addition of small quantities of alkali on the end of a thin glass rod.

In the case of the ferric iron experiments, the exact point where precipitation commenced was taken as the point where a particulate precipitate of ferric hydroxide was first observed under illumination with a bright light. With magnesium salts,

however, the translucent nature of magnesium hydroxide made this method of detection impossible. As an alternative, determination of the quantity of magnesium remaining in solution at a range of pH values was made. A similar technique was also applied to ferric iron in a later stage of investigation.

All experiments were repeated several times to ensure accuracy.

It is worth noting at this point that on addition of the weighed quantities of sugar to the ferric chloride solution, an increase in colour intensity was observed. The intensity of this colour increased as the quantity of sugar present increased, the slope of this curve being dependent on the sugar used (Fig.5) By measuring the absorbence of a ferric chloride solution and a ferric chloride plus fructose solution, both at pH 2·35, over the visible region, the spectra shown in Fig. 6 were obtained. Increased absorption at 388 mμ in the fructose containing solution, is clearly demonstrable, despite the similarity of the spectra. This is suggestive of the presence of a complex between sugar and iron at a pH value of 2·35, similar to the effect noted by Saltman et al. (54)

Exp. No. 4. Investigation of the pH of precipitation of ferric hydroxide in the presence and absence of sugars.

From solubility product data, the theoretical pH of

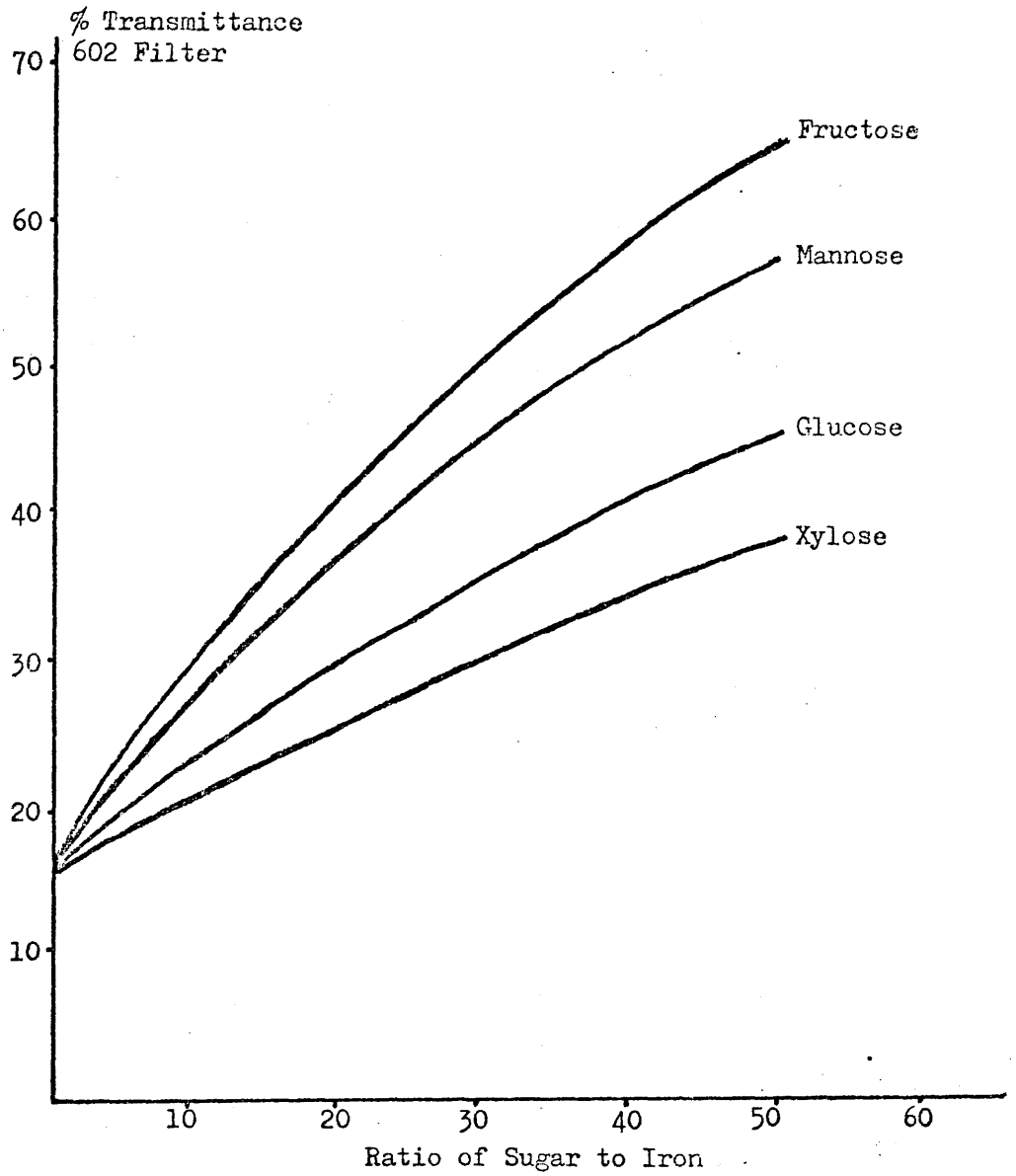

% Transmittance
602 Filter
70
60
50
40
30
20
10
Fructose
Mannose
Glucose
Xylose
10
20
30
40
50
60
Ratio of Sugar to Iron

Figure 5

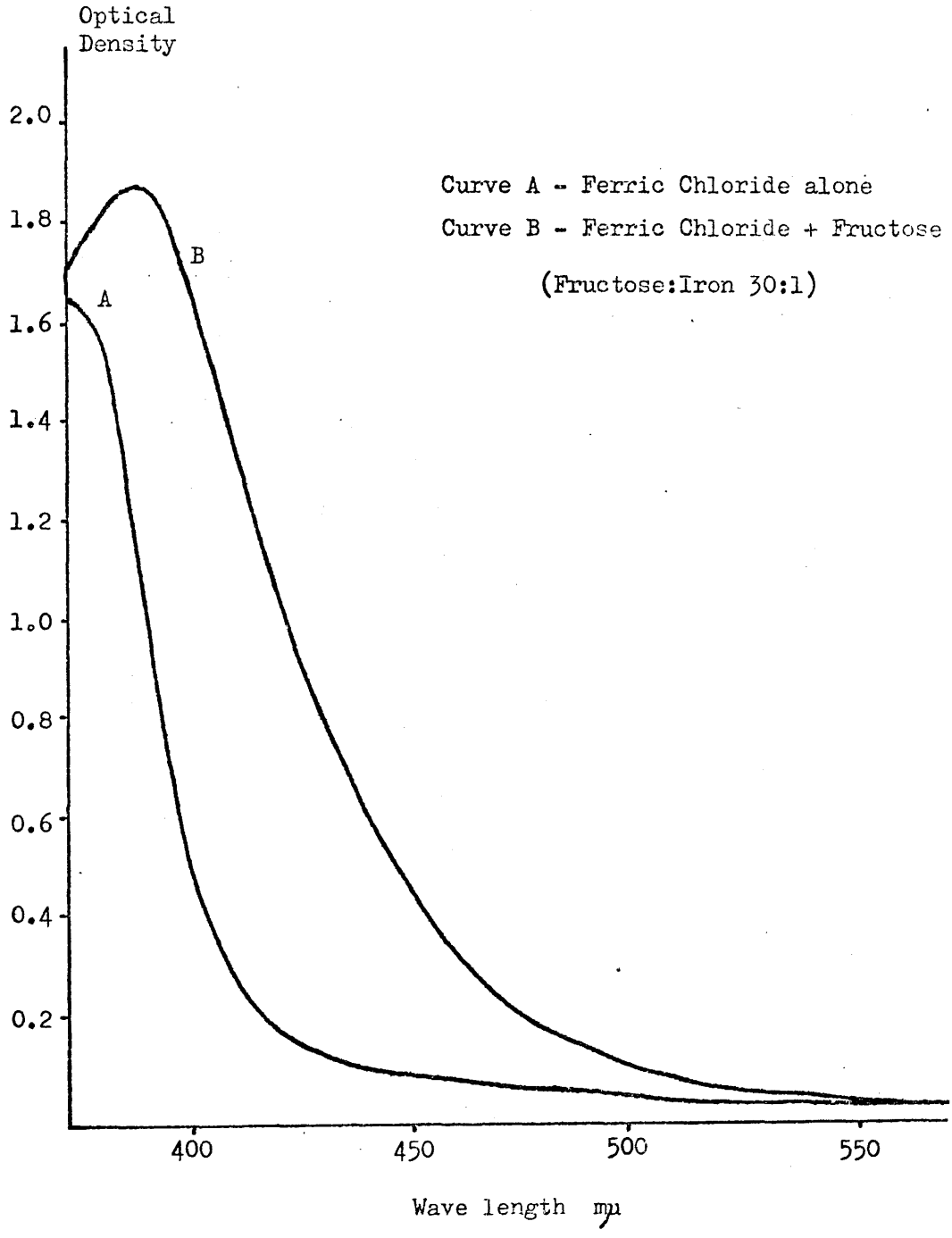

Optical
Density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A
B
400
450
500
550
Wave length mμ
Curve A - Ferric Chloride alone
Curve B - Ferric Chloride + Fructose
(Fructose:Iron 30:1)

Figure 6

ferric hydroxide precipitation from a 0·02 Molar solution was calculated to be pH 2·58.

$$\left[Fe^{+++}\right]\left[OH^{-}\right]^{3} = K = 1{\cdot}1 \times 10^{-36}$$

Now $\left[Fe^{+++}\right] = 0{\cdot}02$

$\therefore \left[OH^{-}\right]^{3} = 1{\cdot}1 \times 10^{-36} \times 50$

$= 55 \times 10^{-36}$

$\therefore \left[OH^{-}\right] = \sqrt[3]{55} \times 10^{-12}$

$= 3{\cdot}8 \times 10^{-12}$

$\therefore$ pOH $= -(-12 + \log 3{\cdot}8)$

$= -(-12 + 0{\cdot}58)$

$= 11{\cdot}42$

$\therefore$ pH $= 2.58$

A similar value was obtained in practice. The presence of sugar effectively inhibited the precipitation of ferric hydroxide from solution, thereby increasing the pH which had to be attained before precipitation began. As the ratio of sugar to iron was increased, so did the pH at which precipitation occurred increase, the greatest individual increase being observed with the first small increment of sugar.

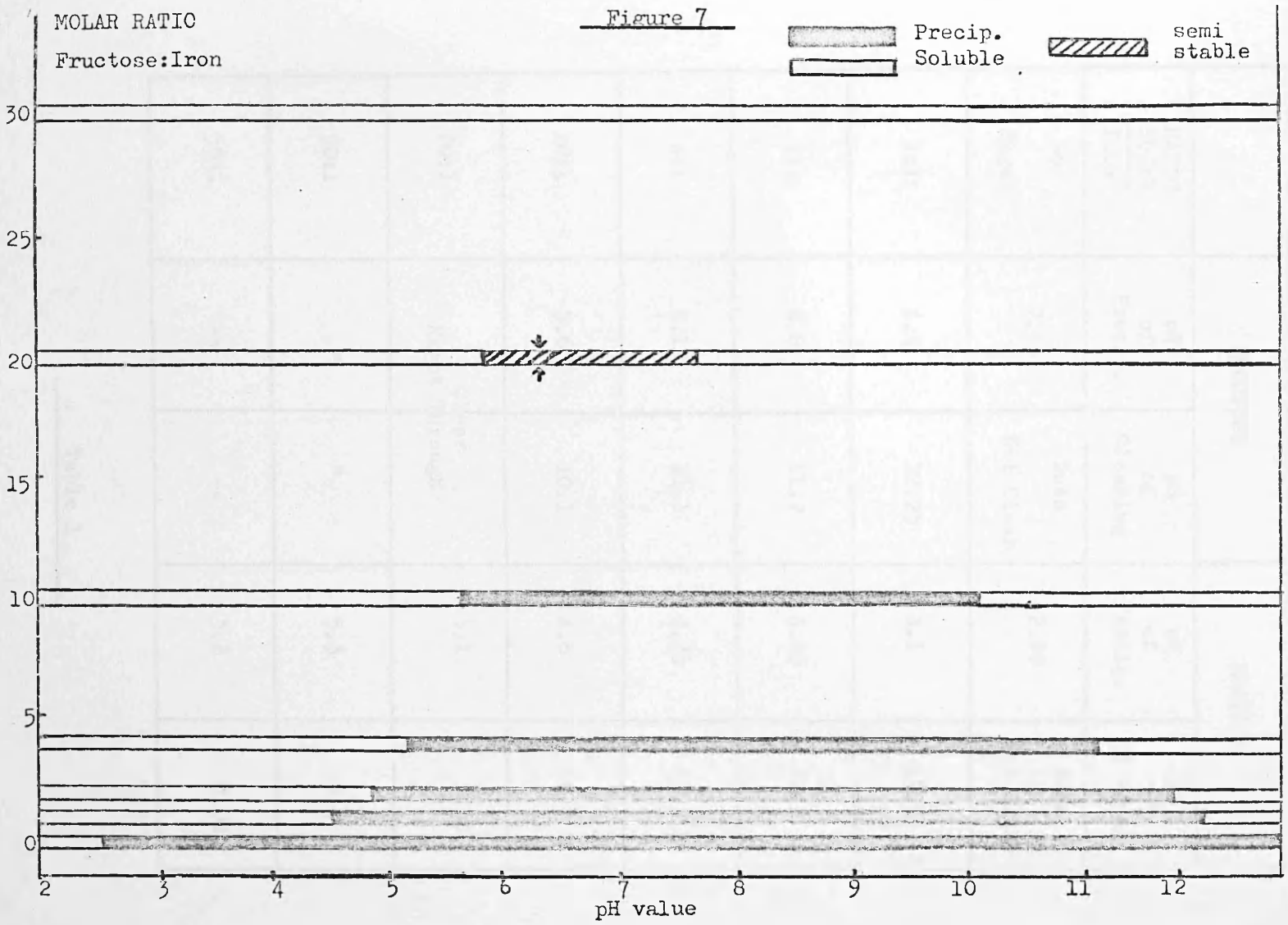
In addition, the presence of sugar also resulted in the precipitate redissolving on increasing the pH above the precipitation point, the pH at which re-solution occurred being lowered as the sugar to iron ratio was increased. Thus the phenomenon of precipitate inhibition as the sugar to iron ratio was increased was found to take the form of a continually narrowing pH range over which a precipitate existed. (Fig.7)

All the sugars tested demonstrated this feature to a varying degree, the results being summarised in table form in Tables 1, 2 and 3. Only in the case of fructose was there a ratio reached where complete precipitate inhibition over the entire pH range was achieved. The order of decreasing effectiveness was:- fructose; mannitol; mannose; glucose; xylose; lactose. Lactose did, however, appear to be more effective than xylose when present in high concentration.

Comparison of the results obtained here for fructose, mannose, glucose and xylose, with the colour intensity levels produced on addition of ferric chloride to those sugars (Fig.5) demonstrated that they are in exactly the same order:-

fructose > mannose > glucose > xylose

This is further evidence that the cause of the spectral change was the formation of a complex between the sugar and the metal ion, and that the feature of colour intensification may act as a rough guide to the effectiveness of complexing.


MOLAR RATIO
Fructose:Iron
Figure 7
Precip.
Soluble
semi
stable
30
25
20
15
10
5
0
2
3
4
5
6
7
8
9
10
11
12
pH value

	FRUCTOSE		MANNOSE	
RATIO SUGAR / Iron	pH of Precip.	pH of Clearing	pH of Precip.	pH of Clearing
No Sugar	2.65	Does Not Clear	2.60	Does Not Clear
1:1	4.4	12.25	4.1	12.55
2:1	4.8	11.7	4.25	11.5
4:1	5.1	11.1	4.35	11.5
10:1	5.6	10.1	4.6	10.8
20:1	Clear Right Through		5.1	9.55
30:1	"	"	5.3	9.4
50:1	--	--	5.8	9.08

Table 1

	GLUCOSE		LACTOSE	
Ratio Sugar / Iron	pH of Precip.	pH of Clearing	pH of Precip.	pH of Clearing
No Sugar	2.60	Does not Clear	2.65	Does not Clear
1:1	3.95	13.0	3.75	No Clearing 13.0
2:1	4.1	12.0	4.0	No Clearing 13.0
4:1	4.4	11.6	4.25	13.0
10:1	4.6	10.9	4.4	11.9
20:1	4.7	10.5	5.0	10.65
30:1	4.9	10.3	5.8	10.4
50:1	5.5	9.8	Incomplete Solution of Lactose	

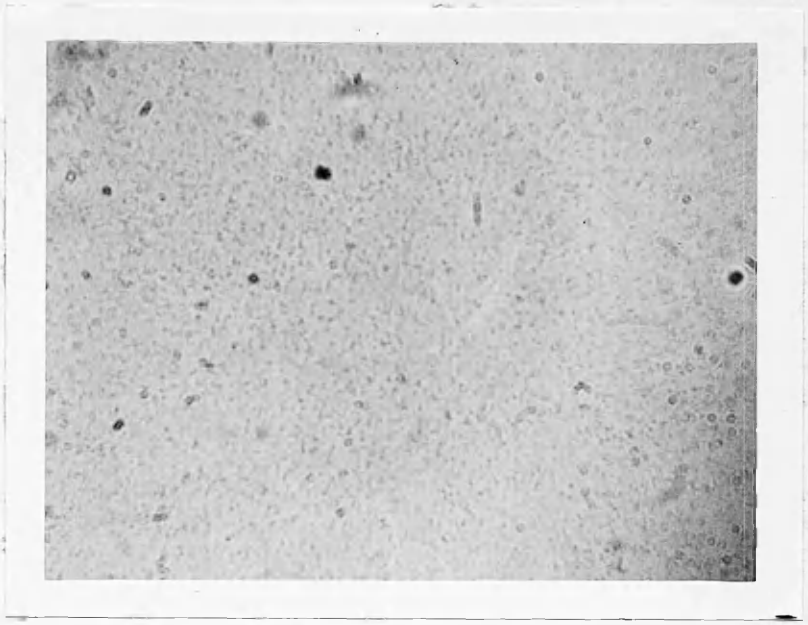
Table 2

	XYLOSE		MANNITOL	
Ratio Sugar / Iron	pH of Precip.	pH of Clearing	pH of Precip.	pH of Clearing
No Sugar	2.70	Does not Clear	2.65	Does not Clear
1:1	4.0	No Clearing 13.0	4.25	11.8
2:1	4.2	12.75	4.8	11.05
4:1	4.25	12.25	5.1	10.4
10:1	4.5	11.8	5.35	10.05
20:1	4.7	10.9	5.4	9.55
30:1	4.9	10.45	5.75	9.1
50:1	5.4	10.05	Incomplete Solution of Mannitol	

Table 3

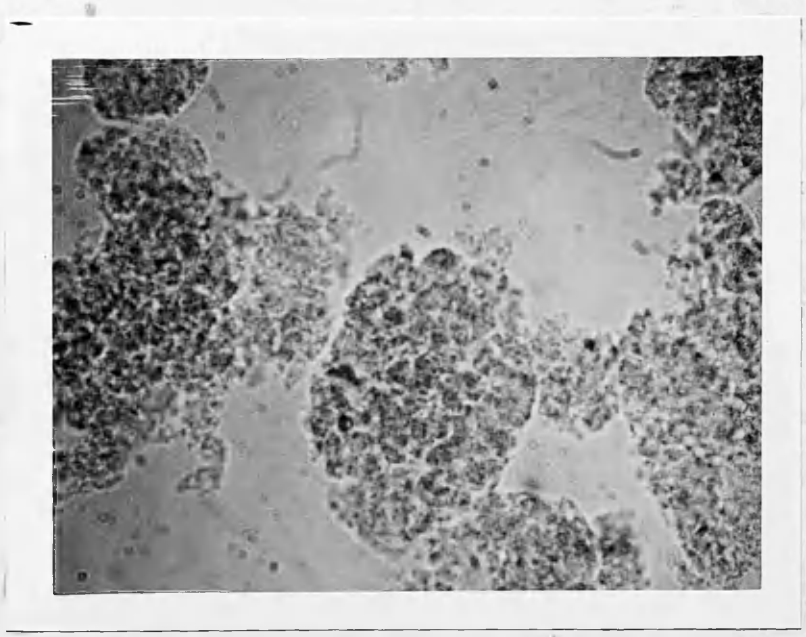
The diagramatic representation of the 20:1 fructose to iron ratio in Fig. 7. indicates that pH adjustment of mixed fructose and iron at this ratio results in the formation of a semi-stable solution over part of the pH range. During several repeats of this experiment no precipitation was observed over the entire pH range, but where the solution was allowed to stand at pH 6.2 for a longer period, a light 'milky' precipitate was shed. On subsequent slight pH increase, by addition of sodium hydroxide, this precipitate disappeared and the solution cleared. Precipitates of similar physical appearance were generally observed with all sugars at the commencement of precipitation where the sugar to iron ratio was high. A sample of the precipitate was removed and examined under a microscope, comparing its appearance with that of the floculated precipitate obtained when ferric chloride alone is adjusted to this pH. It can be seen (Fig. 8 and Fig. 9) that where fructose is present the precipitate is more finely divided than the typically floculated precipitate of ferric hydroxide alone.

A more detailed examination of the behaviour of solutions having this ratio (20:1) of fructose to iron was made by adjusting a series of such solutions to pH values in the region of 6.2. The observations made are summarised in Table 4.



Appearance of precipitate obtained at pH 6.2 with fructose present

Figure 8



Appearance of precipitate obtained at pH 6.2 no fructose

Figure 9

pH to which 20:1 soln. adjusted.	Remarks
5.8	clear, but became slightly cloudy after 15 mins. standing
6.05	became slightly cloudy and precipitated after 15 mins.
6.2	around 1-2 minutes delay followed by precipitation
6.4	very slight precipitation after 3 hours
6.9	clear

Table 4

The results indicate that the pH value at which precipitation is most likely to occur, is about 6.2. At values slightly above or below this 'critical' pH, the iron-fructose complex is stable for some time before ultimate precipitation. At no point, however, was precipitation complete, the solution always retaining some colour characteristic of ferric iron.

Exp.No.5. Measurement of the quantity of iron remaining in solution at a range of pH values, with increasing molar ratios of fructose to iron

Since precipitation from the 20:1 solution was observed to be only partial, the extent of precipitation at all pH

values and all ratios of fructose to iron was examined. This was carried out by making up solutions similar to those used in the previous experiment, and adjusting them to a definite pH value. An aliquot was then taken, which, after standing for half an hour was centrifuged, and the amount of iron remaining in the supernatant determined, (G.M.3). The results are summarised in Table 5. As will be seen, over one third of the iron remained in solution at pH 6.3, where the ratio of fructose to iron was 20:1. This value increased rapidly above and below this pH. As expected, the amount of iron held in solution by lower fructose to iron ratios was less than this amount, although still considerable when compared to the solution containing no sugar. A further feature demonstrated, is that precipitation from solution on pH increase was a gradual process. That is, a quantity of insoluble hydroxide was shed, since only a proportion of the total present could be effectively complexed and held in solution, the quantity shed increasing as the pH was increased, and as the ratio of fructose to iron was reduced.

The results up to this point have shown that sugars, especially fructose, are capable of inhibiting the precipitation of ferric hydroxide, and the pH values at which this is achieved have been well defined, both for a variety of sugars and a range of sugar to iron ratios. It can be concluded that

pH of Sample	gamma per mililetre of iron in solution at various ratios of fructose to iron					
	20	10	4	2	1	0
Original	945	1045	1045	1055	1065	1100
2.6	S	S	S	S	S	1045
2.8	S	S	S	S	S	1020
3.0	S	S	S	S	S	749
4.0	S	S	S	S	1066	136
4.5	S	S	S	1050	99.7	5.2
4.8	S	S	S	-	-	0
5.0	S	S	1040	20.3	16.8	0
5.5	S	1055	49.5	13.5	8.8	0
6.0	940	27.6	12.5	4.2	4.2	P
6.3	385	10.9	-	-	-	P
7.0	450	13.1	1.05	-	-	P
8.0	710	97.5	1.05	-	-	P
8.5	958	-	-	-	-	P
9.0	S	1032	23.7	-	-	P
10.0	S	1058	175	93.6	27.5	P
11.0	S	S	990	552.5	143.0	P
12.0	S	S	S	1039	-	P

S = completely soluble - = No data P = completely precipitated

Table 5

complexing readily occurs at pH values which prevail in natural environments. Contrary to the comments of earlier workers regarding the necessity for excess alkalinity, complex formation has been shown to occur at low pH values. Although the spectral evidence indicates that complexing may occur at pH 2.35 between sugar and ferric ions, evidence of complex formation above pH 2.6, the pH value at which ferric hydroxide precipitation normally takes place, has been demonstrated by a number of techniques.

In addition to raising the pH at which precipitation commences, the presence of sugar results in the precipitate formed at pH 6.2 returning into solution on continued pH increase. It was clearly of interest to discover whether such an effect was also demonstrated on reducing the pH from the precipitation point.

Exp.No.6. Behaviour of precipitates on pH decrease from pH 6.2

Solutions were made up as in Exp.No.4. Initially a solution with a fructose to iron ratio of 20:1 was tested. This was adjusted to pH 6.2, and then by continual monitoring on a pH meter, the pH was reduced to 4.0 and maintained at this value by addition of minute quantities of dilute hydrochloric acid. Within half an hour the precipitate had dissolved completely, although clarification commenced almost immediately. No such effect was observed in the absence of sugar.

To test whether a similar effect would be displayed by

solutions containing less fructose, two solutions with a fructose to iron ratio of 1:1 were made up and adjusted to pH 6.2. The pH values of each were then reduced to 4.5 and 3.5 respectively, and maintained at this level by means of an automatic titration assembly. This equipment delivered dilute acid from a syringe as necessary, if the pH drifted upwards from the set pH value. Similar treatment was also given to a ferric chloride solution containing no sugar. The results are summarised in Table 6.

Solution	pH level to which adjusted	Remarks
1:1	4.5	After 1 hour, no redissolving noted. Precipitate was more finely divided than previously
1:1	3.5	Seen to be clearing after 1 hour. Completely clear after $1\frac{1}{2}$ hours
No fructose	3.5	No clearing after 6 hours

Table 6

Comparing these results with those obtained previously (Table 1 and Table 5) it can be seen that the pH at which the precipitate will redissolve, presents agreement with the pH at which it is first shed from solution. That is, ferric hydroxide will be shed from a solution containing 1:1 fructose at a pH of 4.4, thus it can not redissolve at pH 4.5, but will

redissolve at pH 3.5 with little difficulty. Similarly, ferric hydroxide alone will precipitate from solution at pH 2.6, and therefore will not redissolve at any pH value above this.

Exp.No.7. Fructose addition after precipitation

It has been demonstrated here that when sugar is added to ferric chloride solution and the pH then adjusted to precipitation point, the precipitate formed may be redissolved by raising or lowering the pH within the range where a precipitate of ferric hydroxide would normally exist. It was of interest to discover if a similar phenomenon was exhibited on addition of sugar after the precipitate has formed at pH 6.2.

Aliquots of ferric chloride were adjusted to pH 6.2 and fructose was added to give a range of fructose to iron ratios from 1:1 to 20:1. The pH levels were then adjusted, both upwards and downwards, as in the previous experiments, and the suspensions observed. No obvious redissolving effect was noted even over a period of time. It appears, therefore, that sugar must be present in the ferric chloride solution before addition of alkali is made, in order to exert any influence on the behaviour of the ferric hydroxide precipitate.

It appears likely that the difference in behaviour is due to the possibility that the precipitate shed at pH 6.2 in the presence of sugar, may not consist of pure ferric hydroxide, but may be an intimate association of ferric hydroxide and sugar. This is reinforced by the fact that the precipitate shed

in the presence of sugar possessed a different physical appearance to that of pure ferric hydroxide. This possibility will be examined by analysis of the precipitate at a later stage. (Exp.No.12).

Exp.No.8. Precipitate inhibition in more dilute solution.

A number of short precipitate inhibition experiments were carried out using 0.002 Molar ferric chloride, instead of the customary 0.02 Molar solution, and using the same procedure as that described in Exp.No.4. The behaviour observed deserves brief mention here.

As before, 25 ml aliquots of ferric chloride were added to weighed quantities of sugar to provide the desired Molar ratios of fructose to iron. On subsequent addition of sodium hydroxide, little difference was observed in the behaviour of solutions containing sugar, and those not containing sugar. Where fructose was absent, precipitation of ferric hydroxide did not occur until above pH 5.0 ; in addition, ratios of fructose to iron of up to 100:1 were ineffective in completely inhibiting precipitation over the entire pH range.

To test the accuracy of these observations several 0.02 Molar ferric chloride solutions with fructose to iron ratios of 30:1 and 50:1 were made up. These were adjusted to pH 6.2 and allowed to stand for some time. No precipitation occurred. However, when a 10 times dilution with water was made, immediate precipitation occurred in both test solutions.

