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CONTENTS

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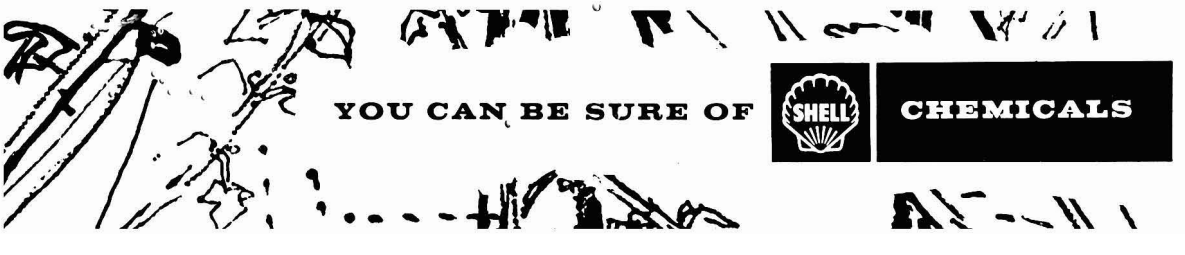

NATURE AND MODE OF WEATHERING OF SOIL-POTASSIUM RESERVES*

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In most partially weathered mineral soils only a small part of the total potassium is readily exchangeable to other ions; the bulk occurs in non-exchangeable (or difficultly-exchangeable) forms in potash feldspars, micas and micaceous clays, all of which are potential sources of potassium for plants.

Some of the principles underlying the weathering processes in feldspars, trioctahedral and dioctahedral micas are discussed. The ease with which freshly cleaved potash feldspar releases potassium to water cannot be taken to indicate that the feldspar weathers easily; in soil the feldspar is protected by a surface covering of its own decomposition products. Trioctahedral micas are less stable to weathering than dioctahedral micas, particularly during the early stages of potassium depletion; the stabilities of these two main groups of micas differ more than can be accounted for in terms of ferrous iron contents. For micas and micaceous clays the relationships between parent lattices and weathering products must depend very largely on the extent to which layer-charges have decreased on weathering. As more becomes known about the potassium-containing minerals which occur in soils, we should be better able to understand the factors which influence the long-term ability of soils to supply potassium to crops.

Introduction

SOILS are complex materials and the chemistry of the potassium they contain is not fully understood. Potassium, like most other plant nutrients in soils, exists in forms which range from the water-soluble to the extremely inaccessible. The division of soil potassium into readily-exchangeable and non-exchangeable categories is convenient and useful in practice, but depends to some extent on the ion or ions used for displacing the exchangeable potassium and on the experimental conditions used. Although the amount of readily-exchangeable potassium in soil is often a good index of the amount of potassium available to plants, plants can sometimes use non-exchangeable or difficultly-exchangeable reserves, depending on a variety of factors which include the nature of the soil, the type of crop, the climatic conditions and the general level of manuring. In most partially weathered soils about 99% of the total potassium is usually not readily exchangeable to other cations in neutral salt solutions. This bulk of the soil potassium has attracted much attention in recent years, particularly in studies on the potassium-supplying powers of soils. The inherent potassium fertility of soils, which at best contain sufficient exchangeable potassium for only a few years' intensive cropping, depends on the potassium released from non-exchangeable or difficultly-exchangeable sources. Partially weathered soils tend to register a level of exchangeable potassium which reflects an equilibrium condition between at least a part of the non-exchangeable reserves and the exchangeable potassium. This concept has been discussed by many authors including Reitemeier[1] and Wiklander.[2] There is indirect evidence[3] with some British soils that the clay fraction releases most of the non-exchangeable potassium, but the silt, particularly the fine silt (2–10 μ), can also contribute significantly towards the release.

In many partially weathered soils the clay and silt fractions cannot merely be regarded as colloidal frameworks on which cation-exchange reactions take place. An understanding of the behaviour of lattice potassium is as important as the understanding of the behaviour of the readily-exchangeable potassium. Soils at an intermediate stage of their weathering contain few types of potassium-containing minerals and nearly all their potassium occurs in potash feldspars, in micas and in micaceous clays. It is to these groups of minerals that attention can profitably be directed and particularly to the weathering transformations the minerals can undergo.