It can be concluded that the complex formed in dilute solution is not very strong, and is unable to maintain ferric hydroxide in solution.

Exp.No.9. Inhibition of ferric hydroxide precipitation with ryegrass fructosan

A series of solutions exactly equivalent to those in Exp.No.4 was used. Dry fructosan isolated from ryegrass as described in Section III was weighed out using the same quantities as used for fructose, without correcting for anhydro-residues. On analysis of the fructosan sample (Section III, 4) a 'fructose' content of 68.2 per cent was found, with a total sugar content of 77.7 per cent; the difference between these two values representing the glucose content of the fructosan sample. In the tabulated results, therefore, both the ratio of 'fructose' to iron, and the total sugar to iron, are given. These molar ratios were calculated according to the molecular weights of the component simple sugars of the fructosan. The results are presented in Table 7.

Although rather ineffective in very low concentrations, fructosan exhibited an exceedingly powerful complexing action in that complete precipitate inhibition was achieved at a 'fructose' to iron ratio of around 4:1. This figure indicates that fructosan is approximately six times more effective as a complexing agent than its monomer fructose.

Ratio Fructose / Iron	Ratio Total Sugar / Iron	pH of Precipitation	pH of Clearing
0.682:1	0.777:1	Milky Ppt. 2.7	No clearing
1.364:1	1.554:1	Milky Ppt. 2.7	11.5
2.728:1	3.108:1	Milky Ppt. 2.7	Almost clear 8.6
3.410:1	3.885:1	Milky Ppt. 2.7 No Particles	Almost clear 6.5
4.092:1	4.662:1	Clear right through	

Table 7

Detailed analysis of the fructosan was carried out (Section III, 4) and it was ascertained that no other moiety capable of complexing was present in the polysaccharide sample. The effect exhibited here is, therefore, attributable only to the action of the fructosan.

Exp.No.10. Inhibition of magnesium hydroxide precipitation.

It proved impossible to determine the pH of precipitation of magnesium hydroxide by observation, due to its translucent appearance. Determination was therefore made of the quantity of magnesium retained in solution at a range of pH values, and a variety of sugar to metal ratios. The procedure adopted was exactly the same as that used in the equivalent iron experiment (Exp.No.5) quantitative determination of magnesium being

carried out as detailed in G.M.4. From solubility product data, the theoretical pH of magnesium hydroxide precipitation from a 0.02 Molar solution, is 9.39, so that only rather high pH values can be used in this series. The results obtained are tabulated in Table 8.

It can be seen that the effect of fructose on the inhibition of magnesium hydroxide precipitation was less powerful than the effect exhibited with ferric hydroxide. Neither was the effect as consistent as that demonstrated with iron (pH 10.9 sample Table 8). However, since a degree of precipitation inhibition was exhibited, it can be said that complexing between magnesium and fructose occurred, although it has only been possible to demonstrate this effect at rather elevated pH values.

Exp.No.11. The effect of alkali on fructose.

Alkali is known to epimerise or degrade fructose, indeed carbohydrates in general (81, 82), the extent of any transformation being dependent on the degree of alkalinity. Although many of the foregoing experiments were conducted over a short period of time, it remained necessary to ascertain that no degradative or other change that would give rise to products which could conceivably have been responsible for some of the effects noted, had occurred during the course of each experiment. For this reason many samples were removed from solutions which

	mgs. of Magnesium present in 25ml. Aliquots at Various pH's		
Ratio Fructose / Iron	pH 10.7	pH 10.9	pH 11.1
No Sugar	0.78	0.70	0.27
1:1	3.1	1.6	0.55
2:1	4.2	2.2	0.81
4:1	6.95	2.0	1.0
10:1	8.1	1.5	1.0
20:1	12.0	3.0	1.24
30:1	12.0	5.2	2.2
Total Magnesium present in 25ml of Solution = 12.16mgs.			

Table 8

had been adjusted above pH 9.0, and examined by paper chromatography (G.M.1). In addition, fructose solutions were adjusted to pH values of 9.0; 9.5; 10.0; 10.5; 11.0; 11.5, both in the presence and absence of ferric chloride. Samples were then chromatographed at $\frac{1}{2}$ hour; 12 hours; 36 hours; 72 hours and 120 hours.

All tests indicated that no degradative or other change, detectable with the silver nitrate reagent, had taken place at pH values up to about 10.5, as fructose was the only entity detected. At pH values higher than this, however, transformation took place fairly rapidly, the main product being glucose,,although saccharinic acids are also known to be produced under strongly alkaline conditions (83). The presence of iron appeared to inhibit the epimerisation to a certain extent.

These facts do not discredit the results obtained, as it has been adequately demonstrated that complex formation with subsequent precipitation inhibition occurs outwith these alkaline pH values. Moreover, it is the results obtained at these lower pH values which are of greatest interest to this study.

The foregoing experiments have indeed demonstrated that complexing between ferric iron and sugars can occur at pH values prevalent under natural conditions. Precise details of

the conditions of pH, etcetera , under which complexing takes place, have been provided. Such an effect has not been demonstrated with magnesium, however, as the precipitation of its hydroxide does not take place until a pH value in excess of those normally found in nature.

Isolation and analysis of the complex was next attempted, at the same time carrying out analysis of the precipitate obtained at pH 6.2. By doing so it was hoped to obtain an explanation for the different effects observed when fructose was added after precipitation, as opposed to addition before pH adjustment had taken place.

4. Isolation, purification and analysis of the complex formed between ferric iron and fructose.

The information detailed in Exp.No.4 demonstrated that the behaviour of ferric chloride with added sugar, can be divided into three distinct phases as the pH is increased.

i) From the commencement of alkali addition up to the precipitation point, a clear dark coloured solution is present.

ii) The precipitate.

iii) From the point of re-solution of the precipitate, with increase in pH, a clear dark coloured solution is again present.

The pH range over which each individual phase exists,

depends on the quantity of sugar present, and on the actual sugar used. Any study of the composition of the complex will require to include one aliquot from each phase.

Exp.No.12. Isolation, purification and analysis of the complex

Two series, each containing a different ratio of fructose to iron were used to determine the effect of the initial sugar concentration on the ultimate analytical figures procured. The procedure used for isolation, purification and analysis was the same for each phase sample.

Aliquots of 0.02 Molar ferric chloride were added to weighed amounts of fructose to give three samples with a 1:1, and three with a 10:1 fructose to iron molar ratio. One sample of each series was adjusted to pH 4.0, and maintained at this pH for five minutes to ensure that equilibrium had been reached. In a similar way, one sample of each was adjusted to pH 6.2. The remaining 10:1 sample was adjusted to pH 10.0, and the 1:1 sample to pH 12.0, so that both solutions were clear of precipitate. The resultant solutions were poured, with stirring, into ethanol, and ether added. The precipitates formed by this treatment were collected by centrifugation, washed three times with a 50:50 ethanol/ether mixture, once with anhydrous ether and dried, to provide dry, brown powdered samples of the six test solutions. Water was then added to each sample, and observations made (Table 9). Measurement of the pH of the solutions, or suspensions, indicated that they were

very close to the pH values at which they had initially been precipitated.

No.	Sample	Behaviour of isolated and purified samples on addition of water
1	1:1 pH 4	No redissolving
2	1:1 pH 6.2	No redissolving
3	1:1 pH 12	Redissolved immediately
4	10:1 pH 4	Redissolved immediately
5	10:1 pH 6.2	No redissolving
6	10:1 pH 10	Redissolved immediately

Table 9.

The conclusion may be drawn from these results that solutions 3, 4 and 6 have retained sufficient fructose to permit redissolving at their respective pH values.

All samples were then dissolved by adjustment to pH 1.4 with hydrochloric acid, so that the content of iron (G.M.3) and fructose (G.M.6) in each could be determined. After calculation of the number of milli moles of each substance present per ml, the molar ratios were worked out.

Table 10 summarises the results of two analyses carried out by the above procedure, essentially similar results being provided by further repetition.

The ultimate ratio of fructose to iron is clearly influenced by the initial sugar to iron ratio, and by the pH at

No.	Sample	1st Isolation Ratio $\frac{\text{Fructose}}{\text{Iron}}$	2nd Isolation Ratio $\frac{\text{Fructose}}{\text{Iron}}$
1	1:1 pH 4	1:11	1:11.8
2	1:1 pH 6.2	1:16.5	1:13
3	1:1 pH 12	1:4	1:3.5
4	10:1 pH 4	1:4	1:4.6
5	10:1 pH 6.2	1:4	1:3.86
6	10:1 pH 10	1:2.5	1:2.4

Table 10

which the complex was isolated. A closer association of fructose and iron exists at higher pH values, indicating that the complex will be more stable under such conditions. This finding presents agreement with the electrophoretic data (Exp. No.3) where it was found that greater mobility of iron was exhibited at higher pH values.

Comparison of the results in Table 9 and Table 10 indicates that a fructose to iron ratio of as little as 1:4 is sufficient to promote redissolving of ferric hydroxide at pH 4.0 ; pH 10.0, and pH 12.0. That is, that very little fructose is required to retain ferric hydroxide in a soluble form at pH values where it would normally be precipitated.

Although Saltman et al (54) isolated a complex from a 10:1 fructose to iron solution at pH 9.0, analysis of which suggested a 2 Fructose: 2 Iron relationship, no such definite

stoichiometric relationship was indicated in this study. However, repetition of the procedure provided relatively constant results, indicating that the ratios found were not mere chance due to incomplete washing of, or occlusion in, the precipitate. The only fact that can be stated with certainty is that fructose is bound to ferric hydroxide at pH values from 4 to 12, indicating that complex formation occurs over this range.

The samples adjusted to pH 6.2, before isolation, did not redissolve on addition of water. They did, however, exhibit the properties of fructose-iron complexes on pH adjustment. For example:-

The brown powder obtained on isolation of the 10:1 pH 6.2 sample was redissolved by addition of dilute hydrochloric acid. On subsequent addition of alkali, precipitation did not occur until pH 3.2, followed by re-solution of the precipitate at pH 12.0.

Clearly then, the previously noted behaviour of precipitates shed at pH 6.2 from solutions already containing sugar, as compared to solutions adjusted to this pH prior to sugar addition, is due to the fact that the precipitate in the former does not consist of pure ferric hydroxide. It is, in fact, an intimate association of ferric hydroxide and sugar. Moreover, the proportion of fructose present in this precipitate is fairly constant, each time the isolation is repeated. This

tends to rule out the possibility that sugar is present merely as an occlusion in the precipitate.

5. Further investigation of complexing between sugars and metals by a titration technique.

A brief summary of the principles on which this technique is based is given here, although a more detailed account has already appeared in the text. When complexing takes place between a metal and an organic ligand, weakly bonded acidic protons are released, resulting in depression of pH, a depressed titration curve being obtained. The extent of this depression is a measure of the stability of the complex, and by observation of the point at which divergence from the normal titration curve commences, the pH at which complexing becomes effective may be determined. This feature is demonstrated in conventional complex formation where a ligand combines with a metal ion, but no reference has been found stating that a similar feature is demonstrated when the ligand combines with the metallic hydroxide. Despite the fact that the results of the foregoing experiments have indicated that complex formation takes place between the sugar and the metallic hydroxide, it was decided to conduct a number of short experiments to investigate the titration behaviour of the complex.

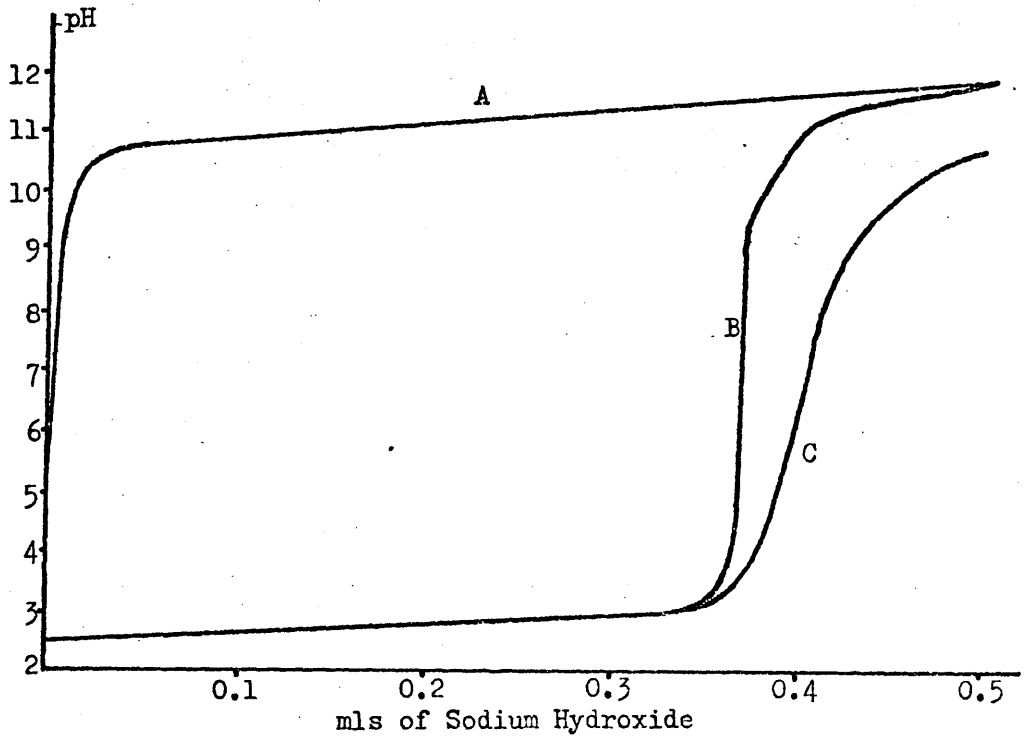
Solutions similar to those used in Exp.No.4 were slowly titrated with sodium hydroxide delivered at a constant rate from

an accurate syringe mounted in a motor driven micrometer assembly. This enabled slow and accurate addition of alkali to be made, and the quantity added could be measured. By changing the rate of turn of the micrometer, the rate of alkali addition could be altered if desired. The pH was recorded on a moving chart instrument, the graph produced representing pH on one axis, and volume of alkali added on the other. Since the syringe held only 0.5 mls of alkali, it was necessary to use 5 Normal sodium hydroxide to avoid irregularities in the titrations. By adjusting the rate of alkali addition to 22 microlitres per minute and stirring rapidly, smooth, entirely reproducible, curves were obtained.

Exp.No.13. Titration of ferric chloride and fructose, alone and mixed.

25 ml aliquots of 0.02 Molar ferric chloride with fructose added to give the desired ratio of sugar to iron were used, several titrations being carried out to ensure reproducibility.

Fig.10 shows the curves obtained for ferric chloride alone, fructose alone, and ferric chloride and fructose mixed to give a fructose to iron ratio of 20:1. The titration curve of mixed fructose and iron is depressed below that of either of the two entities when titrated separately, demonstrating, therefore, that complexing, of a sort accompanied by proton release, has taken place.



Comparison of titration curves

Curve A - Fructose alone (at the same concentration as in C)

Curve B - Ferric chloride alone

Curve C - Ferric chloride plus fructose (fructose: iron 20:1)

Figure 10

Comments upon the ferric chloride titration curve.

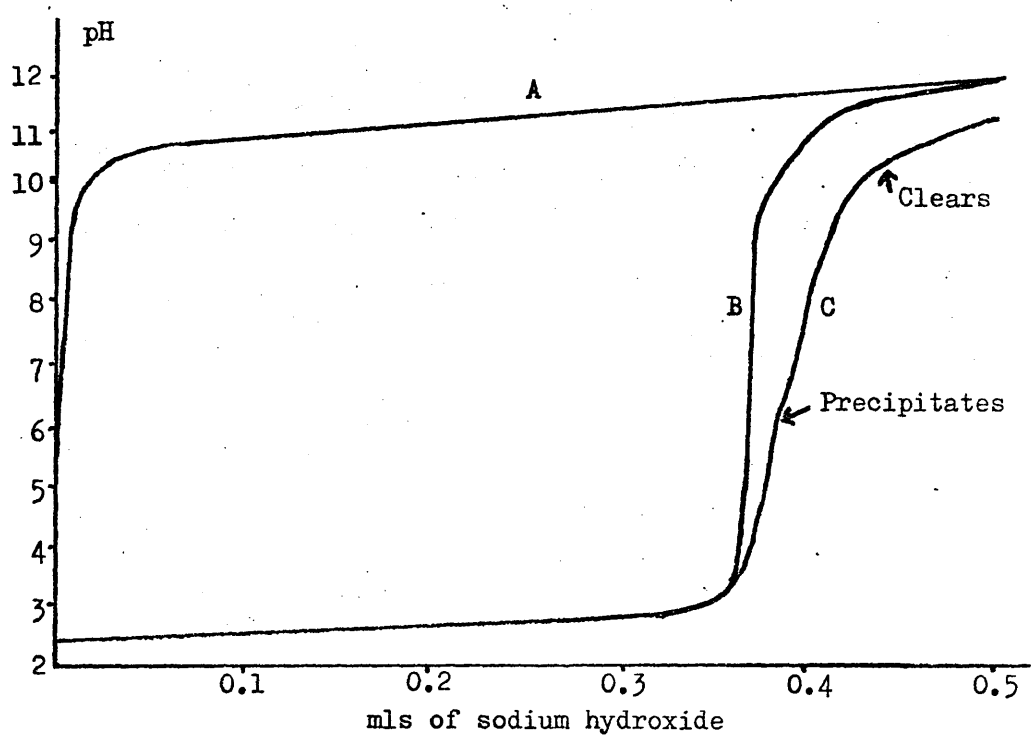
The long straight portion of the curve corresponds to the titration of the free acid present. Precipitation commenced at pH 2.7, followed by a rapid increase in pH.

Comments upon the ferric chloride/fructose titration curve.

This exhibited the same features as the ferric chloride curve up to pH 3.5, due again to titration of the free acid, and showed no evidence of complex formation. Above pH 3.5 however, the curve diverged from the ferric chloride curve, showing that acid was being produced. This phenomenon was consistent with a complexing effect. Complexing continued to take place up to the limit of the titration.

A similar titration was conducted with a fructose to iron ratio of 10:1 (Fig.11). In this case, the curve for mixed iron and fructose, although exhibiting divergence above pH 3.5, more closely resembled the ferric chloride curve than did the 20:1 fructose to iron solution. As had been previously demonstrated (Exp.No.4) a precipitate was shed at pH 5.6, and redissolved at pH 10.0, with a solution of this composition. Insufficient fructose was present to maintain all the ferric hydroxide in a soluble form, so that some of it had to be shed from solution. Consequently, the lesser quantity of complexed iron present produced the lower depression of pH noted.

It can be stated that complexing between fructose and ferric iron occurs over the entire pH range above pH 3.5, although



Comparison of titration curves

Curve A - Fructose alone (at the same concentration as in C)

Curve B - Ferric chloride alone

Curve C - Ferric chloride plus fructose (fructose: iron 10:1)

Figure 11

with lower ratios of fructose to iron the relative stability of the complex is fairly low.

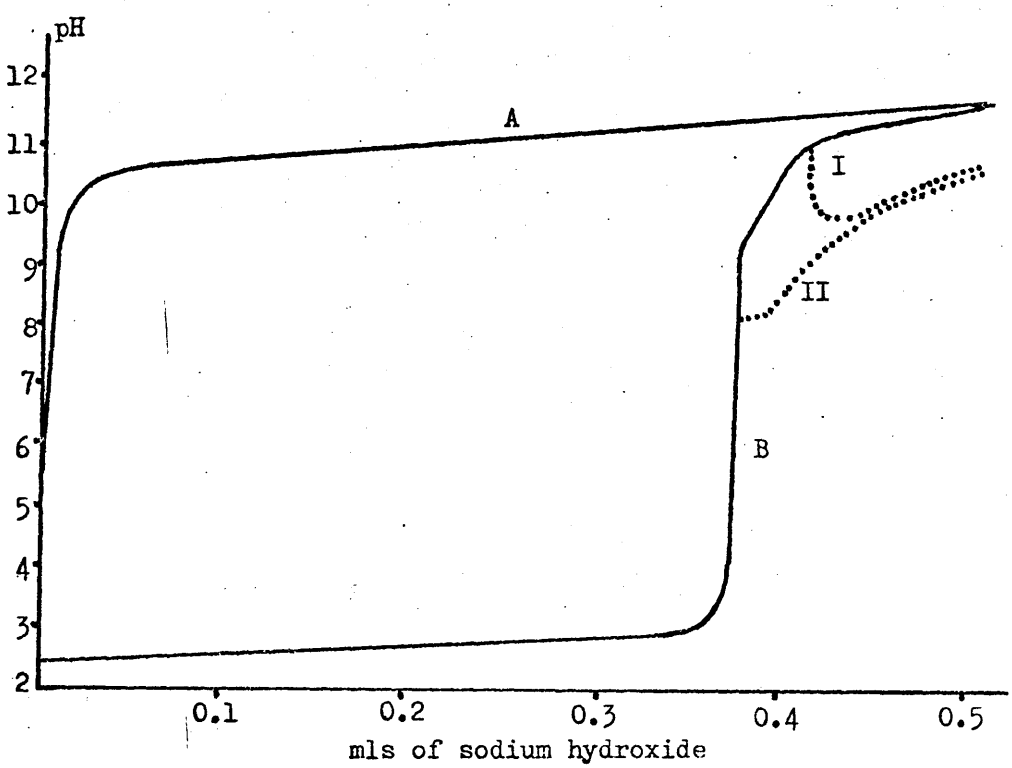
In some preliminary experiments, essentially the same results were obtained when titrations were carried out using glucose as the sugar.

Exp.No.14. Addition of fructose after precipitation of ferric hydroxide.

Although Exp.No.7 indicated that fructose does not promote the redissolving of ferric hydroxide if added after precipitation, this titration technique could provide information as to whether any complexing between fructose and ferric hydroxide takes place, as opposed to actual redissolving.

Aliquots of ferric hydroxide were taken as before and titrated with sodium hydroxide. Fructose, to provide a ratio of 20:1 was then added at various points during the titration. (Fig.12). When fructose was added at pH 11.0, a sudden pH drop occurred, the titration curve thereafter following the same course as the curve obtained when fructose was present before pH adjustment (Fig.10). When fructose was added at pH 8.0 the pH stopped increasing and thereafter followed the same course as that noted for the pH 11.0 addition.

In both cases, therefore, release of acidic protons immediately upon fructose addition was clearly demonstrated, and the extent of the pH depression was equivalent to that previously noted (Exp.No.13). This indicated that complexing between



Comparison of titration curves

Curve A - Fructose alone

Curve B - 0.02 M Ferric chloride alone

I Fructose added to give 20:1 ratio at pH 11.0
II " " " " " " " pH 8.0

Figure 12

fructose and precipitated ferric hydroxide does, in fact, occur, although no immediate dissolving action takes place. That it is easier to prevent a precipitate,than to redissolve it,by complexing agents has been noted by others, for example Smith (84).

The previous experiment demonstrated that divergence from the normal titration curve did not occur until a pH value at which iron would normally be present as its hydroxide. It therefore appears, from these last experiments, that complexing does not take place between sugar and the ferric ion, but between sugar and ferric hydroxide.

Exp.No.15. Titration of magnesium chloride and fructose, alone and mixed.

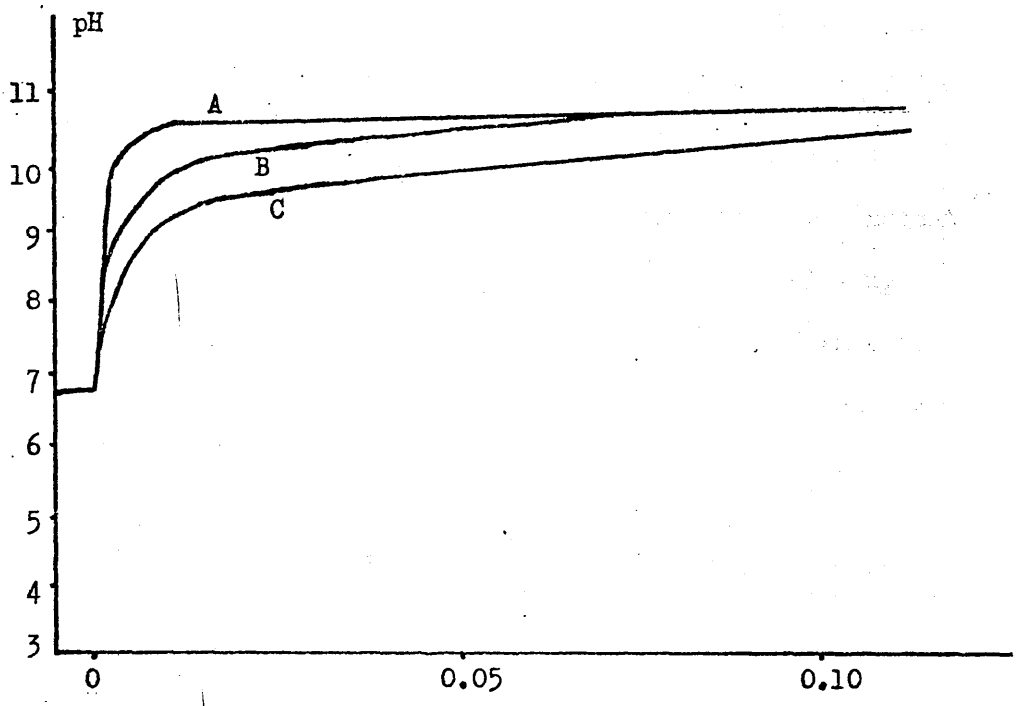
Using magnesium chloride exactly the same procedure was carried out. The results obtained (Fig.13) demonstrated that depression of pH occurred, although not to such a great extent as with iron. This effect was exhibited at pH values above 7.0 indicating that complex formation occurs above this point.

Exp.No.16. Attempted titration of ferrous sulphate.

It would have been of interest to obtain similar data for ferrous iron, but due to the rapidity with which oxidation took place, this proved impossible.

6. Summary.

The foregoing experiments have provided a considerable quantity of information regarding the formation of complexes between sugars and metals. Examination of precipitation



Comparison of titration curves

Curve A - Magnesium Chloride alone

Curve B - Fructose alone (at the same concentration as C)

Curve C - Magnesium chloride plus fructose (fructose: magnesium 20:1)

Figure 13

inhibition demonstrated that the most effective simple sugar was fructose, although fructosan was around six times more powerful as a complexing agent. In addition, it was shown that precipitation inhibition was effected by very small amounts of sugar, since the greatest individual increase in the pH at which precipitation occurred was observed with the first small increment of sugar. This series of experiments also indicated, as suggested by the electrophoretic data, that complexing occurred over a very wide pH range. This was confirmed by analysis of the isolated complex, where an isolable complex was found to exist at pH 4,0; pH 6.2 ; pH 10.0 and pH 12.0. Additional information was also supplied by the titration experiments, which demonstrated that complexing occurred from pH 3.5 upwards.

Although the precipitate shed at pH 6.2 when sugar was present could be redissolved either by pH adjustment into the alkaline or acid range, such an effect was not demonstrated when sugar additions were made subsequent to pH adjustment. Analysis of the precipitate shed in the presence of fructose, indicated that it consisted, not of pure ferric hydroxide, but of fructose and iron, presumably ferric hydroxide, in close association, thereby explaining the difference in behaviour. However, it was later shown (Exp.No.14) that although no redissolving effect was observed, complexing between fructose and precipitated ferric hydroxide did occur.

The results of the titration experiments indicated that complex formation was only apparent at pH values where iron would be in the form of its hydroxide, suggesting that complex formation occurred between sugar and the hydroxide, rather than between sugar and the ferric ion. This fact has been indicated by all the results, demonstrating a certain agreement with the results of the early workers, although the severely alkaline conditions believed to be necessary for complex formation are not required. The only contra-indication to this was the spectral change observed on addition of sugar to ferric chloride, which suggested that complex formation could take place between sugar and ferric ions, at low pH.

Complex formation between sugars and magnesium has also been demonstrated. Although this was only shown to occur at high pH values by the precipitation inhibition experiment, the titration data has shown that complexing is effective above pH 7.0, this being the pH at which divergence in the titration curves took place.

7. Discussion.

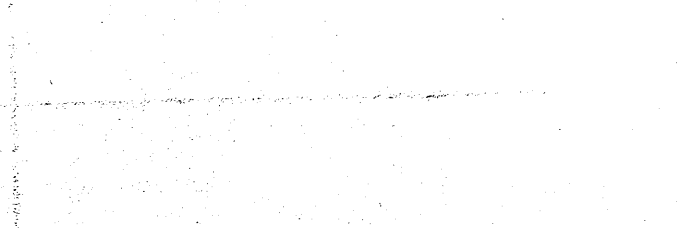
Although relatively high concentrations of sugar were required to inhibit the precipitation of ferric hydroxide over the entire pH range, it has been shown that small amounts of sugar are capable of exhibiting complexing phenomena. In a consideration of soil environments where large ratios of sugar to iron would not be found, this may be significant, in that small

quantities of sugar may act to keep iron in a soluble form, whereas normally, precipitation would occur. A similar phenomenon may be exhibited by fructosan, which is found in the soil (61, 105) and has been shown to be a more powerful complexing agent than any sugar tested.

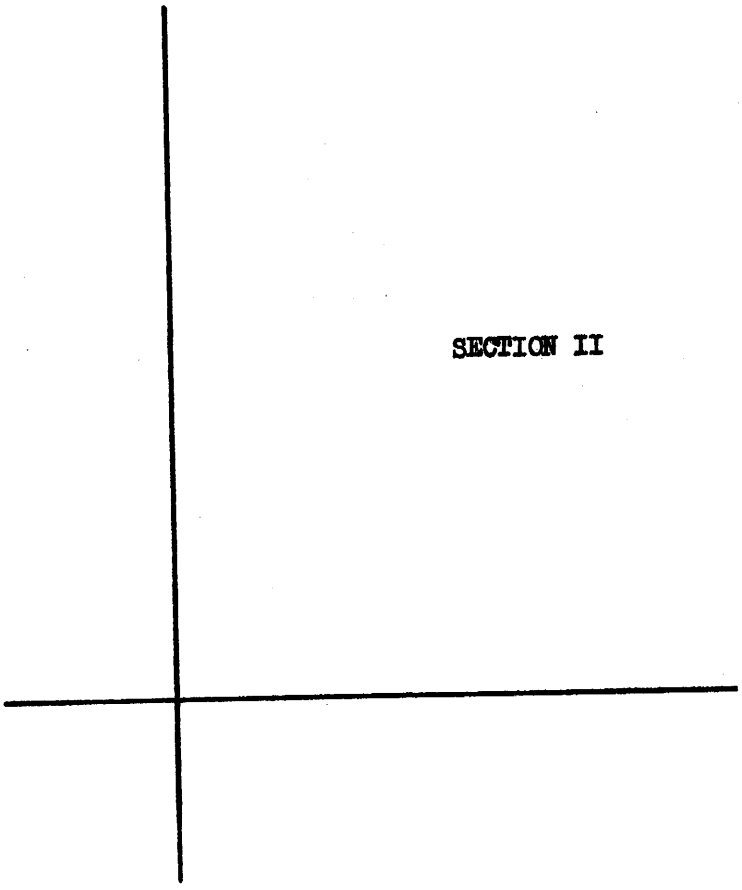
Although the action of sugars on precipitates of ferric hydroxide did not result in any immediate dissolving effect, the fact that complexing actually occurs between these entities may indicate some long-term dissolving action. This, of course, would be the type of conditions found in the soil, where a slow continual passage of carbohydrates down the profile could have a pronounced effect on the translocation of iron. Some preliminary experiments to test this possibility will be described in Section II. As the results have indicated, any dissolving effect at pH 6.2 will be minimal, so that experiments will require to be conducted above and below this value.

Unfortunately no results have been obtained for ferrous iron, and this form of iron may well be more important than the higher valency form. Many soils which exhibit spectacular iron translocation features contain a high proportion of their iron in the ferrous form, due to waterlogging and the accompanying anaerobic conditions. Some studies of the effect of sugars on the redissolving of ferrous iron will therefore be described later (Section II).

From the point of view of the effect of sugars on the mineral intake of animals, it seems unlikely that some effect will not be exhibited. Herbivores ingest large quantities of carbohydrates, especially fructosan, so that very high ratios of sugar to metal will be prevalent in the intestine. It has indeed been shown that high sugar to metal ratios have a pronounced effect on the behaviour of both iron and magnesium. That effects similar to those detailed above will be exhibited during dietary processes therefore seems likely, since complexing with magnesium has been shown to occur at pH values above 7.0, with similar slightly alkaline conditions present in the small intestine, where magnesium absorption takes place.



SECTION II



Section II. Examination of the capacity of sugars to promote the solution of various water-insoluble compounds.

1. Introduction

The ability of sugars to form complexes with metals under conditions normally found in natural environments, indicates that sugars may play a hitherto unappreciated role in natural processes. It is proposed to examine here the possibility that sugars present in the soil may have a solubilising action on some of the water-insoluble components of soils. Special emphasis is again placed on iron compounds, as there are a number of interesting soil features in which iron figures prominently.

This introductory subsection will be devoted to a brief description of these features of interest, and an account of previous research to determine the processes which lead to the formation of such features. For sugars to exhibit any pronounced dissolving effect in soils they must be present in adequate concentration. Thus an account of the level of carbohydrate material in soils is also included.

The main morphological features of interest pertaining to soil iron are characteristically associated with poor drainage conditions, often resulting in waterlogging or intermittent waterlogging of soils. These in turn result in reducing, or alternating reducing and oxidising conditions in the upper horizons of the soil. Crompton (85) has shown that podzolic soils, thin iron-pan soils, and gley soils can all be associated with such

conditions, the factors determining the type of soil being the severity of the poor drainage, and the topography of the region. Common to all three soil types is evidence of extensive mobilisation and translocation of iron under reducing conditions, with subsequent redeposition where more oxidising conditions prevail. Thus all soils exhibit evidence of considerable mineral loss in the upper horizons, with typical redeposition features at a lower level.

The view that translocation of sesquioxides occurred in the form of complex organic compounds was first proposed by Jones and Wilcox (86). They postulated that hydroxy acids are responsible for the solubilisation of the free oxides of iron and aluminium, which are then transported as complexes and finally precipitated as basic salts. Gallagher and Walsh (87) and Halvorson and Starkey (88) reached similar conclusions, the latter using fermented sugar as the iron dissolving agent, and suggesting that reprecipitation occurred due to bacterial assimilation of the ligand.

Bloomfield examined the ability of a number of organic agents to dissolve insoluble oxides of iron and aluminium. His work included a study of fermented and completely sterilised grass extracts (89) where, after 10 days, some 60 mgs of iron were dissolved per litre of extract by the former, and 8 mgs per litre by the latter. Much of the iron extracted became

reduced, and considerable fixation of the dissolved iron on ferric oxide occurred, with subsequent oxidation. These findings were discussed with respect to the gleying process, where in intermittently waterlogged soils the surfaces of structural elements become bleached due to the drainage water carrying plant decomposition products. The area of iron enrichment always found below such a horizon was thought to be due to fixation and oxidation as above.

Similar investigations were carried out using water extracts from Scots pine needles (90); Kauri leaves and bark (91); Rimu leaves (92); Larch leaves (93), and Aspen and Ash leaves (94). All extracts were treated with toluene to stop bacterial action, and all dissolved considerable quantities of iron and aluminium. The amount extracted was greater under anaerobic conditions than when oxygen was present. Similarly, less iron was dissolved at pH 7.0 than at lower pH values. However, the quantity dissolved under aerobic and neutral conditions was still considerable. In addition, Bloomfield found (95) that these iron solutions were strongly adsorbed onto ferric oxide; the greater the quantity of this present, the more rapid the decrease of iron in solution. Adsorption also occurred on aluminium oxide, kaolin and slightly on silica.

Bloomfield has therefore demonstrated a possible mechanism for the leaching of horizons under poorly drained conditions, and

also for the redeposition of iron and aluminium at a lower level. Other workers have conducted similar experiments using grass and leaf extracts (96, 97, 98, 99), all showing high solubilisation of iron.

The disadvantage of these experiments is that no attempt has been made to determine the entity in the extracts which was responsible for the dissolving action. Water extracts from leaves will contain a wide range of compounds, and any one of these could be responsible for the phenomenon. However, one of the most important water soluble constituents of plants, especially grasses, are carbohydrate materials, both monomeric and polymeric. Such substances would therefore be present in the water extracts used, and where care was taken to inhibit their breakdown by bacteria, they would be present in their original form. That part of the effect observed in these experiments might be due to the action of carbohydrate material is, therefore, not unlikely.

Reports have shown that soil carbohydrate levels are higher than may be at first supposed. Acton (100) reported levels of from 10 to 15 per cent of soil organic matter, and Gupta (101) levels of 12.6 to 16.1 per cent. Parsons and Tinsley (63) reported that carbohydrates make up at least 10 per cent of soil organic matter, rising to as high as 25 per cent in meadow soils. It is generally accepted that the proportion of carbohydrates in soil organic matter increases as the organic

matter content of the soil rises (62). In addition to the presence of monosaccharides in the soil (102), various workers have found considerable quantities of polysaccharides in soils of high organic matter content (103, 104). Such polysaccharides have included levans (61, 105), similar in structure to the fructosans of grasses.

The main primary source of soil carbohydrate matter is plant material, of which carbohydrates comprise more than 50 per cent of the dry matter. Micro-organisms are believed to act on this primary carbohydrate material, thereby synthesising the major part of the soil carbohydrate found in an aerated soil (106, 107, 108). However, Nykvist (109) has reported that when water soluble organic substances from leaf and litter are leached into the soil, sugars constitute a fair proportion of the material, and that their breakdown is decreased with lack of aeration. In addition, Kornev (110) has reported that the process of breakdown of polysaccharides and other carbohydrates decreases with depth. A further reference to factors which influence the bacterial breakdown of carbohydrates was given by Martin et al (111) who reported that breakdown decreased when these compounds were associated with inorganic ions.

Soil carbohydrate is, therefore, a mixture of primary carbohydrate and bacterially synthesised carbohydrate, the proportions of each varying with the conditions prevalent in the soil.

As with the grass and leaf leachates, soil fulvic and humic acids, which comprise over 50 per cent of soil organic matter (62), have been shown to possess considerable complexing ability, and to be capable of iron mobilisation (112, 113, 114). All reports agree that fulvic acids are more effective than humic acids in exerting this effect. In addition, Duchaufour (113) reports that mobile iron-fulvic acid complexes are abundant in the B horizons of podzols.

Fulvic acids are a complex mixture of compounds and the reports listed above made no attempt to isolate the entity or entities responsible for the dissolving action. However, more recent reports have demonstrated that humic and fulvic acids contain a fair proportion of polysaccharide material (115). Values of over 5.5 per cent have been obtained for fulvic acids, which contain a higher proportion of carbohydrates than humic acids (116). This in itself demonstrates a correlation between effectiveness as a complexing agent and content of carbohydrate. A number of reports published by Florjanczyk in 1965 (117, 118, 119) are of interest to this study. He separated so-called fulvic acids on a strongly basic anion exchanger, and found ferric-glucose compounds comprising an integral part of this soil organic matter fraction. They consisted of ferric-/βpolyglucose chelate rings, and were found only in the accumulation horizon of forest podzols. Also found was what was thought to be aluminium bound to glucose, which was

somewhat less stable than the corresponding ferric complex, and was found both in the bleached and accumulation horizons of forest podzols. These recent reports represent the first instances of sugar-metal complexes having been found in, and isolated from, the soils. Thus the suggestion that carbohydrates may have a pronounced effect on the mobilisation of iron does not appear to be as unlikely as may at first be thought.

The text of this section comprises an account of the investigation of the dissolving action of sugars on magnesium oxide, ferric hydroxide, and ferrous hydroxide, together with the ferrous containing minerals biotite, tremolite and siderite.

2. Investigation of the dissolving action of sugars on magnesium oxide.

Only a brief note on the action of sugars on magnesium oxide will be given here, since the conditions used were rather alkaline. One feature demonstrated by the experiment was, however, of interest. After shaking 1 gram aliquots of powdered Analar magnesium oxide with 25 mls of 1 per cent fructose and 25 mls of water respectively for 24 hours, little increase in the quantity of magnesium dissolved was noted; being 8.2 mgs in the former and 3.2 mgs in the latter, both at pH 10.0. However, the physical appearance of the remaining solids differed markedly in each case. The solid in the water blank still retained its original appearance, but that in the 1 per cent

fructose appeared to possess a greater volume, was slightly yellow in colour, and was slippery, almost gelatinous, to the touch.

Fructose determinations (G.M.6) carried out on the supernatant, and organic carbon determinations (G.M.7) on the solid, revealed that two thirds of the fructose present was tightly bound to the magnesium oxide, despite extended washing with water. This indicated that rather than dissolving the magnesium oxide, the fructose had been strongly adsorbed onto the powder, probably due to the fact that too great an excess of magnesium oxide over fructose was present, the molar ratio of fructose to magnesium being 1:18.

Thus a strong interaction between magnesium oxide and fructose has been demonstrated, although the quantity of magnesium dissolved by the sugar was slight.

3. Investigation of the dissolving action of sugars on ferric hydroxide.

The ability of sugars to dissolve precipitated ferric hydroxide was of interest, since the titration experiments demonstrated that complex formation between these two entities can occur. On the basis of earlier results, no rapid redissolving was expected, but some degree of slow solution on a long-term basis was considered to be a possibility.

The effect of fructose on ferric hydroxide was examined by means of a batch extraction technique, since the gelatinous

nature of the precipitate precluded the use of a column extraction method. Sugar was weighed out into glass bottles, followed by addition of a suspension of freshly precipitated ferric hydroxide, the pH of this having already been adjusted to the desired level by bubbling carbon dioxide. A small quantity of toluene was added to each bottle to prevent bacterial breakdown of the sugar. After being securely stoppered the bottles were shaken on an end-over-end shaker, samples being removed from time to time. After centrifugation, the iron content of the supernatant was determined (G.M.3) and the total quantity of iron dissolved, calculated.

Precipitation of ferric hydroxide

Freshly precipitated ferric hydroxide was used, as it is known that the precipitate exhibits certain ageing characteristics (120). The quantity of precipitate used was lower than that used in the magnesium oxide experiment, enabling ratios of fructose to iron from 1:1 to 30:1 to be employed without necessitating the use of very concentrated sugar solutions. Ferric hydroxide was precipitated from a 0.05 Molar solution of ferric chloride by a slight excess of sodium hydroxide, and this resulting suspension diluted ten times with water. This procedure was adopted, as Mellor (121) reports that gelatinous ferric hydroxide is thrown down when alkali is added to a ferric salt solution which is not too dilute. After dilution 13.96 mgs of iron were present as ferric hydroxide in

each 50 ml aliquot of the suspension. As stated pH adjustment was accomplished by bubbling carbon dioxide through the suspension after dilution. The suspension was kept uniformly distributed in the mother liquor by means of a rapidly spinning magnetic stirrer, pipetted into the shaking bottles and treated as previously detailed.

Exp.No.17. Dissolving action of sugars on ferric hydroxide.

Initially only two ratios of fructose to iron were tested, determining the quantity of iron dissolved at pH 4.0, pH 7.0, and pH 10.5, after 1 hour and 16 hours of shaking. The results (Table 11) show that the relative quantities of iron dissolved at these pH values are in agreement with previous findings, in that more iron was dissolved at pH 10.5 than at pH 4.0, with no iron dissolved at neutrality. Although the actual quantity of

	Gamma of iron in solution in the total volume of 50 ml		
Sample	No Sugar	10:1 Fructose	30:1 Fructose
pH 10.5 1 hour	0	960	950
pH 10.5 16 hours	0	1,800	1,470
pH 7.0 1 hour	0	0	0
pH 7.0 16 hours	0	0	Trace
pH 4.0 1 hour	150 ?	140	150
pH 4.0 16 hours	0	350	550

Table 11.

iron dissolved in the presence of sugar was small, it represented a considerable increase on that dissolved by the water blank. Little difference in the quantity of iron dissolved by the two fructose ratios was detected.

Chromatograms run on the high pH samples indicated that no alkaline transformation of fructose had occurred after 1 hour, but there was some evidence of very slight epimerisation after 24 hours, as evidenced by the appearance of a trace of glucose.

Exp.No.18. Dissolving action of a range of sugar concentrations on ferric hydroxide at pH 10.0

The procedure used in this experiment was similar to that previously detailed. Although the pH of the suspension was originally adjusted to pH 10, it showed a distinct drop when measured immediately after addition to the sugar-containing bottles, the extent of this drop increasing with the quantity of sugar present (Fig.14). This was similar to the effect noted during the titration experiments (Exp.No.14) and was thought to be due to the immediate formation of a complex, with accompanying release of acidic protons.

Aliquots removed from the shaking samples after 4 hours, 2 days, 3 days, 5 days and 10 days respectively, were then centrifuged and the iron contents determined (Table 12). As before, the actual quantity of iron dissolved by fructose was not large, but the figures obtained indicated that the presence

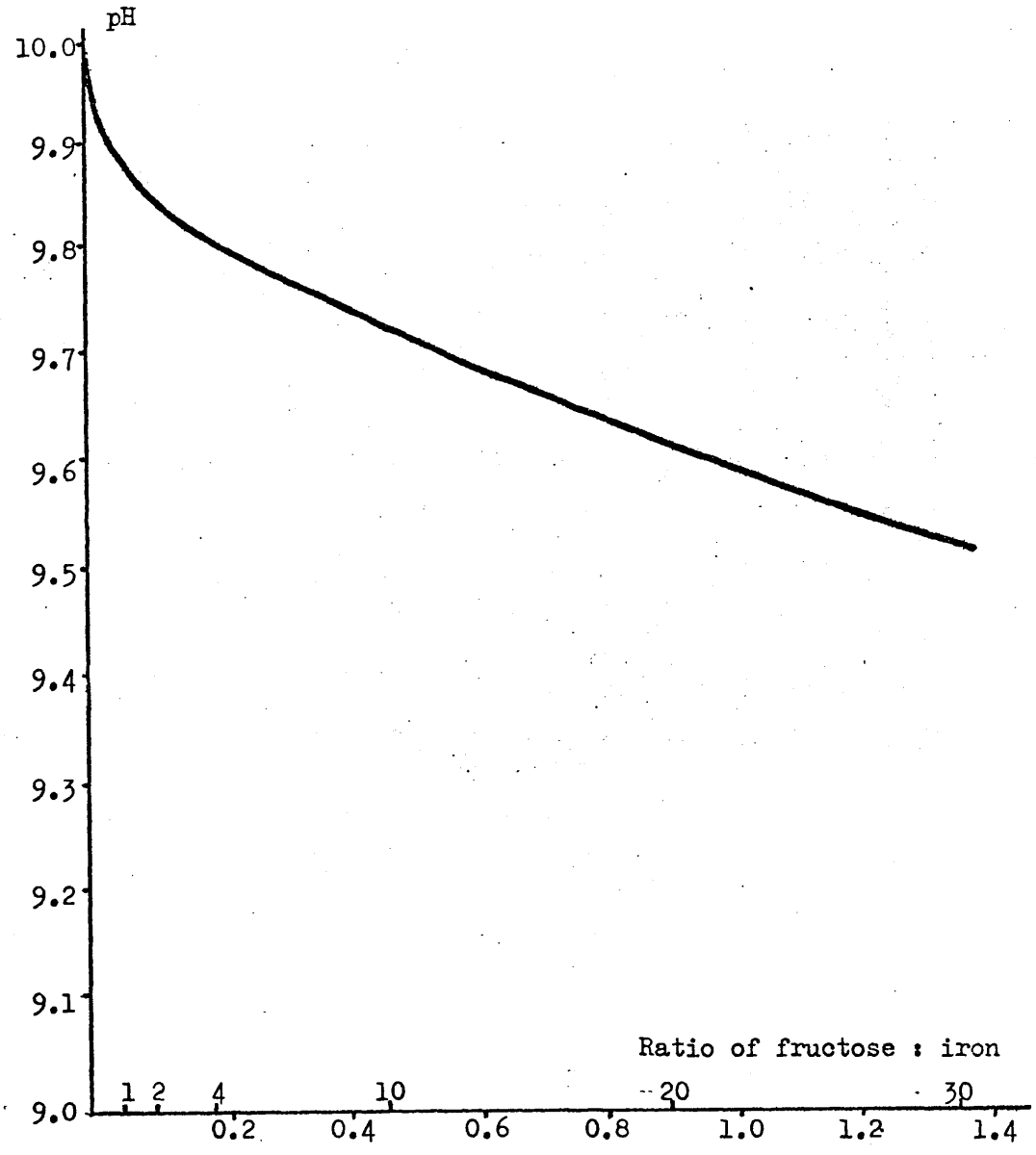

pH
10.0
9.9
9.8
9.7
9.6
9.5
9.4
9.3
9.2
9.1
9.0
Ratio of fructose : iron
1 2 4 10 20 30
0.2 0.4 0.6 0.8 1.0 1.2 1.4
weight of fructose added to 50 ml aliquot
of a suspension of ferric hydroxide

Figure 14

	gamma of iron dissolved in total volume of 50 ml at various time intervals				
Ratio Fructose / Iron	4 Hours	2 Days	3 Days	5 Days	10 Days
No Fructose	0	0	0	0	0
1:1	130	350	600	520	300
2:1	200	300	520	670	300
4:1	160	330	480	670	850
10:1	170	330	560	900	1,100
20:1	170	420	550	1,250	850
30:1	190	360	550	1,000	1,500

Table 12

of sugar considerably enhanced the solution of ferric hydroxide, since no iron whatsoever was dissolved in the water blank. Although the quantity of iron dissolved tended to increase with the duration of shaking and with increase in sugar to iron ratio, this effect was not consistently demonstrated.

Previous to iron determination of each aliquot removed from the samples, the pH was measured. It was found that the pH of the sugar-containing samples fell steadily and slowly throughout the course of the experiment, to a value of around pH 8.0 after 10 days. The pH of the water blank demonstrated a lesser fall to pH 9.3, indicating that part of the fall could be attributed to a naturally occurring process, possibly carbon dioxide interference. The increased pH fall in the sugar-containing solutions was likely to be due to continued slow release of acidic protons from complex formation. Chromatography indicated that no fructose breakdown had occurred, ruling out the possibility of acidic degradation products inducing the pH fall.

The experiment has shown that low concentrations of fructose (Table 13) promote the redissolving of insoluble ferric hydroxide over the pH range 8.0 to 10.0, although the total quantity dissolved is not large.

Exp.No.19. Dissolving of ferric hydroxide at lower pH values.

In this experiment the pH of the ferric hydroxide suspension

was adjusted to pH 5.8 with carbon dioxide before shaking. Aliquots were removed from the bottles after 4 hours, and after 3 days' continuous shaking they were centrifuged, and the iron content of the supernatants determined (Table 14).

Ratios of fructose:iron used and equivalent concentration of fructose in 50 ml samples.	
Ratio $\frac{\text{Fructose}}{\text{Iron}}$	Percentage Concentration of fructose
1:1	0.09
2:1	0.18
4:1	0.36
10:1	0.9
20:1	1.8
30:1	2.7

Table 13

Again, on measurement of the pH, a downward pH movement was observed during the course of the experiment, falling from the initial value of pH 5.8 to pH 5.2 after 3 days. This may be the reason for the observation that no iron was dissolved during the first 4 hour shaking period, in that the pH was too close to the 'critical' value of pH 6.2. As the pH fell during the shaking period, however, conditions more conducive to dissolving

would be promoted, and a small quantity of iron did in fact come into solution.

	Gamma of iron dissolved in total volume of 50 ml	
Sample	4 Hours	3 days
Blank	0	0
1:1	0	-
2:1	0	400
4:1	0	480
10:1	0	600
20:1	Trace	750
30:1	Trace	850

Table 14

The level of iron present in the supernatants showed a consistent increase as the ratio of sugar to iron increased (Fig. 15). With the first small sugar increment the amount of dissolved iron rose rapidly, thereafter increasing more slowly as the sugar level rose. This effect was similar to that noted in Exp.No.4, where the first small sugar addition resulted in a large increase in the pH of precipitation, with correspondingly lower increases for further sugar increase. This was, however, the only occasion during the ferric hydroxide dissolving experiments, that such a feature was exhibited.

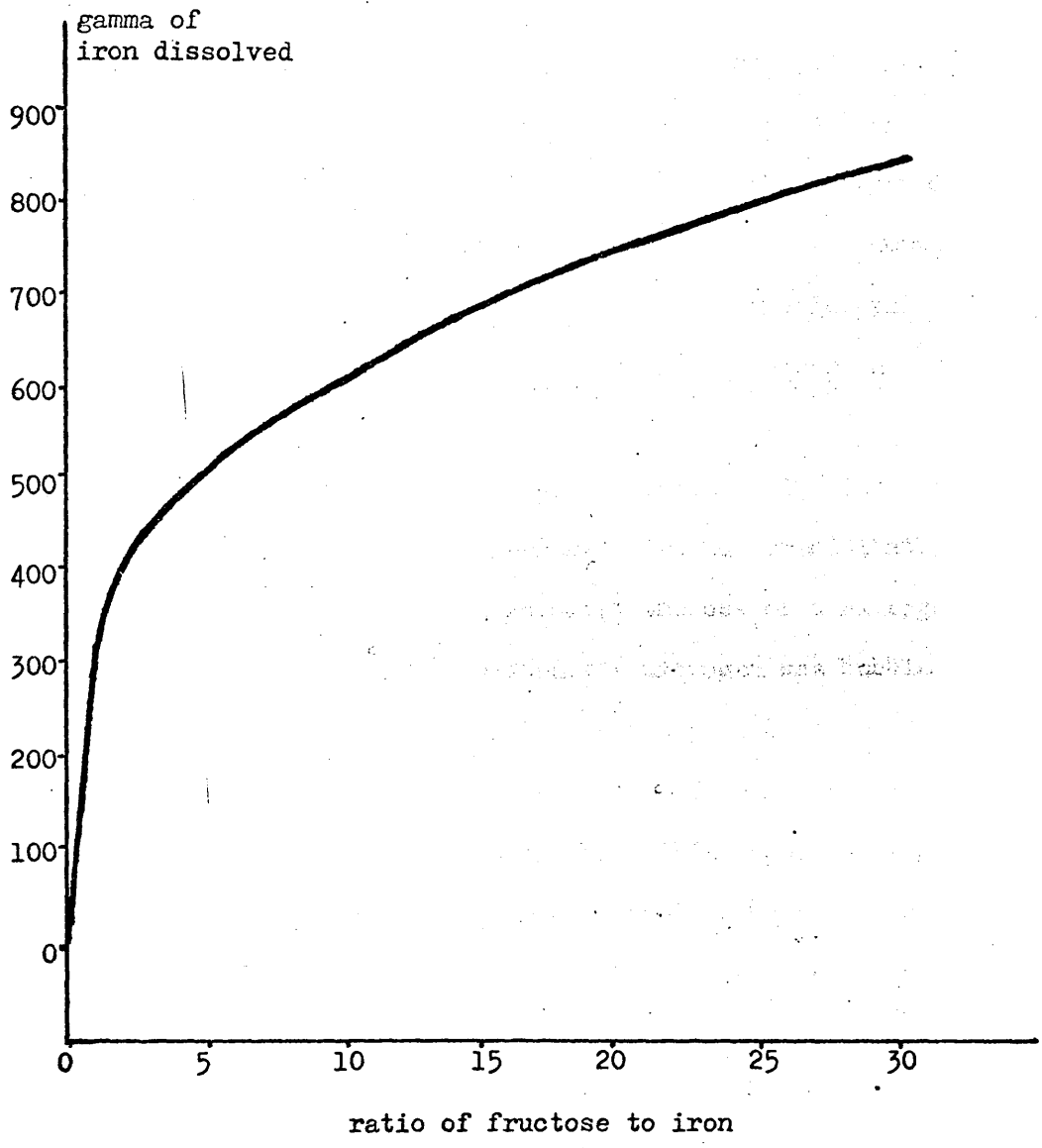

gamma of
iron dissolved
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
ratio of fructose to iron

Figure 15

The conclusion may be drawn from these experiments that the presence of sugar does indeed promote the solution of an otherwise insoluble precipitate of ferric hydroxide. This redissolving effect was slight and slow, as predicted from the results obtained in Section I. In addition, the pattern of redissolving, that is the pH values at which it will occur, follows that predicted from the same earlier experiments.

4. Investigation of the dissolving action of sugar on ferrous hydroxide.

Numerous attempts were made to obtain conditions which limited the oxidation of the ferrous hydroxide precipitate. One attempt to exclude oxygen involved the use of a nitrogen-filled plastic tent, but even when the nitrogen was bubbled through alkaline pyrogallol, sufficient oxygen to promote oxidation still remained in the system. Eventually the procedure set out below was followed. This technique limited the amount of oxidation considerably, but was by no means perfect, as the degree of oxidation of the precipitate varied in each series of extractions, so that the results were not truly comparable.

Procedure

Two litres of water were boiled to remove dissolved oxygen and subsequently chilled by immersion in ice-water, while carbon dioxide was continually bubbled through it. A 0.05 Molar solution of ferrous sulphate was made by dissolving 1.39 grams

of Analar ferrous sulphate in 100 mls of boiled, cooled, water in a chilled beaker from which the air had been displaced by a stream of carbon dioxide. Ferrous hydroxide was precipitated by addition of an excess of sodium hydroxide (0.8 grams) while maintaining rapid stirring and a continuous stream of carbon dioxide onto the surface of the solution. 50 mls of the resultant heavy, slightly blue/green suspension was then pipetted into 450 mls of treated water, again in an ice-water bath with carbon dioxide blowing on to the surface. pH adjustment of this rapidly stirred suspension, containing 13.96 mgs of iron per 50 mls, was effected by addition of a small quantity of sodium hydroxide, or by bubbling in a little carbon dioxide. 50 ml aliquots were then removed and added to bottles containing weighed amounts of sugar from which the air had been removed by a stream of nitrogen, nitrogen being bubbled through the suspension during pipetting. The bottles were then capped with plastic stoppers, using two sheets of polythene film to improve the seal.

The samples were then shaken on an end-over-end shaker, restricting the time of shaking to one hour to limit the extent of oxidation occurring in the bottles. After shaking, the contents of the bottles were centrifuged, an aliquot of the clear supernatant taken, and the quantity of iron in solution determined.

During the development of this procedure it was observed

that ferrous hydroxide was dissolved to a greater extent by sugar, than ferric hydroxide. As before, fructose was the most effective sugar, followed by glucose, in turn followed by sucrose. In addition to dissolving iron, it was found that the presence of sugar tended to maintain the ferrous hydroxide precipitate in a more reduced state than if it were absent. That this was not due merely to the effect of reducing sugars was ascertained by the use of a non-reducing sugar, sucrose. Once again, the ferrous hydroxide showed a lesser tendency to oxidise than in the absence of sugar. A likely explanation for such an effect is that this is a further manifestation of the complexing effect of sugars.

If the extracted and centrifuged sample, containing dissolved ferrous hydroxide, was allowed to stand exposed to the atmosphere, a heavy precipitate of ferric hydroxide began to sediment out. Thus, although sugars are capable of assisting iron to remain in the reduced state where the conditions are fairly reducing, they are not capable of doing so in an oxidising environment. A similar effect was noted by Bloomfield in the experiments conducted on leaf extracts (92), a considerable proportion of the dissolved iron being shed as a precipitate under oxidising conditions. The significance of these observations will be discussed at a later point in the text.

Exp.No.20. Dissolving of ferrous hydroxide by sugars.

Fructosan was used in addition to fructose, no other simple sugars being used, as they had been proved to be less effective than fructose in the preliminary experiments. As was explained earlier (Exp.No.9) fructosan concentration, for the purposes of calculating the ratio of polysaccharide:iron may be expressed either on its fructose content or, alternatively, upon its total hexose content. For the sake of simplicity in what follows here, fructosan ratios and concentrations have been calculated on a total hexose basis. Table 15 demonstrates the relationship of these ratios and concentrations.

Total Hexose		True Fructose	
Ratio $\frac{\text{Fructosan}}{\text{0.005M Iron}}$	Percentage Concentration	Ratio $\frac{\text{Fructosan}}{\text{0.005M Iron}}$	Percentage Concentration
1.554:1	0.14	1.364:1	0.123
3.108:1	0.28	2.728:1	0.246
7.77:1	0.70	6.82:1	0.615
15.54:1	1.40	13.64:1	1.230

Table 15

As already stated, the oxidation/reduction state in each extraction could not be maintained constant. The degree of

oxidation of each extraction, as judged by eye, is therefore given, on a relative basis, along with the tabulated results of the quantity of iron dissolved. The amount of iron dissolved in four such extractions is summarised in Table 16, the degree of oxidation ranging from virtually fully oxidised (Series 1) to almost totally maintained in the reduced state (Series 4). The values obtained for Series 2, 3 and 4, all carried out at the same pH value, indicate that the extent of oxidation of the sample determined the quantity of iron dissolved; the less oxidised the sample the more iron dissolved. The values exhibited such a wide variance, dependent solely upon this oxidation/reduction state, that it was impossible to determine the effect of pH variation on the quantity of iron dissolved. Thus the results obtained in Series 1 represent the effect of increased oxidation, rather than the effect of increased pH, on the amount of iron dissolved by fructose and fructosan.

Further repetition of ferrous hydroxide extractions produced results ranging from the lowest to the highest value in Table 16, depending on the success achieved in preventing oxidation. Various pH values were tested, but the fact emphasised in every case was that the state of oxidation was the overiding factor on which the quantity of iron brought into solution depended. Similarly iron 'concentrations' from 0.005 Molar to 0.020 Molar were used without any significant

	Decreasing Oxidation →			
Sample	Series 1	Series 2	Series 3	Series 4
	gamma of iron dissolved in total volume of 50 ml			
Blank	93.0	93.6	93.5	62.5
2:1 Fructose	93.0	157.5	250	781
4:1 "	157.5	657.5	375	969
10:1 "	231.0	720.0	375	1,437
20:1 "	287.5	720.0	688	2,219
30:1 "	350	407.5?	844	2,919
1.554:1 Fructosan	-	-	-	5094
3.108:1 "	93.0	1,968	3,156	6,313
7.77:1 "	157.5	1,940	4,062	7,000
15.54:1 "	475	1,094	4,062	-
	pH 8.2	pH 6.8	pH 6.8	pH 6.8

Table 16

differences becoming apparent. The fact that less iron was dissolved in the more oxidised samples further reinforced the results obtained for the dissolving of ferric hydroxide (Section III. 3.)

Table 17 summarises the results of a further two extractions, where the features outlined above are again demonstrated. In all the experiments conducted, the fructosan once again proved to be an exceedingly effective solubilising agent, even in low concentrations, dissolving far greater quantities of iron than its monomer. As an example, a 7.77:1 ratio of fructosan to iron dissolved almost 50 per cent of the iron available, (Series 4) whereas fructose used in a similar ratio, dissolved only around 10 per cent. (Series 4) This presented approximate agreement with the relative effectiveness of the two sugars, in inhibiting the precipitation of ferric hydroxide. (Exp.No. 9.)

Once again, when exposed to the atmosphere, the iron solutions obtained during the above experiments, shed iron, presumably as a precipitate of ferric hydroxide. Fig.16 demonstrates the effect obtained when a 7.77:1 fructosan to iron extract is :a), exposed to the atmosphere for some time; ,b) exposed to the atmosphere for a short time and then resealed and c) kept in a sealed vessel with no air allowed to enter. All solutions were kept for a period of three weeks before being photographed. It can be seen that the action of fructosan in

	Decreasing Oxidation →	
Sample	Series 5	Series 6
	gamma of iron dissolved in total volume of 50 ml.	
Blank	31.2	156 ?
2:1 Fructose	156	156
4:1 "	106	156
10:1 "	125	250
20:1 "	437	563
30:1 "	969	3,463
1.554:1 Fructosan	907	4,563
3.108: "	2,094	7,000
7.77:1 "	5,125	7,656
	pH 8.0	pH 7.4

Table 17

dissolving ferrous iron,and maintaining it in a soluble form, is a powerful one under reducing conditions. In addition, the effect of oxygen on the behaviour of the solution can be observed. Similar behaviour was observed in all other sugar extracts.

It has been shown that sugars act on ferrous hydroxide to produce large quantities of soluble iron, exposure to oxidising conditions resulting in the subsequent redeposition of the greater proportion of this dissolved iron. This represents a possible mechanism whereby iron is mobilised under reducing conditions, and immobilised under oxidising conditions, a similar feature to that exhibited in podzols and thin iron-pan soils. By investigating the behaviour of the iron solutions with other entities normally found in such soils, further mechanisms for iron immobilisation and redeposition may be found.

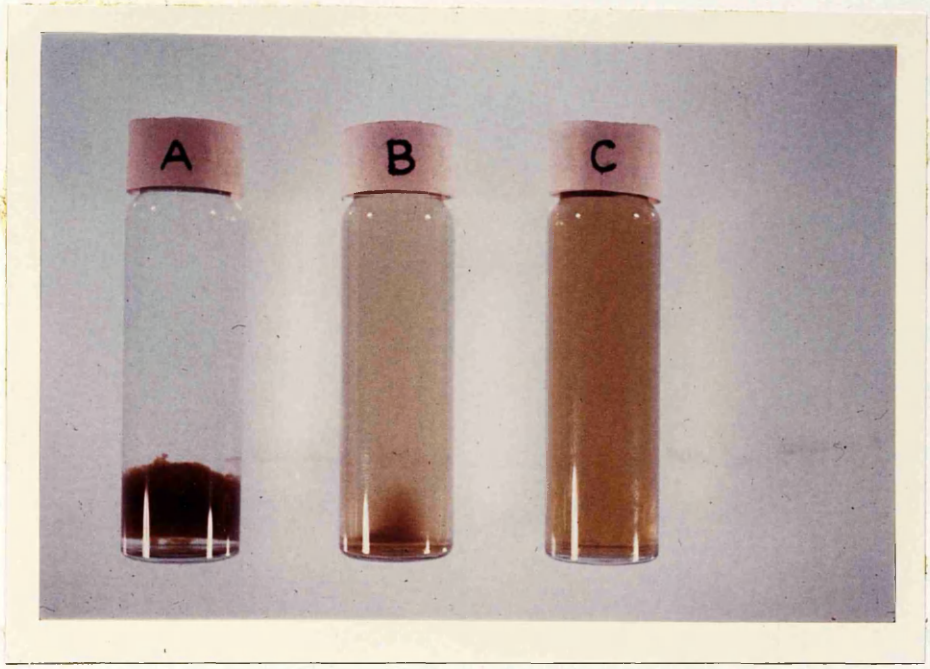
Exp.No. 21 Further examination of the behaviour of the iron solutions obtained on extraction of ferrous hydroxide with fructose and fructosan

The solid materials used in this investigation of the adsorption of dissolved iron, were 1 gram quantities of:-

a) Bentonite clay particles

b) Soil taken from the B horizon of a podzol

c) A red precipitate collected from the drainage water of a poorly drained field, which appeared to consist mainly of ferric hydroxide, and which when suspended



The effect of oxidation on an iron solution obtained after extraction of ferrous hydroxide with fructosan at pH 6.8.

A - exposed to the atmosphere for some time

B - exposed to the atmosphere for a short time and then resealed.

C - No exposure to the atmosphere. Maintained in a sealed condition throughout.

Figure 16

in water gave a pH value of 6.3.

d) Recently oxidised ferrous hydroxide.

The iron solutions were obtained by conducting a bulk extraction of ferrous hydroxide with 30:1 fructose and 7.77:1 fructosan. A water blank was also carried out. 50 ml aliquots of these solutions were added rapidly to one gram amounts of the above mentioned solid in bottles from which the air had been displaced with nitrogen. After sealing and shaking for 1 hour, the samples were centrifuged, and the iron remaining in the supernatants determined. (Table 18) A 50 ml aliquot with no addition of solid material was shaken for the same time to allow for any change that might have occurred to the solution as the result of the brief exposure to the atmosphere during pipetting.

Extensive adsorption on the various solids present occurred, the extent of the loss of iron from the solution being detailed in Table 19. The extent of adsorption was far greater in the case of the fructose-iron solutions than the fructosan-iron solutions, both on a quantitative and a percentage basis. Thus, in addition to dissolving less iron than fructosan, fructose more readily loses the iron which it has solubilised.

The extent of adsorption was seen to be in the order:-

Oxidised ferrous hydroxide $>$ Bentonite $>$ Soil $>$ Precipitate from drainage.

Thus, a series of agents capable of adsorbing and

Treatment	Fructosan		Fructose		Water Blank	
	gamma in 50 mls	pH	gamma in 50 mls	pH	gamma in 50 mls	pH
Original	7,580	7.15	5,187	7.05	, 31.2	6.95
Shaken no Addition	7,455	7.15	5,031	7.05	31.2	6.95
Bentonite	3,082	6.95	218	6.95	31.2	6.8
Sail	4,165	7.05	250	6.95	31.2	6.95
Precipitate from drainage	4,998	7.1	563	7.1	31.2	7.05
oxidised $Fe(OH)_2$	2,665	7.1	156	7.1	31.2	7.0

Table 18

Treatment	Fructosan		Fructose	
	Loss in gamma	Loss per cent	Loss in gamma	Loss per cent
Bentonite	4,768	62.9	4,968	95.7
Soil	3,685	48.6	4,937	95.1
Precipitate from drainage	2,582	34.0	4,624	89.1
Oxidised $Fe(OH)_2$	4,915	64.8	5,031	96.9

Table 19

immobilising have now been shown to exist. An extension of the above experiment was conducted in an attempt to construct a model iron-pan in a column. Bentonite was ideal for this purpose, as it had been shown to adsorb iron strongly. Being non-coloured, any adsorption of iron followed by oxidation would result in the appearance of a red-brown colouration.

Exp.No. 22. Construction of a model iron pan.

A column was set up, introducing firstly a quantity of acid washed sand, followed by a mixture of celite and bentonite, then a further quantity of acid washed sand. The sand was used as a non-adsorbing support medium for the celite/bentonite mixture. Mixed celite and bentonite was used, to increase the permeability of the column. However, the very nature of the two solids, resulted in the rapid sedimentation of the celite, followed by the bentonite. Thus the bentonite formed a very thin band on top of the celite, the latter, therefore, acting in virtually the same way as the sand, by providing a physical supporting medium. All constituents were introduced to the column as a slurry, made up in previously boiled and cooled water, so that reducing conditions prevailed.

A 5 ml aliquot of the fructosan-iron solution used in the previous experiment, was introduced to the top of the column and allowed to percolate slowly through. A water wash followed this addition. The column was then allowed to run dry and air drawn through by means of light suction for a short time.

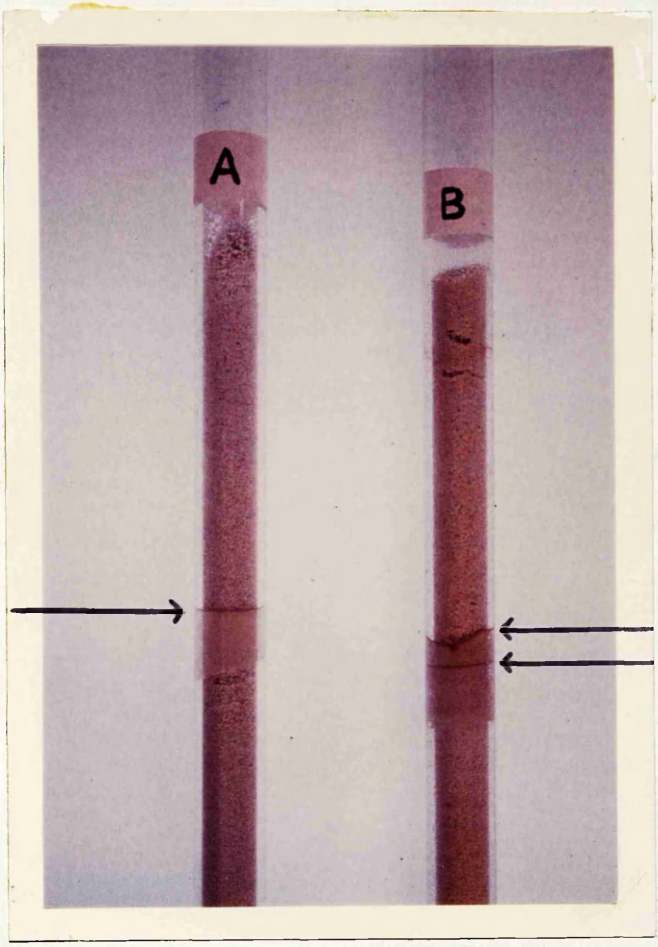
This procedure was repeated a number of times, representative of a rising and falling water table; that is reducing conditions, followed by oxidising conditions. A thin reddish brown band gradually formed at the bentonite layer. The gradual build-up was probably due to the initial adsorption of the sugar-iron complex on the bentonite, followed by oxidation. Further adsorption on the ferric iron thereafter present, then followed.

Figure 17 shows the type of effect exhibited. Column A has one layer of bentonite present, and Column B has two layers, this being the explanation for the presence of two iron-deposition layers in the latter. (arrowed)

Although this was an oversimplified representation of the conditions present in soils, it nevertheless indicated the behavioural pattern of sugar-iron complex solutions. If ferric hydroxide, or podzol soil from horizon B had been used in place of bentonite, a similar picture would have been expected, but any increase in the iron present, could not have been easily observed. In soils the quantity of adsorbent material would far outweigh the quantity of iron-sugar complex, so that adsorption would be more complete.

5. Investigation of the Dissolving Action of Sugars on the Ferrous Minerals, Biotite, Tremolite and Siderite.

Although ferrous iron is present in soils, it would not be expected to exist in such a form as ferrous hydroxide. So



The effect of repeated addition of fructosan - iron aliquots with subsequent aeration of the column.

A - acid washed sand with one layer of celite/bentonite.

B - acid washed sand with two layers of celite/bentonite.

Figure 17

that the results described earlier with ferrous hydroxide could be related directly to natural conditions, samples of the naturally occurring ferrous minerals biotite, tremolite and siderite were used as substrates for sugar extraction. The following is a very brief description of the chemical form of these minerals.

Biotite is a member of the mica group, in which substitution of magnesium by ferrous iron occurs. It is of complex structure and may be represented by the formula:

$$(OH)_4.K_2.(Mg.Fe^{2+})_6(Al_2Si_6)O_{20}$$

Tremolite is another silicate mineral of complex structure, represented by the formula:

$$Ca_2(Mg.Fe^{2+})_5 \quad Si_8O_{22} \quad (OH)_2$$

Again, substitution of magnesium by ferrous iron is found, replacement to a greater extent than 50 per cent being most uncommon (219).

Siderite is the simplest mineral of the three. It is composed of ferrous carbonate, although substitution of iron by magnesium is common.

Analytical data for the sample of siderite was available, giving:-

iron - 34.6 per cent: a total of 346 mgs in 1 gram

magnesium - 0.85 per cent: a total of 8.5 mgs in 1 gram

Exp.No.23. Dissolving of biotite, tremolite and siderite.

5 grams of each finely powdered sample were suspended

in previously boiled and cooled water, the pH adjusted with either dilute sodium hydroxide or hydrochloric acid, and the volumes made up to 250 mls. 50 ml aliquots were pipetted into bottles containing weighed quantities of sugar to provide the desired sugar concentrations. Nitrogen bubbling accompanied this addition to inhibit oxidation. After shaking on an end-over-end shaker, samples were removed periodically and centrifuged. Iron and magnesium analyses were carried out on aliquots of the supernatant as detailed in G.M.3 and 4.

The quantity of iron dissolved on extraction at pH 8.0 (Table 20) was enhanced by the presence of sugar, although to a lesser extent than was observed on extraction of ferrous hydroxide. Only in the case of siderite was there any appreciable increase. Fructose, although previously shown to possess considerable complexing and dissolving powers with iron, was singularly ineffective here, even at a concentration of 2.7 per cent. No increase in the quantity of iron dissolved with time was noted.

In the case of magnesium (Table 21) the solubilising effect of sugar was seen to be far more extensive. Again, little increase with time was observed, and again fructose was rather a poor dissolving agent. Fructosan, however, dissolved a considerable quantity of magnesium from all three minerals.

	gamma of iron dissolved from 1 gram of mineral at intervals of time			
Sample	1 Hour	4 Hours	12 Hours	24 Hours
Biotite				
Blank	438	350	350	365
2.7% Fructose	523	410	450	525
0.14% Fructosan	541	387	450	588
0.70% Fructosan	733	612	695	708
Tremolite				
Blank	125	100	148	143
2.7% Fructose	125	125	148	183
0.14% Fructosan	143	190	148	243
0.70% Fructosan	288	263	290	299
Siderite				
Blank	6.0	40	60	60
2.7% Fructose	50	60	110	180
0.14% Fructosan	288	377	208	245
0.70% Fructosan	310	420	353	450

Table 20

	gamma of magnesium dissolved from 1 gram of mineral at intervals of time			
Sample	1 Hour	4 Hours	12 Hours	24 Hours
Biotite				
Blank	64	62	75	90
2.7% Fructose	94	93	109	133
0.14% Fructosan	573	550	537	564
0.70% Fructosan	2,340	2,573	2,100	2,207
Tremolite				
Blank	296	304	352	374
2.7% Fructose	284	299	347	378
0.14% Fructosan	700	791	795	814
0.70% Fructosan	2,410	2,590	2,478	2,475
Siderite				
Blank	92	110	113	128
2.7% Fructose	78	98	105	104
0.14% Fructosan	464	518	534	501
0.70% Fructosan	1,870	2,148	2,150	2,004

NOTE: The fructosan sample used in these extractions, itself contained a certain proportion of magnesium (Exp. No. 30), so that the actual quantity of magnesium dissolved is less than the value shown in the table.

Table 21

A similar extraction was conducted at pH 5.2, only a water blank and a 0.70 per cent solution of fructosan being used, determining the quantities of iron and magnesium dissolved as before. It can be seen (Table 22) that the solubilisation of ferrous iron by sugars is considerably more effective at lower pH values, a fact which it had proved impossible to determine during the preceding experiments. That this extensive dissolving of iron led to bleaching of the siderite is demonstrated in Fig.18 where the colours after extraction with water, and with 0.70 per cent fructosan, are compared.

Extensive solubilisation of magnesium at pH 5.2 (Table 22) was observed, similar to the quantity dissolved at pH 8.0. The experiments have shown that solubilisation of magnesium can occur over a far wider pH range than was first thought, indicating that some form of sugar-magnesium interaction can also occur at these relatively low pH values.

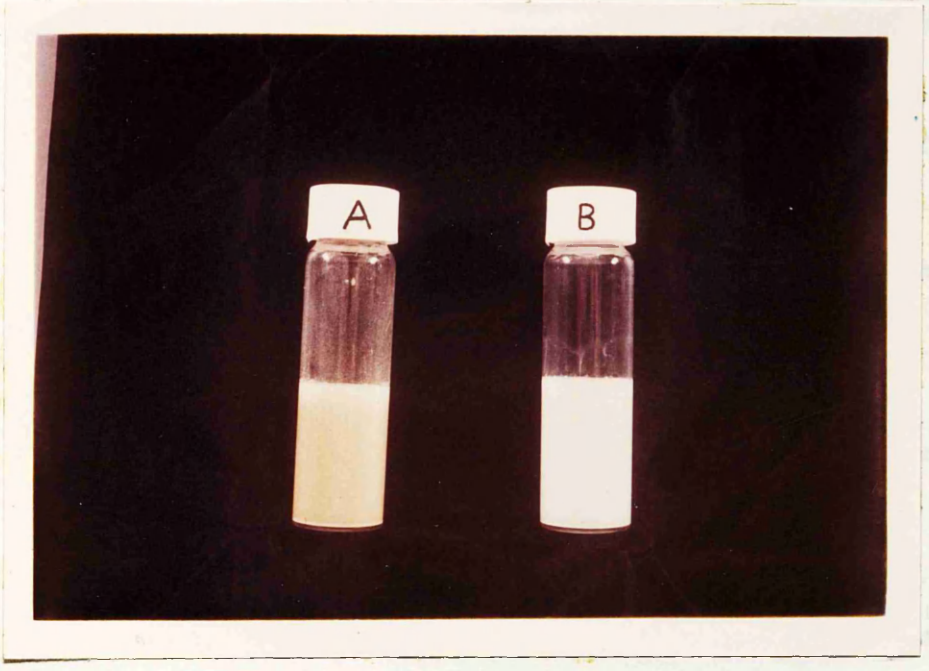
It would have been useful to have obtained the analysis figures for the iron and magnesium contents of all three minerals, so that a more comprehensive comparison between them could have been made. ~~One figure which can, however, be stated with certainty is that the fructosan, in the concentration used here, is capable of extracting some 25 per cent of the total magnesium present in siderite.~~

These results have also indicated that complex formation

	1 Hour	4 Hours	12 Hours	24 Hours
	gamma of iron dissolved from 1 gram of mineral at intervals of time			
Biotite Blank	320	220	210	270
0.70% Fructosan	2,200	2,535	2,550	3,235
Tremolite Blank	125	120	135	180
0.70% Fructosan	540	670	780	981
Siderite Blank	51	91	45	45
0.70% Fructosan	2,173	3,490	3,945	5,300
	gamma of magnesium dissolved from 1 gram of mineral at intervals of time			
Biotite Blank	248	392	392	452
0.70% Fructosan	2,365	2,765	2,950	3,002
Tremolite Blank	410	532	562	591
0.70% Fructosan	2,638	2,980	2,940	3,054
Siderite Blank	149	273	236	260
0.70% Fructosan	2,209	2,247	2,200	2,449

see note on page 117

Table 22



A - Powdered Siderite extracted with water for 24 hours at pH 5.2

B - Powdered Siderite extracted with fructosan for 24 hours at pH 5.2

Figure 18

need not occur solely via the metallic hydroxide. The extensive dissolving of siderite indicated that complex formation between sugar and ferrous carbonate was in operation. In addition, at the pH values used in these latter experiments, magnesium could not possibly be present in the form of its hydroxide, as this does not form until a far higher pH. Thus, sugar has been shown to form complexes with both iron and magnesium when present as compounds other than their hydroxides.

6. Summary and discussion of the results obtained

The foregoing experiments have clearly demonstrated that sugars were far more effective in dissolving ferrous hydroxide than the corresponding ferric compound. Ferric hydroxide was solubilised only to a slight extent, and over a period of time, by sugars, this effect being more noticeable above and below pH 6.2 as predicted. When ferrous hydroxide was extracted with sugars, large quantities of iron rapidly came into solution. It was not possible, however, to examine the effect of pH variation on the quantity of iron dissolved, since the degree of oxidation of each extraction overwhelmingly determined the amount solubilised.

In addition to dissolving ferrous hydroxide, sugars were effective in mobilising ferrous iron and magnesium from naturally occurring minerals, and in this case it was shown that lower pH values promoted increased solution of ferrous iron by the sugar, without increasing the quantity of iron dissolved

in the water blank.

The iron solutions obtained on extraction of ferrous hydroxide with sugar remained completely stable for long periods of time if maintained under reducing conditions, but redeposited the majority of the dissolved iron on exposure to the atmosphere. Moreover, the treatment of such iron solutions with typical soil constituents, resulted in adsorption of a large proportion of the dissolved iron, adsorption from fructose/iron solutions being more complete than from fructosan/iron solutions.

Thus, the experiments have indicated that sugars may play an important role in the solubilisation, translocation and subsequent redeposition of the mineral constituents of the soil.

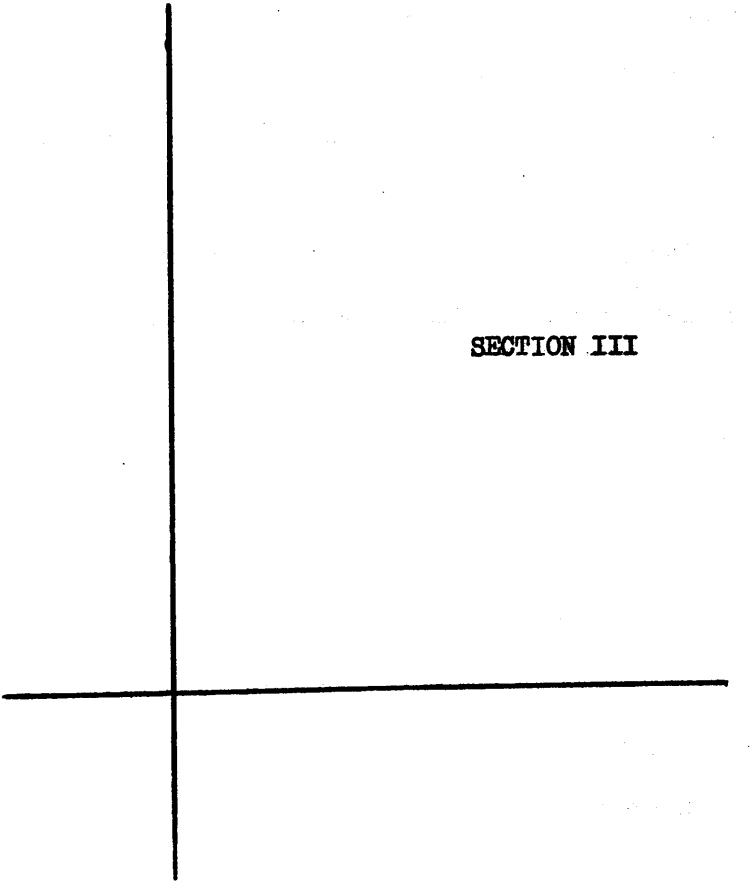
Although constant elution of the upper horizons of the soil with dilute sugar solutions could result in the solubilisation of considerable quantities of otherwise insoluble ferric iron, it is obvious that sugars will have a more pronounced effect in the solubilisation of ferrous iron. It is of interest to note, as stated in the introduction to this section, that most of the features associated with extensive iron mobilisation are prevalent under predominantly reducing conditions. That translocation of the dissolved iron after solubilisation can occur has been demonstrated by the fact that the iron solutions obtained on extraction of ferrous

hydroxide with sugars, are stable for long periods of time when reducing conditions are prevalent.

Iron redeposition features in the soil are associated with conditions of increased aeration, and the experiments have shown that under such conditions, the iron dissolved by sugars is deposited as a precipitate, the proportion shed being determined by the length of exposure to oxidising conditions.

In addition to exhibiting the above effects on oxidation, the ferrous/sugar solutions were strongly adsorbed onto clay and sesquioxides. It is of interest to note that thin iron-pans and the B horizons of podzols, that is, the deposition zones of these soil types, are enriched with these very constituents.

In conclusion it can be stated that the results indicate that sugars may play a hitherto unappreciated role in the formation of certain soil features. Mobilisation of other constituent soil minerals, in addition to iron, is also a distinct possibility, as has been shown by the intense solubilising effect of sugars on magnesium at a wide range of pH values.



SECTION III

Section III. Fructosan investigation

1. Introduction

An excellent review of the discovery and subsequent investigation of the fructosans of monocotyledons is given by Archbold (122). The fructosan reserves of this class of plants differ from those of the compositae, where the fructose residues are linked 1,2, in that the fructofuranose units are linked 2,6, resulting in an elongated molecule of high solubility. Each chain is terminated by a sucrose residue, which results in a small quantity of glucose being present per molecule.

A considerable amount of work has been carried out on the distribution of the water-soluble carbohydrates of grasses (123, 124, 125, 126, 127, 128, 129, 130, 131) much of this work being summarised in Archbold's review. Fructosan is the main constituent, and is stored mainly in the stems of grasses and cereals (128). Relatively smaller amounts are found in the leaves (132).

The seasonal variation of grass fructosan reserves, which has been closely investigated (133, 57) appears to account for most of the seasonal changes in the water-soluble carbohydrate content of grasses, the content of other soluble sugars varying very little (134). Waite and Boyd (133) and Waite (57) demonstrated that different varieties of grass exhibit different seasonal patterns of fructosan accumulation. Timothy,

cocksfoot, and fescue exhibit two maxima during one growing season, ryegrass only one. In both cases a maximum is reached just previous to the change-over from vegetative to floral development, with a further maximum in the former group occurring at a later stage of growth. The fructosan content during these maxima can be as high as 25 per cent of the dry matter, this being an increase from only 1 per cent early in the growing season.

It is of interest to note that other factors influence the level of fructosan reserves. Defoliation markedly reduces the fructosan content (130, 135, 136, 137, 138, 139, 140, 141, 142) as do high applications of nitrogenous fertilizers. (130, 136, 139, 140, 143, 144, 145).

2. . Fructosan extraction

Fructosan was extracted from mixed Italian and Perennial ryegrass obtained from Garscube Estate. Cutting was carried out on 10th August 1966, at the time of full emergence of the head, during the second growth of the season. The material was cut close to the ground to obtain the maximum amount of stem tissue.

The subsequent handling and extraction of the grass essentially consists of drying; macerating the tissue with water, and precipitating the polysaccharide with alcohol. Several alternative methods of drying have been reported. Archbold (130) found that if the drying conditions were sufficiently rapid to

avoid enzymic hydrolysis of the fructosan, there was little or no inactivation of the enzymes themselves. Laidlaw and Wylam (146) stated that high drying temperatures resulted in fructosan breakdown, and found that freeze drying of the plant tissue gave a fructosan content close to the original value. Colins and Shorland (147) examined all the various methods of drying and preservation, and found that boiling ethanol treatment was the most effective in inhibiting enzyme action.

Modifications of the water extraction and purification procedure have been many and varied. These include treatment of the water extract with lead acetate (130, 148, 149) or charcoal (130); treatment with cadmium sulphate and sodium hydroxide at 95°C, followed by deionising by passage over Amberlite IR 100 and IR 4B resins (150); and formation of the insoluble barium complex of the fructosan, subsequently breaking down the complex with carbon dioxide at pH 8 (151).

In the present work, since the product was to be used to examine the possibilities of complex formation between fructosan and metals, it was important to select a technique designed to avoid any change in the properties or constitution of the polysaccharide. For various reasons none of the methods quoted above were entirely suitable. High temperatures as in Laidlaw and Reid's method (150), carried some danger of breakdown, and the addition of any compounds which could possibly form complexes with the fructosan had to be avoided.

The method finally adopted (G.M.8) involved the treatment of approximately 10 kilograms of freshly cut herbage with boiling ethanol, the grass being cut in small batches and immediately plunged into 10 litres of the boiling solvent in a 20 litre, parallel-walled, heated glass extraction vessel. After 5 minutes immersion, the grass was removed, oven dried at 60°C and hammer milled. The 2 kilograms of dry powder obtained was extracted twice with water to give 18 litres of extract. After filtration through Hi Flo Supercel, this was reduced in volume to 3 litres under vacuum, and then precipitated in 3 volumes of cold absolute alcohol. The material obtained was then redissolved in water, discarding any substances which would not redissolve, followed by reprecipitation in 3 volumes of ethanol. This procedure was repeated a further two times, and the product dried by use of dry ethanol, dry acetone and 40-60 petroleum ether. 159 grams of an off-white powder of fructosan was obtained.

3. Experiments conducted using fructosan

The experiments conducted to examine the complexing powers of the fructosan obtained by the above procedure, have appeared earlier in the text, but a short summary of the results obtained is provided at this point. Fructosan was found to be extremely effective in low concentration in:-

1. Inhibiting the precipitation of ferric hydroxide from a solution of ferric chloride on addition of sodium

hydroxide. (Section I Exp.No.9)

2. Redissolving freshly precipitated ferrous hydroxide at various pH values. (Section II Exp.No.20)

3. Dissolving considerable quantities of magnesium and ferrous iron from siderite and biotite. (Exp. No.23)

4. Effectively bleaching siderite at pH 5.2 (Exp.No.23)

Originally this was the only work for which the fructosan was planned, and the results demonstrated the exceptional complexing powers of the polysaccharide. To ascertain that these powers were not being wrongly attributed to the fructosan, a number of tests were conducted to determine the purity of the sample.

4. Initial analytical investigation

1. Moisture content

A sample of the fructosan was dried for 24 hours under vacuum, at 60°C, over phosphorous pentoxide (G.M.9). The moisture content of the fructosan was 8 per cent.

2. Acid hydrolysis

A well known feature of fructosans is the ease with which they are broken down by comparatively weak acids (122, 123, 152). Consequently, $\frac{N}{700}$ to $\frac{N}{10}$ sulphuric acid, (123, 152, 133) and 1 per cent oxalic acid (153) are usually used to effect hydrolysis. The acid solutions are then neutralised by addition of saturated

barium hydroxide, before chromatography.

Since carbohydrates form complexes with alkaline-earth metal hydroxides (2, 154, 155, 156) neutralisation of the hydrolysing acid with barium hydroxide, introduced the risk of removing some of the hydrolysis products as insoluble barium complexes or adducts.

The method finally adopted involved hydrolysis of the polysaccharide with dilute formic acid (G.M.10). A sample of fructosan was hydrolysed with 5 mls of 0.2-N formic acid at 100°C for 1 hour. After addition of water, the excess acid was removed by distillation of the azeotrope (157) under vacuum. By repetition of this procedure three times, the pH of the solution was brought to neutrality with little risk of loss of hydrolysis products. The technique worked so well in this case that it may be applicable to the investigation of other polysaccharides.

Samples of the hydrolysate were chromatographed (G.M.1) in aqueous phenol and ethylacetate-pyridine-water solvents, and the spots located using Trevelyan's silver nitrate method (158). The sole hydrolysis products were fructose and a trace of glucose, which is in agreement with all previous results (159,130).

3. Quantitative fructose analysis

The fructose content of the sample was accurately determined (G.M.6) by means of Bacon and Bell's (160) modification

of Roe's specific ketose determination (161). Since one component solution is a strong acid, hydrolysis of the polysaccharide before analysis was unnecessary. The fructosan contained 68.2 per cent of fructose.

4. Total hexose content

In order to determine quantitatively the trace of glucose present, analysis of the total hexose content was carried out using the phenol sulphuric method (G.M.19). The accuracy of the figure obtained was checked by constructing standard curves using both fructose and glucose as standards, very little difference between them being found. The total hexose content was 77.7 per cent. Subtraction of the figure obtained above for the fructose content gave a glucose content of 9.5 per cent. This was slightly higher than the figure reported by Archbold (130) who found a 6 per cent content of glucose in barley fructosan.

5. Total nitrogen content

Total nitrogen was estimated by the Kjeldahl method (G.M.11) and a value of 0.364 per cent obtained, equivalent to a crude protein content of 2.27 per cent.

6. Reducing power

On conducting a Fehling's test (G.M.12) on a sample of the fructosan, no reducing capacity was detected.

As a result of these tests, the fructosan was considered to be sufficiently pure for use in the previously detailed

experiments, and no further analytical tests were necessary at this stage.

5. Further investigation of the properties of the fructosan.

Fructosan has been shown to be an effective agent in dissolving ferrous iron from a variety of sources. This property would be more significant if resistance to microbial attack could be demonstrated. To investigate the speed with which fructosan is broken down by airborne micro-organisms, and whether this is inhibited by the presence of iron, the following experiment was conducted.

Exp.No.24. The breakdown of fructosan by airborne micro-organisms.

Fructosan concentrations of 1, 2, 4 and 6 per cent were examined, one series being dissolved in water, the other in 0.02 Molar ferric chloride. After exposure to the atmosphere for several days the non-iron solutions had developed a strong microbial growth, whereas the iron-containing aliquots remained completely clear for a number of weeks. This was apart from a slight, but constant, cloudiness in the 2 per cent solution in ferric chloride. The pH values of the eight solutions used are listed in Table 23, where it can be seen that the protective action of iron could have been due to lowering of the pH, rather than a complexing effect.

Using 1, 2 and 4 per cent fructosan concentrations, the following solutions were made up:

Strength of solution	pH value in water	pH value in ferric chloride
1 %	6.8	2.25
2 %	6.75	2.25
4%	6.65	3.0
6%	6.6	4.0

Table 23

1. Fructosan plus water - pH 6.75
2. Fructosan plus ferric chloride - pH 2.25
3. Fructosan plus ferric chloride - pH 6.75

A duplicate series of solutions, overlayered with toluene, an antimicrobial agent, were also set up to verify that the cloudiness and microbial growth were indeed due to the action of airborne micro-organisms.

After standing for a number of days, the observations in Table 24 were made. It was concluded that the microbial growth in the aqueous solutions was due to airborne micro-organisms and that the protective action of ferric chloride was probably due to complex formation, similar to the effect noted by Martin et al (111) with a number of complexed polysaccharides in 1966. No explanation for the slight, constant cloudiness of the 2 per cent fructosan/iron solution was found, although it was evidently not due to microbial attack, as it also occurred under toluene.

Sample	1% and 4%		2%	
	Open to air	Toluene covered	Open to air	Toluene covered
Water pH 6.75	Bacterial growth	clear	Bacterial growth	clear
$FeCl_3$ pH 2.25	clear	clear	slightly cloudy	slightly cloudy
$FeCl_3$ pH 6.75	clear	clear	clear	clear

Table 24

Iron, therefore, appears to be capable of protecting fructosan from microbial attack over the most important physiological pH range, from around pH 2 to around pH 7.

Since no sterilisation of vessels or solutions was carried out, this experiment would not satisfy a microbioligist; nor was it intended to conduct such an experiment, as only a gross effect was being sought.

Exp.No.25. The behaviour of fructosan solution when adjusted to pH 10.0

Two samples of 4 per cent ryegrass fructosan, one in solution in water, the other in 0.02 Molar ferric chloride, were adjusted to pH 10, to examine the capacity of iron to protect fructosan from microbial attack under alkaline. conditions. This original aim was not pursued, in view of the observations made.

As expected from the inhibition of precipitation

experiments (Exp.No 9), the solution of fructosan in ferric chloride remained completely clear during the whole adjustment procedure. The aqueous solution, however, immediately upon addition of sodium hydroxide, deposited a precipitate which increased in bulk until pH 10 was reached.

Fructosan solutions of varying strengths all exhibited this same phenomenon when sodium hydroxide was added, the quantity of precipitate increasing with the strength of the solution.

Conducted with more closely controlled addition of alkali, precipitation was found to commence as soon as the pH was raised to pH 7.25, at this point being apparent as a slight haze. On increasing the pH slowly, the amount of precipitate increased, reaching a maximum at pH 10. This was ascertained by raising the pH to 10, centrifuging off the precipitate, and adding alkali to pH 11.5. No further precipitation was observed.

The grey, flocculent precipitate redissolved upon acidification, and was obviously an impurity which had not been detected by the previous analytical tests.

To verify that the complexing powers attributed to fructosan on the results of earlier experiments were still valid, five grams of fructosan were dissolved in 50 mls of water, adjusted to pH 10, and the precipitate removed by centrifugation. The resultant fructosan solution was

precipitated in alcohol, collected and dried, and a repeat of the inhibition of precipitation experiment (Exp.No.9) conducted. Fructosan minus the precipitating fraction gave results exactly comparable to the original complete fructosan. The polysaccharide, after removal of this fraction at pH 10, still retained its complexing powers.

6. Examination of the precipitate obtained on adjusting a fructosan solution to pH 10

Exp.No.26. Observations on the quantity precipitated.

1 Gram of the fructosan was dissolved in 15 mls of water, adjusted to pH 10,and the precipitate centrifuged, washed several times with water adjusted to pH 10 with sodium hydroxide and dried. 29.1 mgs of precipitate were obtained.

When 1 gram of fructosan was dissolved in 10 mls of water, and the same procedure carried out, the weight of precipitate obtained was 33.62 mgs. Because of this variation 10 per cent solutions were used in all subsequent experiments in order to standardise the procedure.

Exp.No.27. Attempted characterisation of the precipitate

Approximately 150 mgs of precipitate were obtained by dissolving 5 grams of fructosan in 50 mls of water and adjusting the solution to pH 10. After collection by centrifugation, and washing several times with water adjusted to pH 10 with sodium hydroxide, the material was used in the various qualitative analytical tests.

Hydrolysis of an aliquot of the precipitate by the formic acid method (G.M.10) was followed by chromatography in aqueous phenol solvent. No spots were detected using Trevelyan's silver reagent.

The presence of carbohydrate in the precipitate was tested for by the Molisch test (G.M.13) with negative results. Similarly, the material gave a negative reaction to the biuret (G.M.14), Fehling's (G.M.12), Millon's (G.M.15) and sodium cobaltinitrite/acetic acid (G.M.15) reagents.

Hence, a qualitative test for phosphate was carried out using King's method (G.M.16). This test showed appreciable quantities of phosphate present in the precipitate, so that quantitative estimations were then carried out, both total and inorganic phosphate being determined. Similar estimations were also performed on the original fructosan material, and on a sample of fructosan from which the precipitating fraction had been removed by adjustment to pH 10. The values obtained are presented in Table 25, and represent the quantity of phosphate in one dry gram of fructosan.

It can be seen that 66 per cent of the total phosphate present was precipitated at pH 10, and moreover, was not associated with organic matter. No charring occurred on oxidation of the sample with perchloric acid during the total phosphate estimation.

Fraction	Test	mg as P	mg as PO_4
Original Fructosan	Total P	6.95	21.3
	Inorganic P	5.30	16.24
Precipitated Fraction	Total P	4.6	14.0
	Inorganic P	4.6	14.0
Unprecipitated Fraction	Total P	2.35	7.3
	Inorganic P	0.7	2.24

Table 25

The majority of the phosphate present in the original fructosan was inorganic in nature, although some 24 per cent of it registered as being organic, and therefore must be bound either directly or indirectly to the fructosan molecule. The question arises as to whether this inorganic phosphate was indeed true orthophosphate, or whether it had been split off under the acid conditions of King's determination.

To avoid the strongly hydrolysing conditions presented by the reagents used in the King method for phosphate estimation, the method of Lowry and Lopez was utilised (G.M.16). In this method the pH of the test solution is pH 4.0, as compared to pH 0.65 for the King method; the molybdate concentration is lowered from 0.25 per cent to 0.1 per cent, and ascorbic acid is substituted for amidol as the reducing agent.

A lower result for inorganic phosphate with this test would indicate labile bonds attaching the phosphate residue to the fructosan; an equivalent result would tend to indicate the absence of any phosphate-fructosan linkage.

The determination was carried out on the original fructosan alone, since it had already been adequately demonstrated that all the phosphate present in the precipitate was inorganic. The content of true orthophosphate in one gram was found to be 4.64 mgs expressed as phosphorous, which is equivalent to 14.2 mgs of phosphate (PO_4). Comparison of this value with the figures listed in Table 25 shows that 2.04 mgs of acid labile phosphate were detected. However, the majority of the phosphate designated as inorganic by King's method was truly inorganic, the value being almost exactly equivalent to the quantity of phosphate precipitated at pH 10.

There are therefore three types of phosphate present in 1 gram of the original fructosan:

a) acid labile phosphate - 2.04 mgs

b) inorganic phosphate - 14.0 mgs

c) bound phosphate - 5.06 mgs

7. Investigation of the form of phosphate in the fructosan

Several possibilities of the source of phosphate existed.

a) The small quantity of acid labile phosphate may have belonged to a nucleotide type of compound. Compounds of this type have already been found associated with carbohydrate polymers (162).

b) Almost exact correlation between the true inorganic phosphate present and the amount shed by precipitation at pH 10 was observed. Thus the existence of inorganic compounds such as calcium and magnesium phosphates or basic salts weakly bonded to the fructosan but not completely protected at alkaline pH values, is a possibility.

c) The remaining 23.74 per cent of the phosphate not hydrolysed by King's inorganic determination, indicated the presence of strong bonding between the fructosan and the phosphate grouping. It was likely to be in the form of inorganic compounds strongly bonded to the fructosan, unlike the more weakly bonded compounds which precipitated at pH 10 and which gave a positive inorganic phosphate test.

A convenient explanation for the last two categories would be the presence of basic salts, where half of the molecule is bonded to the fructosan, the other portion possessing no organic-inorganic linkage, as shown in Fig.19.

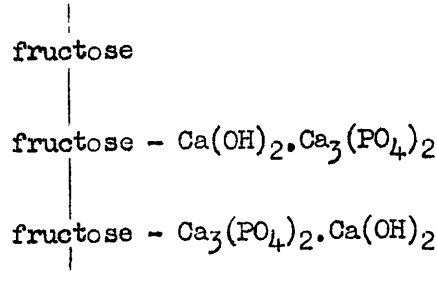


Figure 19

Exp.No.28. The source of the acid labile phosphate

The possibility of the presence of a small quantity of nucleotide material in the fructosan was examined by measuring the ultra-violet absorption of 1 gram of fructosan in 250 mls of water, over the range 220 to 450 mμ. The absorption spectrum obtained, (Fig.20) showed an inflexion point at 260 mμ, indicative of the presence of nucleotide material, as this feature is given by all compounds of this type.

The quantity of nucleotide material present was calculated on a typical nucleotide. Assuming the spectrum was due to U.M.P. :-

$$\text{concentration} = \frac{\text{O.D. x M.W.}}{\Sigma \times \ell} \text{ grams per litre}$$

where O.D. = optical density; M.W. = molecular weight;

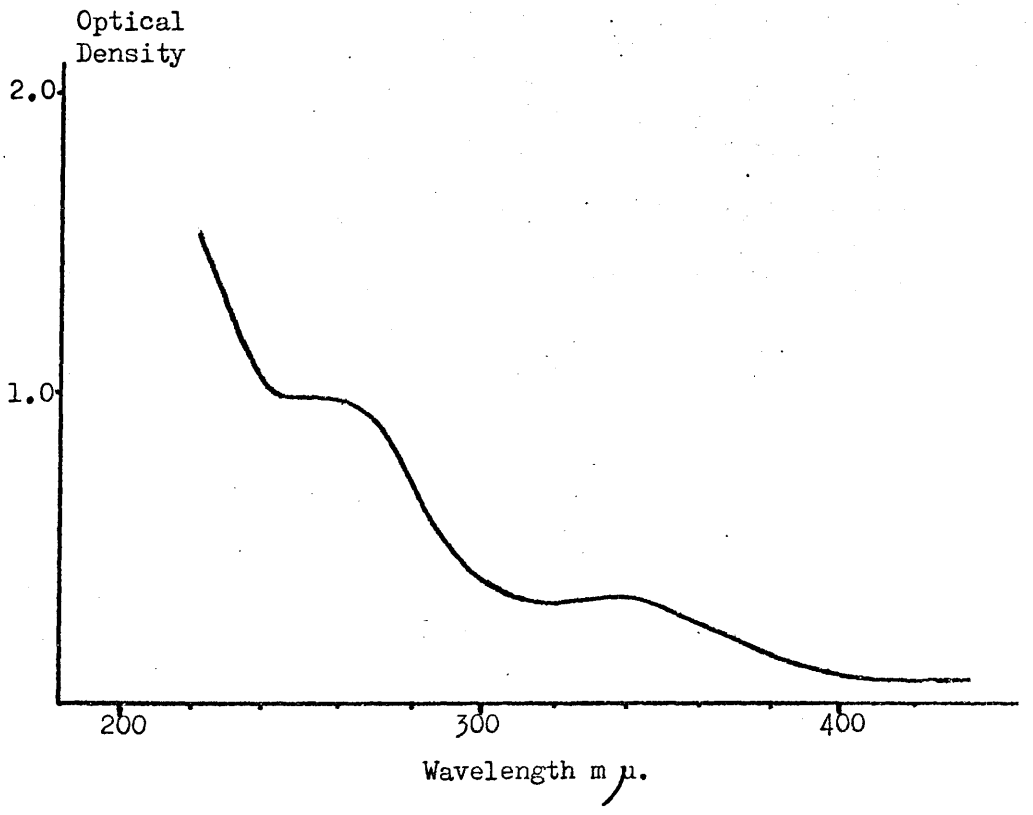
Σ = molar extinction coefficient;

ℓ = path length of cell

$$\therefore \text{ concentration} = \frac{1.0 \times 324}{10^4 \times 1} \times \frac{250}{1000} \text{ grams}$$

$$= \underline{8.1 \text{ mgs in } 250 \text{ mls}}$$

There are 8.1 mgs of nucleotide (calculated as UMP) present in 1 gram of the fructosan. This is equivalent to 2.3 mgs of phosphate (PO_4), indicating that the source of the acid-labile phosphate (2.04 mgs as PO_4) was probably a nucleotide type of compound.



Absorption spectrum of Fructosan

Figure 20

Further investigation of this entity was not pursued, but its presence is noted.

Exp.No.29. Mineral analysis of the precipitating material

A cation analysis of an acidified solution of the precipitate was carried out, (G.M.18) positive tests for calcium magnesium and iron being obtained. Quantitative measurement of these metals by means of the atomic absorption spectrophotometer (G.M.3, 4, 5) gave the values shown in Table 26.

Metal	mgs in ppt. from 1 gm fructosan
Calcium	5.44
Magnesium	2.1
Iron	0.027

Table 26.

Exp.No.30. Ash determination and mineral analysis of the original fructosan

The ash content of the fructosan was determined using Humphrie's method (G.M.17) a result of 20.0 per cent being obtained. No silica was present. No report of such an appreciable mineral content has been found in the literature. This may be due to the more extreme procedures used in the early extraction methods, a summary of which has already been

given. Such treatments as passage over ion exchange resins; addition of lead acetate, and formation of barium complexes with subsequent barium removal by carbon dioxide treatment, may have resulted in the removal of any inorganic ions already bound to the fructosan. Schlubach and Blaschke (163) included a figure of 2.5 per cent ash, in an analysis of total water soluble carbohydrates from ryegrass, but there was no suggestion as to where this may have been associated, and the value obtained is very much lower.

During ashing, removal of all traces of carbon from the ash took a considerable time, as shown in Table 27.

Hours at 490°C	Weight Ash	Appearance
6	226.9	considerable carbon
26	207.5	traces of carbon
37	203.7	traces of carbon
45	200.8	slight traces carbon
60	200.0	pure white ash

Table 27

In comparison, one gram of D fructose was completely ashed in $1\frac{1}{2}$ hours at this temperature.

A cation analysis of the ash was carried out (G.M.18) giving positive qualitative tests for calcium, magnesium, and

iron as expected, with in addition manganese and potassium. Quantitative tests were carried out on the calcium, magnesium and iron as before. The results are tabulated in Table 28, along with the results obtained from the precipitate analysis for comparison. As was previously observed with phosphate, the total content of magnesium calcium and iron in the fructosan was far greater than the amount precipitated out at pH 10.

Ion	Weight in Ash mgs.	Precipitated at pH 10.0 mgs.	% of total ppt^{d}.
Calcium	8.4	5.44	64
Magnesium	5.2	2.1	40
Phosphate	21.3	14.0	65
Iron	0.434	0.027	6.2

Table 28

The values obtained for the precipitated fraction would be in accordance with the precipitation of calcium and magnesium phosphates from solution, as demonstrated in Table 29. A similar calculation performed for the calcium and magnesium remaining bound to the fructosan at pH 10, indicated that only 50 per cent of the total requirement of phosphate was present.

This information is suggestive of the presence of basic salts. The only fact, however, which supports this convenient

Salt:-	$Ca_3(PO_4)_2$	$Mg_3(PO_4)_2$
Ratio by weight of M : PO_4	1:1.583	1:2.6
Content of M in precipitate	5.44	2.1
PO_4 required to form salt	8.61	5.46
Total PO_4 required	14.07	
Total PO_4 present	14.0	

Table 29 M = Ca or Mg

explanation, is the absence of other ions in the ash to account for the weight.

The features demonstrated suggest that the appreciable mineral content is due to some form of complexing between the inorganic matter and the fructosan. The noted behaviour of the mineral matter is similar to that observed during the initial investigation of the effect of sugars on ferric hydroxide precipitation (Exps.Nos.4 and 5) where the presence of sugar was shown to inhibit the precipitation of ferric hydroxide, and that precipitation in the presence of sugar was a gradual process. In this case, precipitation of the inorganic material was inhibited by the presence of fructosan, as calcium

and magnesium phosphates could normally be expected to precipitate at pH 6.5, and was, in addition, a gradual process. Even at pH 10.0, the maximum precipitation level, over 80 per cent of the inorganic material was still soluble.

However, in the earlier experiments both fructose and fructosan (Exp.No.4 and 9) exhibited a maximum precipitation level with ferric hydroxide at pH 6.2, the insoluble precipitate redissolving at higher pH values. Here, the quantity of material precipitated increases as the pH is raised from 7.25 to 10.0. The apparent anomaly may be due to the fact that, in this case, the precipitating materials are mainly calcium and magnesium phosphates, rather than ferric hydroxide as in the previous experiments.

A very efficient complexing action has been demonstrated by a naturally occurring polysaccharide, associated with inorganic constituents already present within the plant tissues before extraction. It is not known whether the fructosan was associated with the mineral matter within the cell, or whether the interaction occurred after the cells had been broken down during the extraction process.

8. Further investigation of the behaviour of the inorganic constituents

Further information on the behaviour of this precipitating material was desirable.

Exp.No.31. Titration of the fructosan solution

Titration of a 4 per cent solution of fructosan gave the curve shown in Fig.21. The buffering capacity exhibited, as compared to a 4 per cent fructose solution, was probably due to the high content of inorganic ions.

Exp.No.32. The pH of re-solution of the precipitate

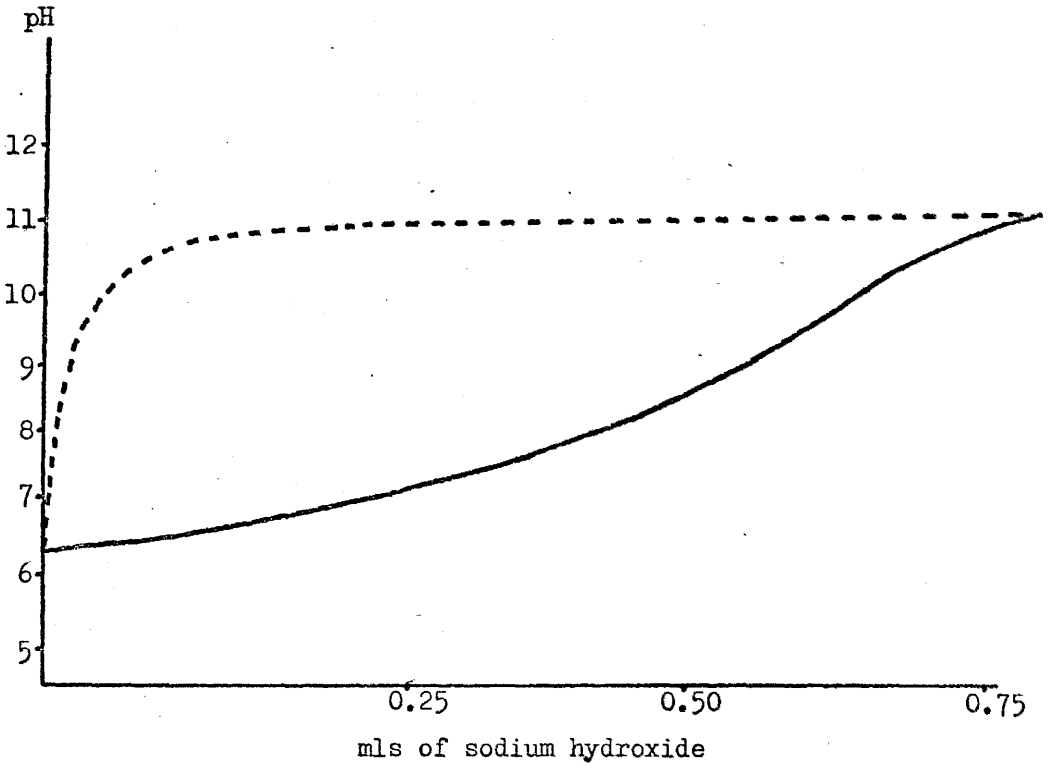
Two 10 per cent fructosan solutions were adjusted to pH 10., the precipitate in one case being left suspended in the alkaline fructosan solution, and in the other, being centrifuged down, washed, and resuspended in an equivalent volume of water. Dilute hydrochloric acid was then slowly added to each and observations were made.

Case 1. Slow disappearance of the precipitate as the pH fell was observed, until the solution was essentially clear at the original pH of 6.55.

Case 2. Little difference was noted in the appearance of the suspension as the pH fell from 10 to neutrality. At this point slow redissolving appeared to commence. The majority of the precipitate appeared to redissolve suddenly at pH 5, the test solution being essentially clear at pH 4.5.

Exp.No.33. Dialysis of the fructosan solution

10 mls of 10 per cent fructosan solution were dialysed against 1 litre of water for 2 days, and the resulting dialysate concentrated to 50 mls for mineral analysis. On analysis for



Titration of fructosan and fructose

——————— Fructosan

- - - - - - - - - Fructose

1 gram in 25 mls water

Figure 21

iron and magnesium, only 0.025 and 0.95 mgs respectively were detected, indicating that the majority of the inorganic material is closely bound to the fructosan. In addition, 48.74 mgs of fructose, presumably short-chain fructosans, were found in the dialysate.

Exp.No.34. Variation in weight of the precipitate obtained at different pH values, and the effect of fructose addition prior to pH adjustment

It has been consistently demonstrated that the effect of sugar on a metal compound is considerably enhanced by increasing the ratio of sugar to metal. An example of this is the observation that less metallic hydroxide is shed from solution at unfavourable pH values if the amount of sugar is increased (Exp.No.5). A similar effect on the weight of precipitate shed by fructosan was expected, on addition of fructose to the solution prior to pH adjustment.

To determine to what extent fructose inhibits the precipitation of material from fructosan at different pH values, three series of 10 per cent fructosan solutions were made up. No addition of fructose was made to the first series, 1 gram was added to the second series, and 2 grams to the third. All volumes were adjusted to compensate for the volume increase accompanying fructose addition. The pH of each solution was then adjusted, the precipitates obtained at these various pH values collected, washed, dried and weighed.

The results (Table 30) demonstrated that the weight of material precipitated increased with the pH, as is also shown in Fig.22. Moreover, fructose increments did indeed reduce the quantity of inorganic material deposited at all pH levels, this effect being more pronounced at lower pH values.

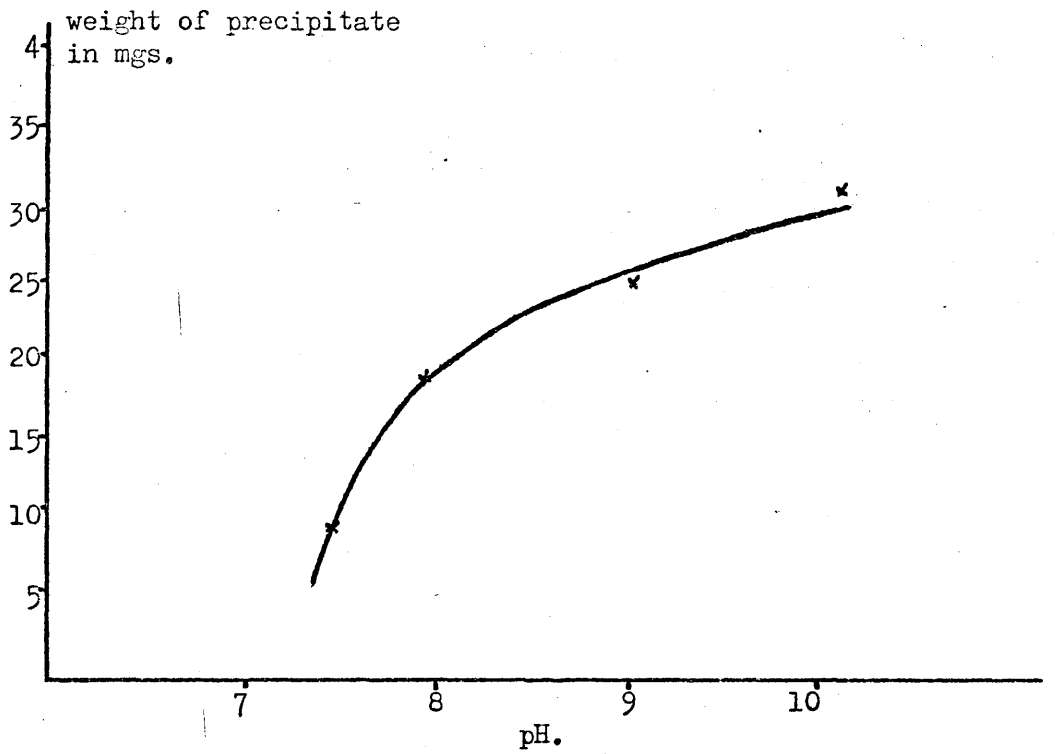
The possession of this ability by fructose indicates that an increase in 'true fructosan' content would be far more effective in reducing the weight of precipitate shed at alkaline pH values. Similarly it could be argued, that a reduced fructosan content would result in increased quantities of inorganic matter being precipitated. Such an effect may relate directly to the suggestion that the level of fructosan in herbage can determine the availability of mineral constituents to animals. This will be further discussed at a later point in this section.

Additions made to 1 gm fructosan	weight of ppt. shed from 1 gm fructosan			
	pH 7.5	pH 8.0	pH 9.0	pH 10.0
No addition	8.9	18.5	25.4	33.47
+ 1 gm fructose	6.5	16.5	24.8	32.8
+ 2 gm fructose	5.2	15.8	23.9	32.4

Table 30

9. Analysis of a further four fructosan samples

To verify that the high content of inorganic material in the extracted and purified fructosan sample was not an



Weight of precipitate shed from 1 gram of fructosan dissolved in 10 mls of water on increasing the pH

Figure 22

isolated chance occurrence, a further four samples of grass were subjected to exactly the same procedure and tests.

The grasses, Italian Ryegrass, Perennial Ryegrass, Cocksfoot and Timothy, were cut at the heading stage on 23rd May 1967. As before, they were obtained from trial plots in Garscube Estate. They had been sown out in June 1966, except the Timothy which had failed to grow at this seeding and was resown in August. Top dressing with Nitro-Chalk was carried out in the spring of 1967.

The extraction procedure was the same as described for the previous grass sample. However, after the same number of reprecipitations the samples of Italian, Perennial and Timothy fructosans became gummy due to uptake of water, and the precipitates had to be redissolved and reprecipitated one further time. Care was taken to avoid the complications which had previously occurred by maintaining a low temperature during the handling process.

pH readings were taken at every stage where the fructosan was dissolved in water (Table 31) to check that the pH level during extraction and purification did not rise above the value where precipitation could be expected, that is pH 7.25. Only the Cocksfoot and Timothy fructosans rose slightly above this value during the latter stages of purification. However, the ultimate criterion will be the actual pH value where precipitation commences, on addition of alkali to the

individual samples.

Sample	pH Value				
	Initial Extraction	1st Rediss.	2nd Rediss.	3rd Rediss.	4th Rediss.
Italian	-	6.25	6.45	6.65	6.93
Perennial	6.1	6.27	6.42	6.65	6.8
Cocksfoot	6.05	6.85	7.15	7.38	-
Timothy	6.2	6.6	6.95	7.30	7.52

Table 31

Table 32 summarises the weights of grasses extracted and the quantities of fructosan obtained. These results are in agreement with the relative quantities found by previous workers (57) for this stage of growth.

Grass	Weight of grass in grams	Weight of Fructosan in grams
Italian	500	33.7
Perennial	500	36.5
Timothy	330	5.0
Cocksfoot	500	6.0

Table 32

The determinations effected on the samples were carried out using the same methods as previously. In the tables summarising the values obtained, the value for the original fructosan has been included for comparison.

1. Moisture content

The moisture contents of the four fructosan samples were all less than 2 per cent.

2. Acid hydrolysis

As before, the only spots detected on hydrolysis and chromatography of the Italian and Perennial ryegrass fructosans, were fructose and a trace of glucose. A further moiety, which may have been a rather resistant polysaccharide was detected on chromatography of the Timothy and Cocksfoot fructosan hydrolysates. The Timothy fructosan contained only a trace of this material, although the Cocksfoot fructosan appeared to have a higher content.

3. Quantitative fructose analysis

The values obtained are summarised in Table 33.

Fructosan	Percentage Fructose
Italian ryegrass	80.6
Perennial "	75.1
Timothy	57.6
Cocksfoot	28.0
Original	68.2

Table 33

4. Total hexose content

The results obtained are listed in Table 34, along with the estimated glucose content, calculated by subtraction of the values in Table 33. The rather high content obtained

for the Cocksfoot fructosan probably includes the other sugars detected on chromatography of the acid hydrolysate.

Fructosan	Percentage content of total sugars	Percentage content of glucose (Subtract Table 33)
Italian	90.3	9.7
Perennial	81.4	6.3
Timothy	63.76	6.16
Cocksfoot	41.2	13.2
Original	77.7	9.5

Table 34

5. Ash determination

In addition to conducting ash determinations on the fructosan samples, the ash contents of the original dried grass powders were estimated, the values being presented in Tables 35 and 36 respectively.

Fructosan	Italian	Perennial	Timothy	Cocksfoot	Original
Per cent Ash	9.5	13.7	21.7	33.5	20.0

Table 35

A fifth reprecipitation step was conducted on the Italian, Perennial and Timothy fructosans, and an ash determination carried out on the dried material. The same

values were obtained, verifying that the number of reprecipitation steps did not affect the ultimate ash content of the fructosan. This was additional evidence that the mineral matter is not present by virtue of occlusion in the precipitated polysaccharide.

Grass	Italian	Perennial	Timothy	Cocksfoot
Ash %	8.95	8.77	9.21	8.88
Silica Free Ash %	5.74	5.43	5.08	5.71

Table 36

The figures shown in Table 37 indicate, on a rough basis, the percentage of the total silica-free ash present as an integral part of the fructosan samples.

Fructosan	Italian	Perennial	Timothy	Cocksfoot
% Total Ash present in Above	12.1	17.0	6.47	7.04

Table 37

Although an increase in the actual mineral content with increasing 'true fructosan' (fructose) content was not observed in the fructosan samples, a higher percentage of the total mineral constituents was found to be associated with samples possessing a high 'true fructosan' content (Table 37).

Clearly, when a mild extraction procedure is used, a high mineral content is consistently to be found associated with fructosans.

10. Examination of the precipitation characteristics of the fructosans

The weights of precipitate shed on adjustment of 10 mls of 10 per cent solutions of the fructosans were determined and are tabulated in Table 38.

Fructosan	Italian	Perennial	Timothy	Cocksfoot	Original
Weight in mgs. of precipitate	5.1	10.7	23.0	71.2	30.2

Table 38

In the case of the Italian and Perennial fructosans precipitation did not commence until above pH 8.5, compared with pH 7.25 with the original fructosan. However, 10 per cent solutions of the Timothy and Cocksfoot fructosans were too darkly coloured for an observation of this nature to be made.

The results also demonstrated that the weight of precipitate shed decreased considerably as the 'true fructosan' content of the samples rose. (Fig.23) Use of the fructose content, rather than the total sugar level, as a measure of the 'true fructosan' content, avoided the complications introduced by the presence of other polysaccharides in the Timothy and

Cocksfoot fructosans.

A similar linear relationship was demonstrated by plotting the percentage of the total inorganic matter which precipitated at pH 10, against the 'true fructosan' content. (Fig.24)

Thus, although the mineral matter present did not increase with increase in 'true fructosan' content, the precipitation characteristics demonstrated a linear relationship, indicating that the inorganic constituents were more firmly bound in samples with a high fructose content.

11. The level of calcium magnesium and iron present in the precipitate and the whole fructosan sample

Analyses were made as before and the results are tabulated in Table 39. As with the original sample, the total contents of calcium, magnesium and iron present were greater than the quantities deposited at pH 10.

As the 'true fructosan' content rose, the quantity of magnesium precipitated from solution decreased (Fig.25) and the percentage of the total magnesium present which was retained in solution increased (Fig.26). In both cases an almost linear relationship was demonstrated.

The results indicate that the inorganic ions are kept in solution by the complexing action of fructosan, with the actual level of 'true fructosan' determining the quantity of metal which will precipitate out.

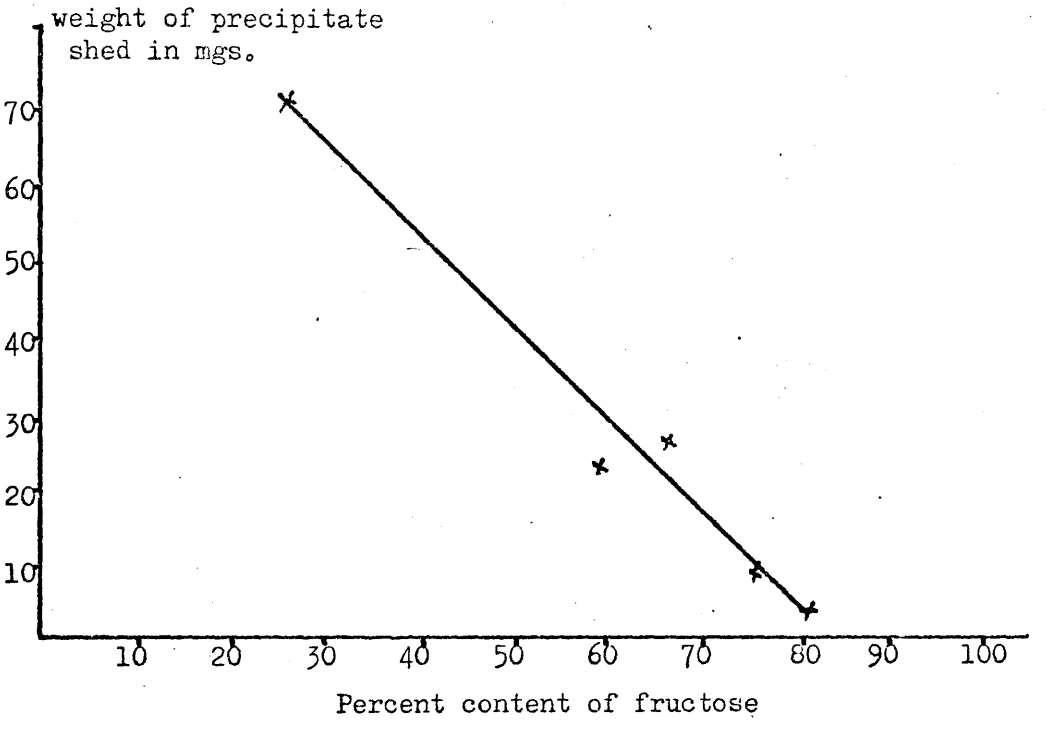


Figure 23

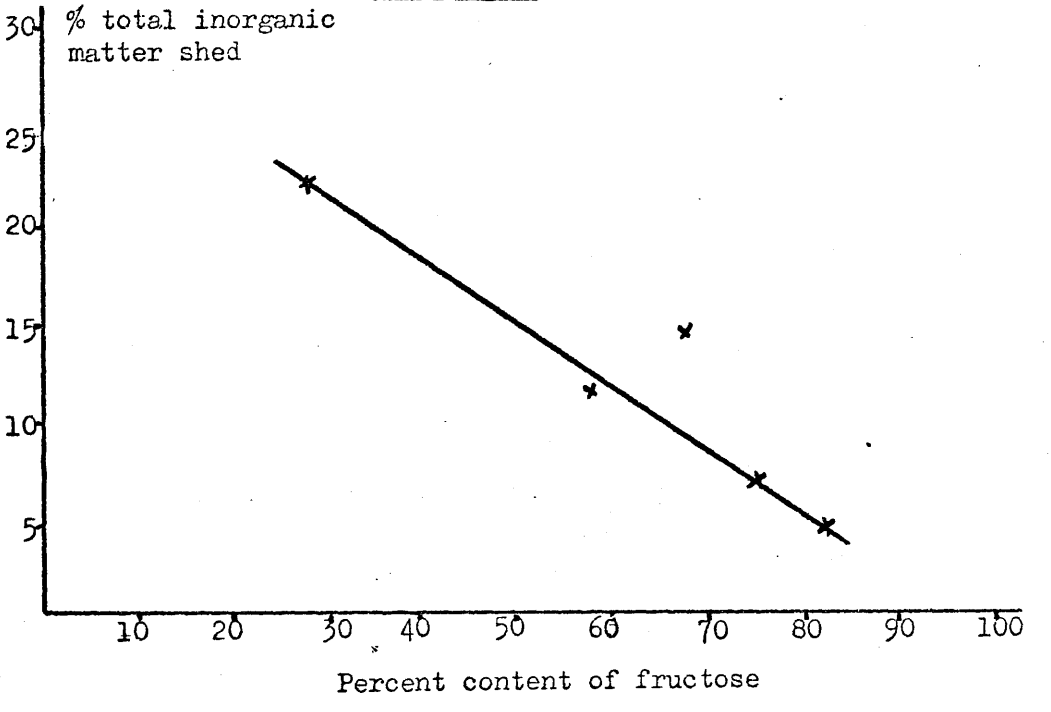


Figure 24

	Fraction	Calcium content in mgs.	Magnesium content in mgs.	Iron content in mgs.
Italian Fructosan	Complete	2.84	1.88	0.203
	Precipitate	1.55	0.33	0.000
Perennial Fructosan	Complete	2.79	3.05	0.183
	Precipitate	2.20	0.77	0.000
Timothy Fructosan	Complete	2.80	4.20	0.051
	Precipitate	1.05	2.75	0.000
Cocksfoot Fructosan	Complete	0.812	9.15	0.081
	Precipitate	0.585	9.15	0.000
Original Fructosan	Complete	8.40	5.2	0.434
	Precipitate	5.44	2.1	0.027

Table 39

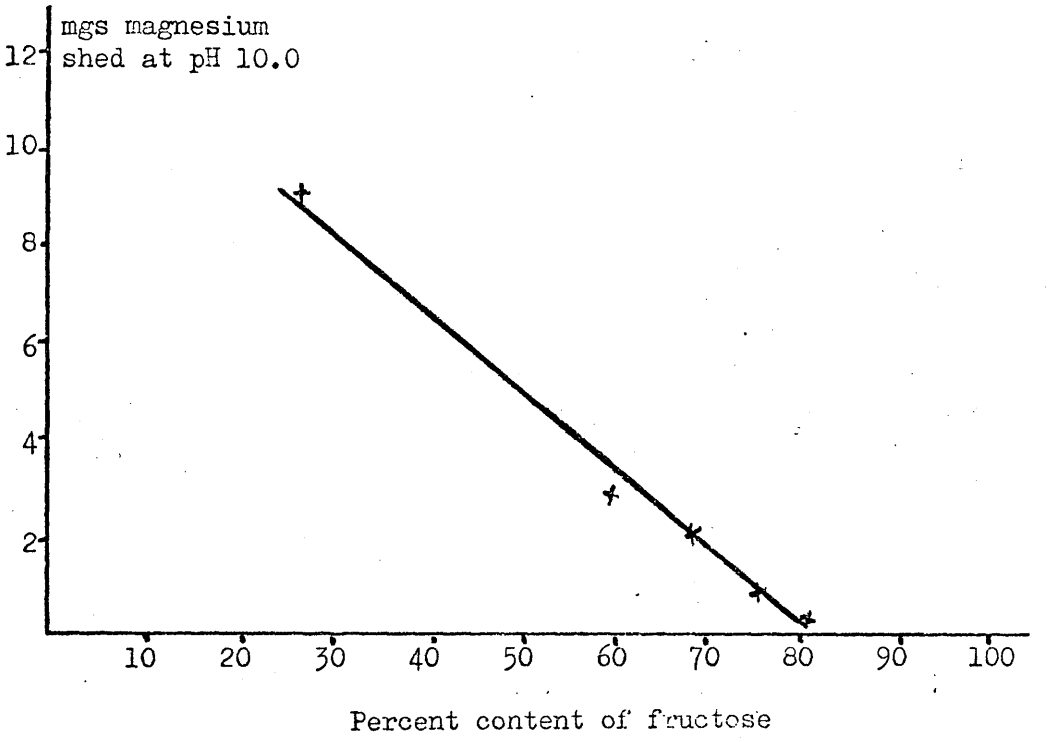


Figure 25

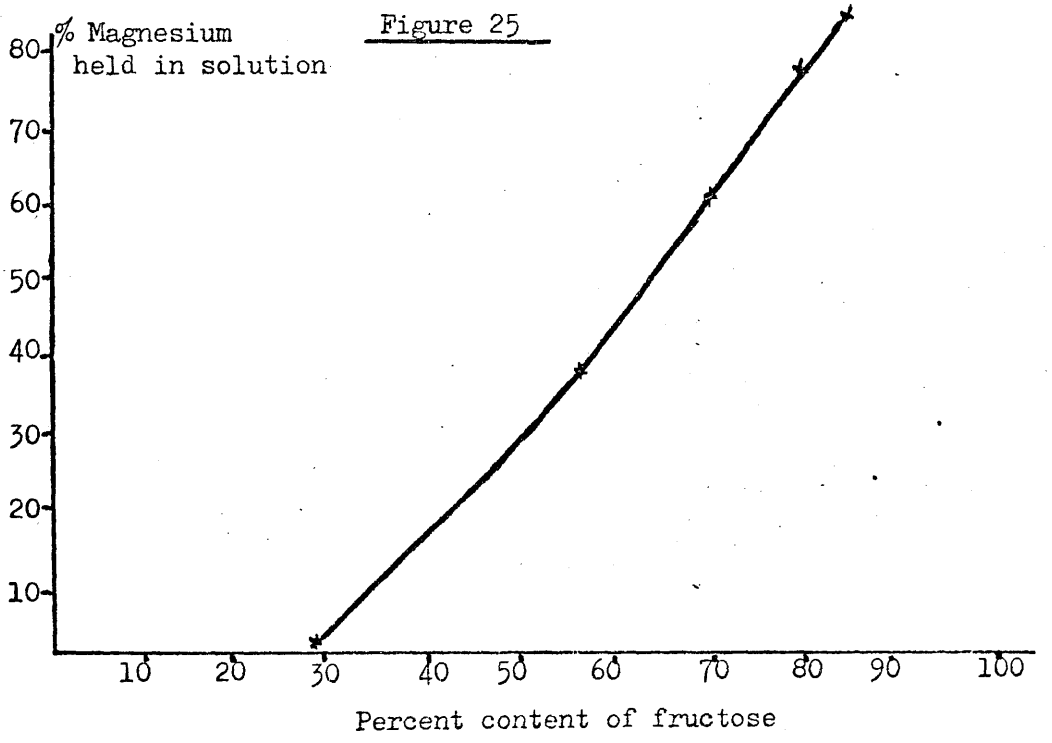


Figure 26

No such obvious correlation was observed in the case of calcium, as the results did not indicate any definite trend.

12. The level of phosphate present in the precipitate and the whole fructosan sample

Phosphate determinations were conducted in the same manner as previously, omitting the Lowry and Lopez determination, and the results tabulated (Table 40). The majority of the phosphate registered as being inorganic with a proportion being present as truly organic, as previously observed.

The weight of phosphate precipitated at pH 10.0 decreased as the 'true fructosan' content rose (Fig.27). In addition, a higher proportion of the total phosphate present appeared as organic, or bound, phosphate, as the 'true fructosan' content increased (Fig.28).

These results indicate that the actual level of 'true fructosan' in a sample of grass fructosan, determines the proportions of the various minerals which will be held in solution at unfavourable pH values. This was especially obvious in the case of magnesium, but was also demonstrated by the general behaviour of the inorganic constituents as a whole.

Sample	Method Used	mgs. as P	mgs. as PO_4
Italian Fructosan (complete)	Kings Total	3.34	10.2
	" Inorganic	2.18	6.68
Precipitate	" Inorganic	0.99	3.03
Perennial Fructosan (complete)	Kings Total	5.27	16.2
	" Inorganic	3.45	10.6
Precipitate	" Inorganic	1.67	4.9
Timothy Fructosan (complete)	Kings Total	12.24	37.5
	" Inorganic	10.6	32.5
Precipitate	" Inorganic	3.06	9.4
Cocksfoot Fructosan (complete)	Kings Total	25.4	77.8
	" Inorganic	24.1	73.8
Precipitate	" Inorganic	9.1	27.9
Original Fructosan (complete)	Kings Total	6.95	21.3
	" Inorganic	5.3	16.24
Precipitate	" Inorganic	4.6	14.0

Table 40

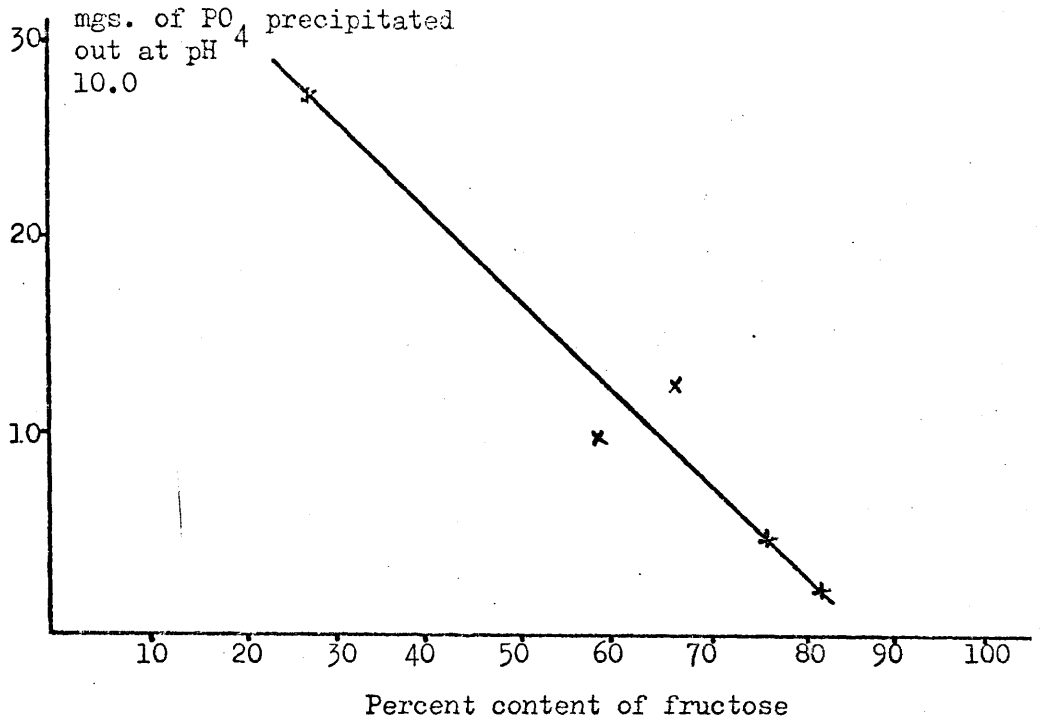


Figure 27

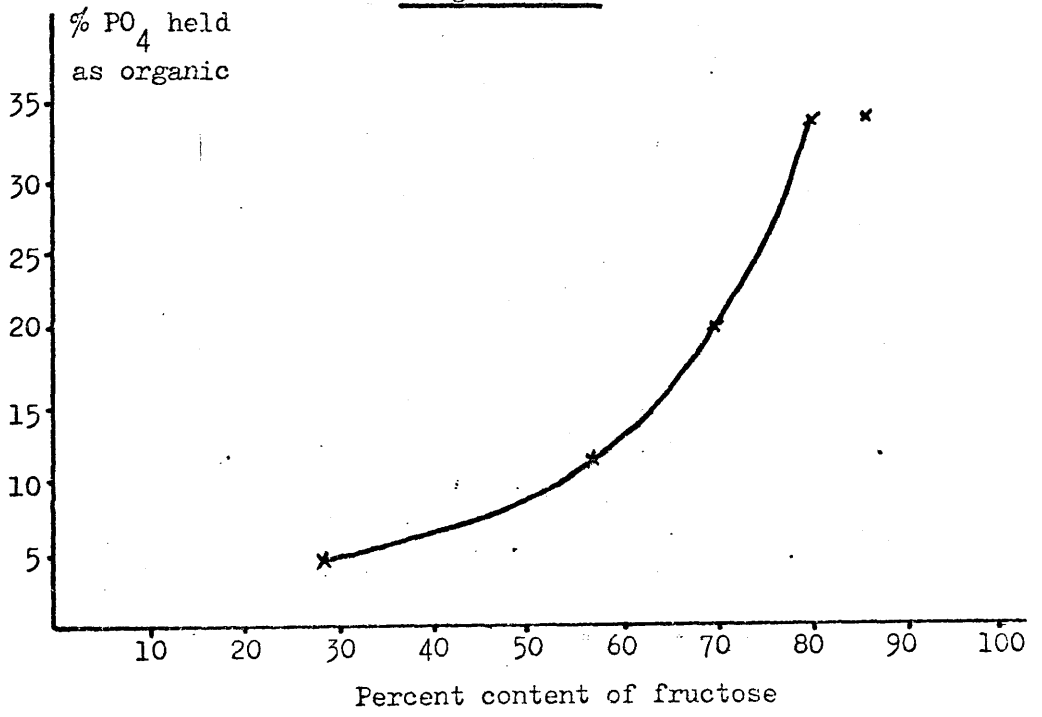


Figure 28

13. Summary

The foregoing tables and figures represent the results of the determinations carried out on the samples of extracted grass fructosan. In each case a high content of inorganic material was found to be associated with the fructosan when extracted by the mild procedure used. The behaviour of this mineral matter was similar in every case, in that a certain proportion precipitated out on adjustment to alkaline pH.

The investigation of the original fructosan sample indicated that the mineral matter was closely associated with the polysaccharide, since only around 15 per cent of the total present, precipitated out on pH adjustment to 10.0. It was further shown that addition of fructose prior to pH adjustment, resulted in less inorganic material being deposited.

This indicated that an increase in the 'true fructosan' content of the fructosan sample would also demonstrate a similar effect, and that a decrease in 'true fructosan' content would result in a greater quantity of insoluble material on addition of alkali.

Analysis of a further four grass fructosan samples indicated that this was indeed the case. As the 'true fructosan' content rose, both the quantity of inorganic material precipitated, and the percentage of the total mineral matter deposited, decreased, exhibiting an almost linear

relationship in both cases. Similarly, the levels of the individual minerals precipitating varied with the 'true fructosan' level. The element which showed the closest linear agreement, both on a weight and a percentage basis, was magnesium.

The pH at which precipitation commenced, also appeared to be influenced by the 'true fructosan' content, being pH 7.25 at a fructose content of 68.2 per cent and pH 8.5 at fructose contents of 75.1 and 80.6 per cent.

A degree of similarity is remarked upon between the results for the behaviour of the metal ions in these experiments, and the earlier experiments investigating the complexing ability of various carbohydrates with ferric iron.

14. Discussion of the possible significance of these findings.

This last section has shown that the polysaccharide fructosan, when extracted from grass using a mild procedure, is in intimate association with an appreciable content of inorganic constituents. By the very nature of this association, the polysaccharide exerts a considerable influence on the behaviour of this mineral matter. It is proposed to discuss here, the possible significance of such effects, in influencing the availability of minerals to animals, with particular emphasis on magnesium. A deficiency, or apparent deficiency, of this element, gives rise to the condition known as hypomagnesaemia, especially common in cattle and sheep.

The suggestion that the onset of hypomagnesaemia could be associated with the variation in herbage fructosan levels, was first put forward by Rees (1) in 1963. It is this suggestion which will be developed in the following discussion.

The conditions prevalent in the small intestine, where magnesium absorption is thought to take place (165, 166, 167, 168), tend to be alkaline, ranging from pH 5.5 to 6.2 in the first section, and from pH 7.4 to 8.0 in the latter section, although higher pH values have been observed under certain conditions. Storry (169) reports that the abomasal pH, usually 2.5, of two sheep transferred to spring grass, rose above pH 5.0 in four hours, and that a pH of 6.5 was noted in the abomasum of a cow which had died from hypomagnesaemia. High abomasal pH values such as these would result in a corresponding pH increase in the small intestine. Storry concluded that tertiary calcium phosphate could be expected to precipitate at pH 6.5, and that magnesium could be shed as magnesium ammonium phosphate, thus rendering the calcium and magnesium unavailable to the animal.

The possible significance of the interrelationship of the mineral constituents and fructosan can now be seen. Firstly, the expected precipitation point of pH 6.5 is raised to pH 7.25 when a 'true fructosan' content of 68 per cent is

present, and is further raised above pH 8.5 with 'true fructosan' contents of around 80 per cent. Secondly, precipitation in the presence of fructosan is a gradual process, the quantity increasing as the pH rises. Thirdly, considerably reduced quantities of mineral matter are shed from solution at high pH as the 'true fructosan' content rises.

Thus, increasing the level of fructosan in the diet of an animal is likely to result in increased availability of magnesium, by raising the pH at which precipitation will commence, and by reducing the quantity of mineral shed from solution and thus made unavailable.

It is recalled that numerous workers have reported enhanced uptake of minerals from the gut when sugars are present (170, 171), the sugars including fructose (172); glucose (67, 47); lactose (48); and the sugar alcohol sorbitol (49, 50, 51). Polysaccharides do not appear to have been studied from this aspect. It is of interest to note that Lengeman (173) has shown that sugars must be present concomitantly with the metal in order to promote uptake.

If increasing levels of fructosan increase the availability of magnesium, then the corollary that decreasing levels of fructosan will decrease the availability, will also hold true. The total loss of magnesium on pH adjustment of

the Cocksfoot sample, which possessed a very low 'true fructosan' content, being an extreme example.

It is pertinent to indicate at this point that the incidence of hypomagnesaemia can be related to low herbage fructosan levels. In dairy herds the peak incidence of hypomagnesaemia occurs during the early growing season, with a smaller peak in some years associated with a flush of grass in the autumn (174). Low levels of herbage fructosan are associated with both these periods, being at their lowest during the early growing season and suffering depletion during any period of rapid growth (133). Hemingway et al (175) concluded that the rise in deaths due to hypomagnesaemia which occurs in early spring, can often be associated with a mild spell of weather inducing rapid herbage growth. Similarly, mild weather early in the growing season and the resultant rapid growth, interrupts the usually rapid build-up of fructosan reserves, which remain at a very low level (133). Cases of hypomagnesaemia are more prevalent on highly manured herbage (176, 177, 178), just as high fertilizer levels, especially nitrogenous fertilizers, have been shown to reduce fructosan levels considerably (130, 136, 139, 140, 143, 144, 145).

Low levels of herbage fructosan can not only be related to the incidence of hypomagnesaemia, but can also be related directly to magnesium availability. Research on balance studies has shown that the generally accepted figure for

magnesium availability is around 20 per cent (179) although this figure can vary considerably. It is probably due to this factor that hypomagnesaemia can occur on pasture which contains as much magnesium as pasture which does not produce tetany (180, 181, 182, 183, 184). There is evidence that young grass cut early in the season possesses lower than average availability of magnesium (170, 185), coinciding with the lowest level of fructosan. Similarly the availability of magnesium is higher in mature grasses; where the level of fructosans is at a peak (133). Herbage dressed with nitrogenous fertilizer possesses magnesium of lower than average availability (170, 185), and protein supplements decrease the availability of magnesium in the diet (170, 186). These last two facts may be compared with the fact that grasses dressed with fertilizer possess a depressed fructosan content, while the protein content is raised considerably.

Todd (187, 188) determined the distribution of magnesium in plant tissue into three fractions: acetone soluble; water soluble, and water insoluble. In young spring grass of low magnesium content, around one third of the magnesium was insoluble, whereas only one quarter to one fifth was insoluble in pasture at the heading stage. Little more than 50 per cent of the magnesium was water soluble in the first case, with a higher proportion in the latter. In

addition he found that over 50 per cent of the total herbage magnesium was associated with the water soluble constituents of grasses which represented only 25 per cent of the total dry matter. This fraction is largely composed of carbohydrate material, which is almost exclusively fructosan. Further, any fluctuations in magnesium content were to a large extent fluctuations in water soluble magnesium, the levels of the other fractions varying little.

The evidence in the last three paragraphs is largely circumstantial, but when considered in relationship to the results obtained in this study, there is a strong indication that the level of fructosan in herbage may play a hitherto unappreciated role in determining the availability of magnesium, and possibly other metals, to animals. Clearly, as stated by Rees (1), the final answer will lie in well designed nutritional experiments with animals.

I N D E X

to

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G.M.1 Paper chromatography

The descending technique of Martin (189) was utilised. Use was made throughout this study of Whatman No.1 filter paper, the solutions for analysis being spotted at one inch intervals, along a line drawn approximately three inches from one end of the sheet.

Generally two papers, each with the same sample spots were run, one in aqueous phenol solvent (160 grams Analar phenol: 40 mls water) and one in ethyl acetate; pyridine; water solvent (120: 50: 40).

After irrigation with the eluting solvent, the paper was withdrawn from the tank (Shandon 500 Chromatank) and, in the case of the latter solvent allowed to dry at room temperature. Chromatograms run in aqueous phenol were dried in a circulating air oven at 60°C.

Sugar detection reagent

Throughout the study the silver nitrate reagent of Trevelyan et al (158) was used. This reagent detects sugar alcohols in addition to aldoses and ketoses. Three separate solutions are required.

1. Silver nitrate in acetone ; saturated aqueous silver nitrate solution (0.5 ml) was diluted with acetone (99.5 ml) and water added dropwise with shaking, until the precipitate of silver nitrate just dissolved.

2. 0.5 Normal sodium hydroxide in ethanol: sodium hydroxide (2 grams) was dissolved in the minimum quantity of water and the solution diluted to 100 mls with ethanol.

3. 6 Normal ammonia.

The technique was as follows:

After dipping in the silver nitrate solution, the paper was allowed to dry, then dipped into the sodium hydroxide solution. When the spots had developed the required intensity, the dark background was removed by immersion of the paper in the ammonia solution. The paper was then washed in running water for several hours and dried. Fresh reagents were made up each time chromatograms were developed.

Iron detection spray

Tests were made with various reagents which form coloured complexes with iron, as to their suitability for use to detect iron on chromatograms. Many were of limited use on paper, the most useful being: 5% acid thiocyanate in ethanol: 5 grams of potassium thiocyanate were dissolved in the minimum quantity of dilute hydrochloric acid, and made up to 100 mls with ethanol.

On spraying, this gave a deep red colouration with iron, which lasted for several hours before fading. It proved to be a fairly sensitive reagent and was used extensively when attempting to locate iron.

G.M.2. Electrophoresis

The method used was essentially the immersed strip method of Smith (190). Strips of Whatman No. 1 filter paper, measuring 15 inches by 4 inches were used. Solutions to be tested were spotted three quarters of an inch apart, along a line drawn 5 inches from one end of the strip.

After spotting, the strip was saturated in a solution of the buffer to be used. Firstly one end of the strip was immersed in the buffer and the solution allowed to saturate the paper, up to the origin, by wick action. This was followed by removal of excess buffer between sheets of filter paper. The other end of the strip was then treated in a similar manner. This enabled the whole strip to be saturated in the desired buffer, without the sample spots diffusing and increasing in area.

The strip was then immersed in Analar carbon tetrachloride, with each end dipping into a vessel containing the buffer being used. The origin was submerged in the carbon tetrachloride, to an extent of one inch. Use of this non polar solvent was made in order to exert a cooling effect on the paper. Electrodes were then inserted into the buffer containing vessels, the negative electrode in the vessel adjacent to the origin, and the positive electrode in the vessel opposite.

On applying a voltage to this system, negatively charged species migrated from the origin, along the paper strip,

towards the positive electrode.

After running the apparatus for 1 hour at a voltage of 500 volts the current was switched off, the paper removed and dried, and fructose and iron detected. The detection methods were those used in G.M.1.

Fresh Analar carbon tetrachloride was used for each electrophoresis run. This procedure has been found to give better results than repeated usage of the same coolant, since it precludes the formation of a skin of buffer on the surface of the coolant. Such a feature arises due to electro-osmosis, and can result in overheating and disruption of the circuit.

Many different buffers were used, of which the following is a list.

1. Borax-Sodium hydroxide - 0.1 Molar in terms of borate (191)

   A - Borax - 19.05 grams in 1 litre (0.2 Molar in terms of Borate)

   B - Sodium hydroxide - 8 grams in 1 litre (0.2 Molar)

   pH 10.0 50 mls of solution A + 43 ml solution B, and make up to 100 mls with water.

2. Boric Acid-Borax 0.1 Molar in terms of borate (192)

   A - Boric acid - 12.4 grams in 1 litre (0.2 Molar)

   B - Borax - 19.05 grams in 1 litre (0.2 Molar in terms of Borate)

   pH 9.0 10 mls solution A + 40 mls solution B, and dilute to 100 mls with water

3. Carbonate - Bicarbonate 0.10 Molar (193)

A - Anhydrous sodium carbonate - 21.2 grams in 1 litre (0.2 Molar)

B - Sodium bicarbonate - 16.8 grams in 1 litre (0.2 Molar)

pH 10.0 27.5 mls of solution A + 22.5 mls of solution B diluted to a total of 100 mls with water

pH 9.2 4.0 mls of solution A + 46.0 mls of solution B diluted to a total of 100 mls with water

4. Bicarbonate - carbon dioxide (194)

pH 8 concentration of sodium bicarbonate - 5.86×10^{-2} Molar

This buffer, to be made up correctly, should have the given concentration of sodium bicarbonate in equilibrium with a gas phase containing 5 per cent carbon dioxide. However, it was impossible to adhere exactly to these conditions during electrophoresis. To approximate these conditions, carbon dioxide was bubbled through the sodium bicarbonate solution until the pH of the buffer was reached. This solution was then utilised for the electrophoresis run, a small quantity of solid carbon dioxide being placed inside the cabinet holding the electrophoresis equipment.

That this was effective was ascertained by checking the pH of the used buffer after the run.

pH 7.0 concentration of sodium bicarbonate 5.86×10^{-3} Molar made up and used in the same way as the above buffer.

5. Phosphate 0.1 Molar (195)

A - Di sodium hydrogen phosphate 35.61 grams in 1 litre (0.2 Molar)

B - Sodium di hydrogen phosphate 31.21 grams in 1 litre (0.2 Molar)

Di-hydrated salt in each case

pH 8.0 94.7 mls of solution A + 5.3 mls of solution B diluted to 200 mls with water.

pH 7.0 61.0 mls of solution A + 39.0 mls of solution B diluted to 200 mls with water

6. Citrate 0.10 Molar (196)

A - Citric acid 42.02 grams in 1 litre (0.2 Molar)

B - Sodium citrate (dihydrated) 58.82 grams in 1 litre (0.2 Molar)

pH 6.0 9.5 mls of solution A + 41.5 mls of solution B diluted to 100 mls with water

pH 5.0 20.5 mls of solution A + 29.5 mls of solution B diluted to 100 mls with water

pH 4.0 33.0 mls of solution A + 17.0 mls of solution B diluted to 100 mls with water

G.M.3. Development of a suitable iron determination method

The iron determination method necessary for this study was required to yield consistent results in the presence of

relatively high concentrations of sugars. Bloomfield (197) and Debs (198) both used colorimetric methods which required the destruction of organic matter before determination. In the former, organic matter was destroyed by the action of nitric and sulphuric acids, and in the latter by hydrogen peroxide and sulphuric acid. This was not entirely suitable for use in this study, since so many solutions had to be analysed for iron that time consumption would have been prohibitive.

Firstly an EDTA titration method was tested, with rather disappointing results. This was based on a method by Welcher (199) and comprised the formation of the deep red coloured thiocyanate-iron complex at low pH, followed by titration with standard EDTA until colourless.

With a high sugar concentration, however, the coloured complex only formed very slowly towards the end of the titration. Presumably this was due to the sugar interfering in the colour reaction. The method was eventually abandoned as being too time-consuming.

A colorimetric method based on the formation of the 1,10 phenanthroline complex with ferrous iron was modified to suit the necessary requirements. The method, quoted by Charlot (200), involved the use of 25 per cent sodium acetate to adjust the solution to pH 3.5, followed by addition of 1 ml of 10 per cent hydroxylamine hydrochloride to reduce the iron

present; and addition of 1 ml of 0.5 per cent 1,10 phenanthroline. This was left for 1 hour, made up to standard volume, and read at 480 - 520 mμ.

To obtain accurate iron analysis figures in many of the experiments, it was necessary to ascertain that any iron present was completely dissolved, and that any sugar-iron complex was dissociated. It was decided, therefore, to carry out the determination at pH 1.4. Consequently pH adjustment of the solution was made with dilute hydrochloric acid, rather than sodium acetate.

The method finally used was :-

An aliquot generally 1.0 ml of iron solution was taken and adjusted to pH 1.4 with dilute acid, 1 ml of 10 per cent hydroxylamine hydrochloride was added, followed by 1 ml of 0.5 per cent 1,10 phenanthroline. After standing for 1 hour, this was made up to standard volume (25 ml) and read at 495 mμ with a Unicam S.P.500 using 1 cm cells.

This gave a linear graph up to 110 gamma of iron and was completely unaffected by the presence of sugars, even up to a 2,000 to 1 excess. A standard graph was constructed each time a batch of solutions was analysed. An extremely reliable method, it was used for all the iron determinations carried out in this study, with the exception of the fructosan analysis work given in Section III.

While this work was being carried out, the department acquired a Unicam S.P.90 Atomic Absorption Spectrophotometer. The results given in Section III were obtained by the use of this equipment. All that was required was dilution of the sample to below 110 gamma per ml, followed by measurement of the absorbance. A standard graph was repeated each time a batch of readings was carried out. The method was found to be very reliable and consistent.

G.M.4. Magnesium determination.

As with the iron determination method given in G.M.3, the magnesium determination method had to give consistent results in the presence of sugar. The method used in this study was an EDTA titration method using Eriochrome Black T as indicator (201).

Reagents.

1. Standard EDTA : 0.02 Normal disodium EDTA made up from standard Volucon ampules supplied by May and Baker.
2. Buffer pH 10.0 : 65.0 grams of ammonium chloride dissolved in water. 570 mls of ammonium hydroxide (S.G. 0.88) were added and the whole diluted to 1 litre with water.
3. Eriochrome Black T : a 1:200 mixture of dye and sodium chloride, ground to a fine powder (202)

Procedure

To an aliquot of solution containing magnesium, 5 mls of buffer solution were added, followed by sufficient solid Eriochrome Black T to impart a distinct wine red colour. This was titrated with standard EDTA until a distinct blue colour was achieved.

Reliable results were obtained with this method, and all the magnesium determinations were carried out using this technique, except for the results obtained during the fructosan analysis.

As in the case of iron determinations associated with this work, the magnesium content was measured by use of the S.P.90 Atomic Absorption Spectrophotometer.

This merely involved dilution of the magnesium containing solution to within the range 0.4 to 1.6 gamma of magnesium per ml. This was effected with an EDTA solution so that the resultant strength of the EDTA solution in the diluted sample was 0.75 per cent. Measurement of the absorbance was then carried out.

A standard graph was constructed for each batch of samples tested. Here again, the ultimate strength of the EDTA solution in the standards was 0.75 per cent.

Addition of EDTA solution was carried out to overcome suppression of the reading by phosphate (203).

G.M.5. Calcium determination

The quantitative measurement of calcium involved in the fructosan investigation experiments was carried out by means of direct reading on the S.P.90. This was based on the method of Willis (203).

The technique involved dilution of the calcium-containing solution to within the range 0.0 to 6.0 gamma per ml. This was effected with an EDTA solution so that the resultant strength of the EDTA in the diluted sample was 0.75 per cent. Measurement of the absorbance was then carried out.

A standard graph was constructed for each batch of samples tested. Here again, the ultimate strength of EDTA in the standards was 0.75 per cent.

As in the case of the magnesium determination, EDTA was added so that suppression of the reading by phosphate would not occur.

G.M.6. Fructose analysis.

Just as determination methods unaffected by sugars had to be found for iron and magnesium analysis, so had a fructose determination method unaffected by iron to be found. Bacon and Bell's modification (160) of Roe's resorcinol method (161) was used to determine the fructose concentration in solutions.

Reagents.

1. 0.15 per cent resorcinol in ethanol
2. Concentrated hydrochloric acid containing 7.5 mg of ferric chloride per litre.

Procedure.

A 2 ml sample of the solution to be analysed was taken, and 3 mls of 0.15 per cent resorcinol added. After 1 minute, 3 mls of the hydrochloric acid reagent was added. The solution was heated in a water bath at 75°C for 30 minutes, in a firmly-stoppered Quick-fit test tube. After heating, the tubes were rapidly cooled in ice-water to prevent any increase in colour intensity. The tubes were then read at 480 mμ on the Unicam S.P.500. This method produced a linear graph up to 150 gamma of fructose.

It was found, however, by conducting extensive tests, that the colour produced was influenced by the quantity of iron present, which suggested that the method would be completely unsuitable for the required work.

By carrying out extensive analyses of standard solutions with various additions of iron, it was found that with additions of iron between 45 and 90 gamma, the graph obtained was constant, although higher than the graph obtained with no added iron.

Thus, where iron and fructose were to be determined, the iron analysis was always carried out first, to find the iron content per ml. The procedure followed then depended on the content of iron.

1. Where the iron content was less than 45 gamma per ml, one ml of the solution to be tested was taken and one

ml of a dilute iron solution was added to bring the iron content up to between 45 and 90 gamma. A complete range of dilute iron solutions was kept for this purpose.

2. Where the iron content fell between 45 and 90 gamma per ml, one ml of the solution to be tested was taken and one ml of water added.

3. If the iron content was above 90 gamma per ml, then the solution was diluted, by a known factor, so that the iron content of the solution to be analysed fell between 45 and 90 gamma per ml. The solution was then treated as in procedure 2.

This procedure replaced the 2 ml sample used in Bacon and Bell's method. The remainder of the determination was carried out as previously detailed.

The modification of the method as above, gave very accurate results which were completely reproducible. As with all other quantitative determinations, a standard curve was constructed for each batch of solutions analysed. Here too, one ml of standard fructose was taken and one ml of dilute iron solution, containing between 45 and 90 gamma, was added.

G.M.7. Organic carbon determination.

The procedure used was a wet combustion method.

Procedure

To 0.5 grams of solid in a 500 ml conical flask, the following were added:

25 mls 0.4 Normal potassium dichromate (Standardised)

25 mls Analar sulphuric acid

12.5 mls Phosphoric acid (S.G. 1.75)

A coldfinger was fitted to the flask which was then boiled gently for $1\frac{1}{2}$ hours. This was followed by cooling and addition of 100 mls of water. The residual dichromate was titrated with standardised 0.2 Normal ferrous ammonium sulphate, using 5 mls of barium diphenylamine sulphonate as indicator, until a bright green colour appeared.

1 ml 0.4 Normal dichromate = 1.2 mg carbon

The indicator was made up by dissolving 0.5 grams of barium diphenylamine sulphonate in water, adding 38.7 grams of barium chloride, (dihydrated) and making the whole up to 1 litre.

Note: 1 equivalent of potassium dichromate is equal to one-sixth of its molecular weight.

G.M.8. Extraction of the fructosan.

10 Kilograms of herbage, freshly cut as close to the ground as possible, were treated with boiling ethanol. The grass was cut in small batches and immediately plunged into 10 litres of boiling ethanol in a 20 litre, parallel-walled, heated glass extraction vessel. After 5 minutes immersion the grass was removed, oven dried at 60°C and hammer milled. The 2 Kilograms of dry powder obtained was made up to a fine slurry with 10 litres of water, allowed to stand for 1 hour with a

small quantity of chloroform added, and was then squeezed dry in a press. This extraction procedure was repeated with a further 10 litres of water, allowing the grass to remain in the extraction solvent overnight at 4°C, again with chloroform added. The supernatant from the first extraction was stored in a similar way. After obtaining the supernatant from the second extraction by means of the press, the extracts were bulked and filtered under light suction through a pad of Hi Flo Super Cel. In all, 18 litres of extract were obtained.

Using a large vacuum distillation apparatus, and a flash evaporator, the filtrate was reduced in volume to 3 litres, at a temperature no higher than 30°C. This was then poured into 3 volumes of cold ethanol, giving a heavy brown rubbery precipitate. By successive retreatments with cold ethanol, this rubbery precipitate was obtained in a more workable condition, and much of the colour was removed.

This was then redissolved in water, using the minimum quantity, again adding chloroform. After shaking for some time the residue was centrifuged down on an M.S.E. Magnum centrifuge at 2,000 revs per minute, using the 6 x 250 swing-out head. The supernatant was decanted and the residue was again shaken with water, followed once again by centrifugation. After bulking the supernatants the polysaccharide was reprecipitated in 3 volumes of cold ethanol.

This reprecipitation procedure was repeated a further two times. After the last reprecipitation the by now light-cream coloured precipitate was collected by centrifugation. During this last step it was found to be most important to keep the temperature low, in order to prevent water pick-up and subsequent tackiness developing in the precipitate.

The precipitate was dried by use of dry ethanol, dry acetone, and 40-60 petroleum ether. This yielded 159 grams of fructosan. The use of a small quantity of chloroform each time the fructosan was dissolved in water performed the function of inhibiting the bacterial breakdown of the polysaccharide. All the stages where the fructosan was dissolved in water were restricted to the minimum time duration possible

G.M.9. Moisture content determination.

The moisture content of the fructosan was determined by weighing out 1 gram of the powder into a silica dish. This was placed in a pistol which was maintained in an evacuated condition by means of a water-pump. Drying at 60°C for 24 hours in the presence of phosphorous pentoxide was then carried out. After this treatment the fructosan was allowed to cool, still being maintained in an evacuated environment in the presence of phosphorous pentoxide. Immediate weighing then followed, and the loss in weight due to moisture was calculated.

G.M. 10. Acid hydrolysis.

The procedure used in this study was as follows:

A small quantity, around 0.1 gram of the fructosan was taken and hydrolysed for 1 hour at 100°C with 5 mls of 0.2 Normal formic acid. After hydrolysis, the solution was transferred to a round-bottomed Quickfit flask, 50 mls of water added, and the whole reduced in volume by vacuum distillation in a Buchi rotary evaporator.

According to Horsley (157) a water and formic acid mixture will distil off azeotropically, the composition of the azeotrope being 77.5 per cent formic acid and 22.5 per cent water.

The addition of water, followed by vacuum distillation, was carried out 4 times. On testing with Universal paper the solution was found to be neutral. This solution can, therefore, be spotted directly onto chromatography paper.

G.M.11. Determination of total nitrogen present.

This was carried out using a modification of the Kjeldahl method. Since this method was first used, many modifications have been tried, a full review of these being given by Kirk (204).

Procedure.

2 Grams of dry fructosan were weighed out and transferred to a dry Kjeldahl flask. After addition of 30 mls of Analar sulphuric acid, 5 grams of sodium sulphate, and a

crystal of copper sulphate, the solution was digested over a low flame. On completion of frothing and charring, the contents of the flask were seen to be quite fluid. The flame was turned up and the mixture boiled until all the brown colouration had disappeared.

After cooling, the contents of the flask were diluted with water until around half full. The flask was then clamped in the distilling apparatus. At the receiving end was placed a conical flask containing 50 mls of standard 0.1 Normal hydrochloric acid, a few drops of indicator, and sufficient water to cover the end of the delivery tube. Sufficient 50 per cent sodium hydroxide to make the solution alkaline was then added to the Kjeldahl flask by means of a filter funnel with a glass tube extending to the bottom of the flask. Finally a piece of granulated zinc was added to the flask, the stopper inserted, and the contents mixed by swirling. The flask was then heated to boiling and boiled for around 30 minutes, or until the ammonia had stopped distilling over into the receiving flask.

The receiving flask contents were then titrated with standard 0.1 Normal sodium hydroxide, and from the amount of 0.1 Normal hydrochloric acid used to absorb the ammonia, the nitrogen content of the sample was determined.

G.M. 12. Test for reducing capacity.

This was tested for by means of Fehling's test (205).

Reagents.

A. 34.6 grams of pure copper sulphate were dissolved in water and diluted to 500 mls.

B. 175 grams of potassium tartrate and 70 grams of pure sodium hydroxide were dissolved in water and diluted to 500 mls.

Procedure.

Equal quantities of A and B were mixed immediately before use and added to the solution to be tested. On heating, the formation of a red precipitate of cuprous oxide would indicate the presence of a reducing substance.

G.M. 13. General carbohydrate test.

The general qualitative carbohydrate test, Molisch's test (206) was used.

Procedure.

To 1 ml of the solution to be tested were added 2 drops of a 15 per cent α-napthol solution in ethanol. This was then underlayered with 1 ml of Analar sulphuric acid. A red-violet ring would appear at the interface if sugars were present.

G.M. 14. Protein test.

The qualitative test used here was the Biuret test (207).

Reagent: 1.50 grams of cupric sulphate and 6.0 grams of sodium potassium tartrate were dissolved in 500 mls of water. To this was added with swirling 300 mls of 10 per cent sodium hydroxide (carbonate free). The whole was diluted with water

to 1 litre and stored in a paraffin lined bottle.

Procedure.

1 ml of solution to be tested was taken, and 4 mls of the biuret reagent added. This was allowed to stand for 30 minutes at room temperature. Slight variations in the intensity and tint of colour produced occur, depending on the material used (208). Generally the colour would be bright violet.

G.M. 15. Tests for phenols.

Since no general test for phenols exists two tests were conducted on the sample.

1. Millon's test (209)

Reagent: 1 part mercuric chloride was dissolved in 1 part of fuming nitric acid, and diluted with 2 parts of water.

Procedure. To 1 drop of the solution to be tested, 1 drop of the reagent was added, and the mixture allowed to stand in a micro-crucible. If no change occurs the mixture is heated briefly to boiling. A red colouration would form in the presence of phenols.

2. Complex cobaltic salts (210)

Procedure. To 1 drop of the test solution, 1 drop of freshly prepared 5 per cent sodium cobaltinitrite, and 1 drop of glacial acetic acid were added. A blank test with water was

also carried out. Both tubes were then heated until the water blank acquired a pink colour. A positive response in the unknown would be given by a brown to yellow colour or a brown precipitate.

G.M. 16. Phosphate determination.

Initially the method used to determine the phosphate content was the modification of King's method (211), as proposed by Allen (212). This involved the substitution of Amidol reagent for the 1-amino-2-sulphonic acid reagent previously used. The method may be used to determine both the total phosphate and the inorganic phosphate.

Inorganic phosphate procedure:

To the sample containing up to 100 gamma of phosphorous the following reagents were added:

A - 1.2 mls 60 per cent perchloric acid

B - 1.0 mls 5 per cent ammonium molybdate

C - 0.5 mls Amidol reagent

Water was then added to give a standard volume of 15 mls, the sample mixed by shaking, and the colour intensity measured at 660 mμ.

Total phosphate procedure.

When the total phosphate was to be determined, combustion was performed in the above amount of perchloric acid. This was followed by addition of the other reagents when cool. A standard curve was constructed for each batch

of solution analysed.

The Lowry and Lopez Method (213)

As was stated in the text, the Lowry and Lopez method for the determination of inorganic phosphate is very much milder than the equivalent King's determination.

Reagents:

A - Acetate buffer pH 4.0 (0.1 Molar in acetic acid and 0.025 Molar in sodium acetate)

B - 1 per cent ascorbic acid

C - 1 per cent ammonium molybdate in 0.025 Molar sulphuric acid.

Procedure.

The sample, of which the inorganic phosphate content is within the range 0.075 to 0.5 milli moles (2.5 to 15.0 gamma per ml of phosphorous) was diluted five times with solution A.

To 10 mls of the resulting solution, 1 ml of solution B, and 1 ml of solution C were added. The solutions were then read at 700 mμ on the Unicam S.P.500, firstly after 5 minutes, then after 10 minutes standing. The two values were extrapolated to zero time to obtain the true inorganic phosphate content. A standard curve was constructed for each batch of solutions analysed.

G.M. 17. Ash determination.

The dry ashing technique of Humphries (214) was applied to this determination.

Procedure.

1 gram of oven dried fructosan was weighed out into a silica dish. The material was then charred preliminarily over a low bunsen flame, previous to placing it in a muffle furnace at 490°C. The time required for complete ashing depends on the material being used, and details of this have been given in the text. Once a pure white ash was obtained the silica dish was cooled under vacuum, and a weighing carried out to determine the weight of ash present in 1 gram of fructosan.

The silica dish was then covered with a clock-glass and 40 mls of dilute hydrochloric acid (1:1) were cautiously added. This was then heated on a boiling water bath for 30 minutes. After this time had elapsed, the clock-glass was rinsed and removed, and heating continued for a further 30 minutes to dehydrate silica. 10 mls of hydrochloric acid (1:1) and 50 mls of water were then added. The solution was kept warm on the water bath to dissolve soluble salts.

The contents of the dish were then filtered through No.44 Whatman filter paper into a volumetric flask, and the residue washed from the silica dish into the filter paper. This was then followed by washing with hot dilute hydrochloric acid, after which the solution in the volumetric flask was made up to volume.

The residue in the filter paper consisted almost entirely of silica. After ignition of the filter paper in the silica dish, a further weighing was carried out to determine the silica content of the fructosan.

G.M. 18. Qualitative analysis of ash constituents.

The solution obtained from the above procedure was subjected to routine cationic analysis by an elementary semi-micro qualitative analysis method (215, 216).

G.M. 19. Total hexose determination.

The method used to determine the total hexose content of the fructosan was the phenol-sulphuric acid method (217).

Procedure.

To 1 ml of fructosan solution, 1 ml of 5 per cent phenol and 5 ml of Analar sulphuric acid were added, and the tube thoroughly shaken to promote mixing. After standing for 20 minutes, the colour intensity was read at 490 mμ.

A standard curve was constructed each time a batch of solutions was analysed.

Experimental Data

Exp.No.1. Paper Chromatography (p.30)

25 ml Aliquots of 0.02 Molar ferric chloride were added to weighed quantities of fructose, glucose, galactose, and mannose (0.09, 0.18, 0.36, 0.90, 1.8, 2.7 and 4.5 grams) to provide molar ratios of sugar to iron of 1:1, 2:1, 4:1, 10:1, 20:1, 30:1 and 50:1 respectively. Samples were then spotted onto Whatman No.1 filter paper, along with 0.02 Molar ferric chloride and sugar samples as standards. In addition, mixed ferric chloride/sugar solutions previously adjusted to pH values from 3.5 to 10.0 were chromatographed.

After irrigation in aqueous phenol (160 grams Analar phenol:40 mls water) and ethyl acetate: pyridine: water (120:50:40) solvents, the papers were dried and iron and sugar located as in G.M.1.

Fructose was the main sugar tested.

Exp.No.2. Chromatography on a synthetic fibre sheet (p.32)

A series of solutions exactly equivalent to those used in Exp.No.1 were spotted onto 'paper nylon' sheets and chromatographed in ethyl acetate: pyridine: water solvent (120:50:40). After drying the sheets were treated with iron and sugar locating reagents.

Exp.No.3. Investigation of the electrophoretic properties of sugars and iron in buffers of different pH (p.33)

25 ml Aliquots of 0.02 Molar ferric chloride solution were added to weighed quantities of fructose (1.8, 2.7 and 4.5 grams) to provide molar ratios of fructose to iron of 20:1, 30:1 and 50:1 respectively. In addition, fructose and ferric chloride were run as standards. Six spots of each sample were spotted onto strips of Whatman No.1 filter paper, and electrophoresis as described in G.M.2 carried out. Fructose and iron were located as in G.M.1. Details of the buffers used are presented in G.M.2.

Similar tests were conducted using fructosan, glucose, galactose, xylose, sucrose and lactose.

Exp.No.4. Investigation of the pH of precipitation of ferric hydroxide in the presence and absence of sugars (p.41)

25 ml Aliquots of 0.02 Molar ferric chloride were added to weighed quantities of fructose (0.090, 0.18, 0.36, 0.90, 1.8, 2.7 grams) to provide fructose to iron ratios of 1:1, 2:1, 4:1, 10:1, 20:1, 30:1 respectively. A 25 ml aliquot of 0.02 Molar ferric chloride with no sugar addition was also used for comparison.

1.0 N sodium hydroxide was added slowly to the rapidly stirred solutions, allowing $\frac{1}{2}$ minute between addition of each drop of alkali for equilibrium to be reached. Fine adjustment

of pH was effected by addition of minute quantities of alkali on the end of a thin glass rod.

Observations were made and the exact point of precipitation in each solution was determined. In addition, by continued addition of alkali, the point of re-solution of the precipitate was observed.

Mannose, lactose, glucose, xylose, and mannitol were all examined by making up equivalent solutions and observing their behaviour on addition of alkali.

Previous to pH adjustment the effect of sugar additions on the colour intensity of the ferric chloride solution was determined by reading the percentage transmittance of each solution, using the 602 filter in an EEL colorimeter. The absorption spectra of a 0.02 Molar ferric chloride solution, and a solution with fructose added to provide a 20:1 fructose: iron ratio, were measured on a Unicam S.P.800 automatic spectrophotometer, over the visible region, in a 1 cm cell, against water as standard.

Detailed examination of the 20:1 fructose to iron solution was effected by making up several such solutions, as previously described, and adjusting them to pH 5.8, 6.05, 6.2, 6.4, and 6.9 respectively, with sodium hydroxide. They were then allowed to stand and observations were made.

Exp.No.5. Measurement of the quantity of iron remaining in solution at a range of pH values, with increasing ratios of fructose (p.52)

25 ml Aliquots of 0.02 Molar ferric chloride solution were added to weighed quantities of fructose to provide ratios of fructose to iron from 1:1 to 20:1. The pH of each solution was then raised by addition of 1 N sodium hydroxide. Aliquots were withdrawn at predetermined pH values, which after standing for ½ hour, were centrifuged, and the quantity of iron in the supernatants determined (G.M.3).

Centrifugation was effected on an MSE 'High-Speed 17' centrifuge at 15,000 r.p.m., developing around 26,000 g.

Exp.No.6. Behaviour of precipitates on pH decrease from pH 6.2 (p.55)

A 25 ml aliquot of 0.02 Molar ferric chloride was added to 1.8 grams of fructose to give a molar ratio of fructose to iron of 20:1. This solution was adjusted to pH 6.2 by addition of 1 N sodium hydroxide. After precipitation the pH was lowered to 4.0 by addition of 1 N hydrochloric acid and observations made.

Similar treatment was given to fructose/iron solutions of 1:1 ratio. After precipitation at pH 6.2, one was adjusted to pH 4.5, and one to pH 3.5, and observations made.

A solution of 0.02 Molar ferric chloride alone, was also

adjusted to pH 6.2, with subsequent lowering to pH 3.5 and observations made, for comparison.

Exp.No.7. Fructose addition after precipitation (p.57)

25 ml Aliquots of 0.02 Molar ferric chloride solution were adjusted to pH 6.2, followed by addition of solid fructose (0.090, 0.18, 0.36, 0.90, 1.8 grams) to provide molar ratios of fructose to iron from 1:1 to 20:1.

This was followed by a) addition of 1 N sodium hydroxide up to pH 12.0 and b) addition of 1 N hydrochloric acid down to pH 3.5. Observations were made over a period of 6 hours.

Exp.No.8. Precipitate inhibition in more dilute solution.(p.58)

25 ml Aliquots of 0.002 Molar ferric chloride solution were added to weighed quantities of fructose (0.009, 0.018, 0.036, 0.090, 0.18, 0.27 grams) to provide molar ratios of fructose to iron from 1:1 to 20:1. pH adjustment with 0.1 N sodium hydroxide was then carried out and observations were made as in Exp.No.4).

Two solutions of 30:1 and 50:1 fructose to iron ratio were made up using 0.02 Molar ferric chloride solution, and the pH of each adjusted to 6.2. After standing for $\frac{1}{2}$ hour a 10x dilution with water was effected and observations were made.

Exp.No.9. Inhibition of precipitation of ferric hydroxide with ryegrass fructosan (p.59)

25 ml Aliquots of 0.02 Molar ferric chloride solution were added to weighed quantities of ryegrass fructosan (0.090, 0.18, 0.36, 0.45 and 0.54 grams). Analysis of the fructosan (Section III, 4) showed that the fructose content was 68.2 per cent, and the total hexose content was 77.7 per cent. Thus using the molecular weights of the component simple sugars the weights of fructosan above represent:-

ratio 'fructose' to iron - 0.682:1, 1.334:1, 2.728:1, 3.410:1 and 4.092:1 respectively.

ratio 'total hexose' to iron - 0.777:1, 1.554:1, 3.108:1, 3.885:1 and 4.662:1 respectively.

Adjustment of the pH with 1.0 N sodium hydroxide was then effected as in Exp.No.4, and observations were made.

Exp.No.10. Inhibition of magnesium hydroxide precipitation (p.60)

25 ml Aliquots of 0.02 Molar magnesium chloride solution were added to weighed quantities of fructose (0.090, 0.18, 0.36, 0.90, 1.8 and 2.7 grams) to provide ratios of fructose to magnesium of 1:1 to 30:1.

Three series were used, adjusting the pH values of each to 10.7, 10.9 and 11.1 respectively with 1 N sodium

hydroxide. After standing for ½ hour, aliquots were withdrawn, centrifuged as in Exp.No.5, and the quantity of magnesium remaining in the supernatant determined as in G.M.4.

Exp.No.11. The effect of alkali on fructose (p.61)

Samples withdrawn from many of the foregoing solutions which had been adjusted above pH 9.0, were chromatographed in aqueous phenol and ethyl acetate: pyridine: water solvents (G.M.1) followed, after drying, by development in silver nitrate reagent (G.M.1)

In addition, 1.8 gram lots of fructose were dissolved in 25 ml aliquots of water and the pH adjusted to 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 respectively. Solutions were also made up using 0.02 Molar ferric chloride, followed by adjustment to the pH values detailed.

Samples were withdrawn at ½ hour, 12 hours, 36 hours, 72 hours and 120 hours, chromatographed in the above solvents and developed in the silver nitrate reagent (G.M.1).

Exp.No.12. Isolation, purification and analysis of the complex (p.65)

Three 50 ml aliquots of 0.02 Molar ferric chloride were added to 0.18 gram lots of fructose (fructose to iron ratio 1:1) and a further three added to 1.8 gram lots of fructose

(fructose to iron ratio 10:1).

One sample of each ratio was adjusted to pH 4.0, one of each to pH 6.2, the remaining 1:1 solution to pH 12.0, and the remaining 10:1 solution to pH 10.0, with 1.0 N sodium hydroxide.

Each sample was then poured into 150 mls ethanol, with stirring, and 150 mls of ether were added. Following centrifugation on an MSE Magnum centrifuge using the 6 x 250 swing-out head at 2,000 r.p.m., the precipitates obtained were washed three times with a 50:50 ethanol/ether mixture, once with anhydrous ether, and dried.

45 ml aliquots of water were added to each precipitate so obtained, observations made, and the pH values measured. Following adjustment to pH 1.4 with 5 N hydrochloric acid, the samples were made up to 50 mls with water, and the quantity of iron and fructose in each was determined (G.M.3 and 6). The molar ratios of fructose to iron were then calculated.

Exp.No.13. Titration of ferric chloride and fructose alone and mixed (p.70)

25 ml Aliquots of 0.02 Molar ferric chloride solution were titrated using a Radiometer Titrator TTT1C, with 5.0 N sodium hydroxide, added from a 0.5 ml Agla syringe at a constant rate of 22 microlitres per minute. During addition the solution

was rapidly stirred, and the pH was recorded on a Bausch and Lombe recording valve voltmeter.

25 ml aliquots of 0.02 Molar ferric chloride solution were added to 0.90 and 1.8 gram lots of fructose (fructose:iron ratios of 10:1 and 20:1 respectively and titrated in a similar way. For comparison, 0.90 and 1.8 gram aliquots of fructose dissolved in 25 mls of water were also titrated.

Exp.No.14. Addition of fructose after precipitation of ferric hydroxide (p.74)

25 ml Aliquots of 0.02 Molar ferric chloride were titrated as previously described (Exp.No.13). During titration with 5.0 N sodium hydroxide, a measured quantity of fructose (1.8 grams, fructose: ratio, 20:1) was added at pH 11.0. This was further repeated, in this case adding the weighed quantity of fructose at pH 8.0.

Comparison of the curves obtained, with the normal titration curve of 0.02 Molar ferric chloride was made.

Exp.No.15 Titration of magnesium chloride and fructose, alone and mixed (p.76)

A 25 ml aliquot of 0.02 Molar magnesium chloride was titrated as above (Exp.No.13). A further aliquot with fructose added (1.8 grams) to give a fructose to magnesium ratio of 20:1 was also titrated. Comparison of the curves obtained was made.

Exp.No.16. Attempted titration of ferrous sulphate (p.76)

Due to the rapid oxidation of ferrous sulphate, no titration data for this solution could be obtained.

Exp.No.17. Dissolving action of sugars on ferric hydroxide (p.91)

1.352 grams of solid Analar ferric chloride were dissolved in 95 mls of water, and 1 gram of solid Analar sodium hydroxide dissolved in 5 mls of water was added with stirring. 50 mls of the resultant ferric hydroxide suspension were diluted to 500 mls with water, and the suspension obtained was treated in the following way.

Three 150 ml aliquots were taken and adjusted to pH 10.5, pH 7.0 and pH 4.0 respectively, by bubbling carbon dioxide through the suspension. 50 ml aliquots of each were then pipetted into bottles containing a) no sugar b) 0.45 grams of fructose (fructose:iron ration = 10:1) and c) 0.90 grams of fructose (fructose:iron ratio = 20:1). The sealed bottles were shaken end-over-end, removing 5 ml samples from each after 1 hour and 16 hours shaking. After centrifugation the quantity of dissolved iron in the supernatant was determined.

Tests indicated that high-speed centrifugation was unnecessary, so that all samples were centrifuged in an MSE Minor centrifuge at 3,000 r.p.m., developing approximately 1,700 g until clear.

Exp.No.18. Dissolving action of a range of sugar concentrations on ferric hydroxide at ph 10.0 (p.92)

Ferric hydroxide was precipitated as described in Exp. No.17 providing 500 mls of suspended precipitate. The pH was adjusted to 10.0 with carbon dioxide and 50 ml aliquots were pipetted into bottles containing weighed quantities of fructose (0.045, 0.090, 0.180, 0.45, 0.90, 1.35 grams) to give fructose to iron ratios of 1:1, 2:1, 4:1, 10:1, 20:1 and 30:1 respectively. In addition, one 50 ml aliquot with no addition of sugar was taken as a water blank.

Immediately after addition of the suspension to the sugar-containing bottles the pH was read, the bottles were stoppered, and shaken on an end-over-end shaker. 5 ml samples were removed from each after 4 hours, 2 days, 3 days, 5 days and 10 days respectively. After reading the pH of each, the samples were centrifuged as in Exp.No.17 and the quantity of iron present in the supernatant was determined.

Exp.No.19. Dissolving of ferric hydroxide at lower pH values (p.95)

An experiment exactly equivalent to that detailed above was conducted, adjusting the pH of the suspension to 5.8 prior to addition of 50 ml aliquots to the shaking bottles.

Exp.No.20. Dissolving of ferrous hydroxide by sugars (p.102)

Adequate details of the precipitation of ferrous hydroxide have appeared in the text. After pH adjustment 50 ml aliquots were pipetted (with the precautions detailed) into bottles containing weighed quantities of fructose (0.09, 0.18, 0.45, 0.90, 1.35 grams) to provide fructose to iron ratios of 2:1, 4:1, 10:1, 20:1 and 30:1 respectively.

50 ml aliquots were also pipetted into bottles containing weighed quantities of fructosan (0.90, 0.18, 0.45 and 0.90 grams) the fructosan to iron and total hexose to iron ratios represented by these quantities being detailed in Table 15.

After shaking on an end-over-end shaker for 1 hour the samples were centrifuged, and the iron contents of the supernatants determined. Centrifugation was carried out in an MSE 'High Speed 17' centrifuge at 12,000 r.p.m. developing around 17,000 g. This was not strictly necessary to obtain clear solutions, but the samples could be centrifuged rapidly, thereby limiting oxidation.

Each separate series of extractions was carried out in exactly the same manner, the pH of extraction being given in Tables 16 and 17.

Three solutions, after extraction of ferrous hydroxide with fructosan (ratio of total hexose:iron = 7.77:1) were centrifuged and a) exposed to the atmosphere for 2 days,

b) exposed to the atmosphere for 2 hours and c) maintained in a sealed condition. After three weeks the resultant solutions and suspensions were photographed.

Exp.No.21. Further examination of the behaviour of the iron solutions obtained on extraction of ferrous hydroxide with fructose and fructosan (p.107)

Ferrous hydroxide was precipitated as previously described using double quantities. The pH of the suspension was adjusted to 6.8 with carbon dioxide, and 300 ml aliquots added to bottles containing a) no sugar, b) 8.1 grams of fructose (fructose to iron ratio of 30:1) and c) 2.70 grams of fructosan (ratio of total hexose to iron = 7.77:1).

After extraction for 1 hour on an end-over-end shaker, the suspensions were centrifuged on an MSE 'High Speed 17' centrifuge at 15,000 r.p.m. developing around 26,000 g., and 50 ml aliquots of the clear supernatants of each were pipetted into bottles containing 1 gram lots of:

a) bentonite clay
b) soil from the B horizon of a podzol
c) precipitate collected from the drainage of a poorly drained field
d) freshly oxidised ferrous hydroxide

A 50 ml aliquot was also pipetted into a bottle containing no solid material so that the effect of brief exposure to the

atmosphere during pipetting, on the iron content of the solution, could be determined. In addition, a 5 ml aliquot was withdrawn to determine the original iron content of the supernatants.

After shaking for a further hour on the end-over-end shaker the contents of the bottles were centrifuged and the iron remaining in the supernatants was determined.

Exp.No.22. Construction of a model iron pan (p.111)

5 gram lots of acid washed sand were added to 1 cm bore columns. On settling, 1 gram lots of a 70:30 celite/bentonite mixture were added, the bentonite forming a very thin band on top of the celite. To one column, one addition of celite/bentonite was made, to the other,two additions were made, resulting in the formation of two thin bands of bentonite. These additions were followed by further 5 gram lots of acid washed sand. All constituents were added as a slurry made up in previously boiled and cooled water. The resultant columns were well washed with similarly treated water, using light suction.

5 ml Aliquots of the fructosan/iron solution used in the previous experiment were introduced to the top of each column, and allowed to percolate slowly through under light suction. A water wash (25 mls) followed this addition. The columns were then allowed to run dry and air was drawn through

by light suction for 15 minutes. After washing with 25 mls boiled, cooled water, further 5 ml aliquots of the fructosan/iron solution were added to the columns, and the process repeated. In all, ten such treatments were carried out, followed by photography of the columns.

Exp.No.23. Dissolving of Biotite, Tremolite and Siderite (p.114)

5 grams of the finely powdered samples of biotite, tremolite and siderite were each suspended in previously boiled cooled water, the pH adjusted to 8.0 with dilute sodium hydroxide, and the volume made up to 250 mls. 50 ml aliquots were withdrawn from each of the rapidly stirred suspensions and pipetted into bottles containing:

a) no sugar

b) 1.35 grams of fructose (2.7 per cent concentration of fructose)

c) 0.090 grams of fructosan (0.14 per cent concentration on a total hexose basis)

d) 0.45 grams of fructosan (0.70 per cent concentration on a total hexose basis)

nitrogen bubbling accompanying the addition.

After shaking on an end-over-end shaker, samples were withdrawn at 1 hour, 4 hours, 12 hours and 24 hours respectively. After centrifugation (in an MSE Minor centrifuge at 3,000 r.p.m., the iron and magnesium contents of the supernatants were

determined (G.M.3 and 5).

A further extraction was carried out at pH 5.2 using only a 0.70 per cent fructosan solution and a water blank. As before, the iron and magnesium contents of the supernatants were determined after extraction and centrifugation.

Note. The analytical determinations carried out on the fructosan sample (Section III 4) are adequately detailed under the respective general methods. All quantitative results quoted are on the basis of 1 dry gram of fructosan.

Exp.No.24. The breakdown of fructosan by airborne micro-organisms (p.131)

0.25, 0.5, 1.0 and 1.25 gram samples of fructosan were added to 25 ml aliquots of a) water b) 0.02 Molar ferric chloride to provide 1, 2, 4 and 6 per cent solutions respectively. The pH value of each solution was read, the solutions allowed to stand exposed to the atmosphere for several days, and observations were made.

0.25, 0.5 and 1.0 gram samples of fructosan were added to 25 ml aliquots of:

a) water - (pH 6.75)

b) 0.02 Molar ferric chloride (pH 2.25)

c) 0.02 Molar ferric chloride adjusted to pH 6.75 with sodium hydroxide

to provide 1, 2 and 4 per cent solutions respectively. These were allowed to stand exposed to the atmosphere for several days, observations being made.

A duplicate set of solutions, all overlayered with 2 mls of toluene were also made up, allowed to stand, and observations made.

Exp.No.25. The behaviour of fructosan solution when adjusted to pH 10.0 (p.133)

1 gram lots of fructosan were dissolved in 25 ml aliquots of a) water b) 0.02 Molar ferric chloride to provide 4 per cent solutions. The solutions were adjusted to pH 10.0 with sodium hydroxide and observations made. A grey flocculent precipitate was deposited from the aqueous solutions.

Fructosan solutions of 1, 2, and 6 per cent in water were all adjusted to pH 10.0 with alkali, to determine whether or not a precipitate was deposited at a range of concentrations.

On acidification of a suspension of the precipitate with dilute hydrochloric acid, the precipitate was found to dissolve.

5 grams of fructosan were dissolved in 50 mls of water, adjusted to pH 10.0 with sodium hydroxide and the precipitate removed by centrifugation. The clear supernatant was

precipitated in 150 mls of cold absolute ethanol, collected by centrifugation, well washed with ethanol and dried with dry ethanol, dry acetone, and 40-60 petroleum ether. Around 4.0 grams of fructosan (minus the precipitating fraction) were obtained. Using this material, a repeat of Exp.No.9 was carried out.

Exp.No.26. Observations on the quantity precipitated (p.135)

Adequate details of the experimental procedure are provided in the text.

Exp.No.27. Attempted characterisation of the precipitate (p.135)

Adequate details of the qualitative tests carried out on the precipitate are provided in the text and in the respective general methods.

1 gram of fructosan was dissolved in 10 mls of water, adjusted to pH 10.0, the precipitate collected by centrifugation, and washed with water adjusted to pH 10.0 with sodium hydroxide. The washings were combined with the original mother liquor and the volume made up to 100 mls with water.

The washed precipitate was dissolved in dilute hydrochloric acid and the volume made up to 25 mls with water.

A further 1 gram of fructosan was dissolved in 100 mls of water.

Quantitative phosphate determinations by King's method (G.M.16) were carried out on all samples, dilutions being carried out where required. All results were expressed as phosphate (PO_4) from 1 gram of dry fructosan.

A phosphate determination using the Lowry and Lopez method (G.M.16) was carried out on an aliquot of the complete fructosan solution.

Exp.No.28. The source of the acid labile phosphate (p.140)

1 gram of fructosan was dissolved in 250 mls of water, and the absorption spectrum measured on a Unicam S.P.800 Automatic spectrophotometer. 1cm cells were used and water was used as the blank. From the absorbence at 260 mμ the content of nucleotide, expressed as UMP was calculated.

Exp.No.29. Mineral analysis of the precipitating material (p.142)

1 gram of fructosan was dissolved in 10 mls of water, adjusted to pH 10.0 with sodium hydroxide and the precipitate collected by centrifugation. After washing with water adjusted to pH 10.0 with sodium hydroxide, the precipitate was dissolved in dilute hydrochloric acid made up to 10 mls with water, and a cation analysis carried out (G.M.18).

Quantitative determinations of the calcium, magnesium and iron present were conducted as in G.M.3, 4, 5.

Exp.No.30. Ash determination and mineral analysis of the original fructosan (p.142)

An ash determination (G.M.17) was conducted on 1 gram of the fructosan. Quantitative determinations of the calcium, magnesium and iron present were conducted as in G.M.3, 4, 5.

Exp.No.31. Titration of the fructosan solution (p.147)

1 gram of fructosan was dissolved in 25 mls of water and titrated with 5.0 N sodium hydroxide, delivered from a 0.5 ml. Agla syringe at 22 microlitres per minute, using a Radiometer automatic titration assembly TTT1C. The graph obtained was recorded on a Bausch and Lambe recording valve voltmeter.

Exp.No.32. The pH of re-solution of the precipitate (p.147)

Two 1 gram lots of fructosan were dissolved in 10 mls of water and adjusted to pH 10.0 with sodium hydroxide. In one case the precipitate was left suspended in the alkaline fructosan solution, and in the other the precipitate was centrifuged down, washed with water, adjusted to pH 10.0 with sodium hydroxide, and resuspended in 10 mls of water, again at pH 10.0.

Dilute hydrochloric acid was slowly added to both solutions, the pH was measured and observations made.

Exp.No.33. Dialysis of the fructosan solution (p.147)

1 Gram of fructosan was dissolved in 10 mls of water, placed in a 20 cm length of Visking tubing with the ends securely tied and dialysed against 1 litre of water for 2 days at 4°C. The resultant dialysate was reduced to 40 mls in a Buchi rotary evaporator, and made up to 50 mls standard volume.

Quantitative determinations of iron, magnesium and fructose were then made as in G.M.3, 4, and 6.

Exp.No.34. Variation in weight of the precipitate obtained at different pH values, and the effect of fructose addition prior to pH adjustment (p.149)

Twelve 1 gram samples of fructosan were dissolved in 10 ml aliquots of water in weighed 20 ml conical centrifuge tubes. To each series of four solutions the following were added:

a) no addition of fructose

b) 1 gram of fructose added

c) 2 grams of fructose added

One sample of each series was adjusted to pH 7.5, one to pH 8.0, one to pH 9.0 and the last to pH 10.0.

The precipitates deposited were collected by centrifugation, washed with water at pH 10.0, and dried at

60 C over phosphorous pentoxide for 24 hours. Weighings were then conducted and the weights of precipitate obtained, calculated.

Note.

During the extraction of the further four fructosan samples the volumes of water and ethanol used were:

a) for Italian, ryegrass, perennial ryegrass, and Cocksfoot, one quarter of those used in the extraction of the original fructosan.

b) for Timothy, one sixth of those previously used.

All determinations carried out on the four grass fructosan samples were exactly as detailed for the original fructosan sample.

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