Without understanding the weathering changes undergone by these rather stable potassium-containing minerals, appreciation of the potassium release and 'fixation' problems of soils

* Read at a meeting of the Agricultural Group, 10th March, 1959

must remain superficial. In this paper is indicated the type of problems encountered and some of the progress towards their elucidation described. Potassium 'fixation' problems in soils should logically be included in the present discussion, but they will be dealt with separately.

Potash feldspars

Although high-potash feldspars differ from one another in their resistance to chemical weathering,[4] as a group they are usually regarded as having a stability to weathering intermediate between those of muscovite and biotite.[5] Because of their resistance to breakdown they have not been the subject of intensive study under non-hydrothermal conditions. The surface reactions of feldspars under atmospheric conditions have been considered by Nash & Marshall.[6] Freshly cleaved potash-feldspar surfaces react rapidly with water to release potassium ions[7, 8] and give the mineral a surface layer of H-feldspar, the presence of which immediately slows down further reaction. The surface H-feldspar or H-aluminosilicate is unstable and breaks down, releasing some of its ions to leave a thin but comparatively stable residue on the mineral.[6, 9, 10] Once this stage is reached potassium is released only slowly because the interior of the particle is rather well protected from further attack by the surface covering and by the sub-surface layer of crystalline H-feldspar which merges into unaltered mineral. The ease with which freshly cleaved K-feldspar releases potassium to water cannot, therefore, be taken to indicate that the feldspar weathers easily in soil. The potassium release from feldspar will depend on the particle size range of the mineral and on the stability of the protective weathering film which will probably vary appreciably in character in different soil types. The thinness of the protective surface covering has prevented progress in discovering its nature.

Most of the K-feldspar in soils occurs in the particles with $>2\ \mu$ diameter. The general paucity of K-feldspar in the $<0{\cdot}2\ \mu$ fractions is thought to be explained by the fact that once particles reach this small size they tend to weather rapidly. Productivity ratings of soils in Belgium,[11] the U.S.A.[12–14] and Puerto Rico[15] have been correlated with feldspar contents with some degree of success, but the subject is still in its infancy.

Micas and micaceous clays

Before discussion of the micas and micaceous clays it can be stated that the dimorphic (or 1 : 1 layered) aluminosilicates, for example kaolinite and halloysite, play a comparatively simple rôle in the behaviour of potassium in soil, because they hold potassium solely as an exchangeable ion. The same is true of soil organic matter, but the latter may indirectly influence the potassium release and fixation properties of some clay minerals. It is easily demonstrated that in truly organic soils all the potassium is present in a readily-exchangeable form.

Micas and micaceous clays play a dominant rôle in determining the potassium behaviour of many types of partially weathered mineral soils. In the discussion a general acquaintance with the more important types of trimorphic (or 2 : 1 layered) aluminosilicate lattices will be assumed. Following the usage of Stevens,[16] the term 'trioctahedral' refers to layer minerals in which all or nearly all possible octahedral positions are filled, while 'dioctahedral' refers to those lattices in which only two out of three octahedral positions are occupied.

Clay minerals, particularly the 2 : 1 minerals occurring in soils, are matters of definition which depend on the diagnostic criteria used to characterise them; their identification invariably depends on a weight of evidence that may not be decisive. Not only do soil clay minerals tend to vary in composition with changing particle size, but even within narrow size limits the composition and properties of particles belonging to one recognised clay type can vary. It is now accepted that the occurrence of regular and random interstratified 2 : 1 lattices is the rule rather than the exception, both in soils and recent sediments. Earlier, although interstratified assemblages were recognised, they were not regarded as so important as they now are.

Based on the assumption that there are 44 negative charges per unit of lattice and that the oxygen–hydroxyl pattern, $O_{20}(OH)_4$, is free from imperfection, the idealised formulae of two

well-known micas, biotite and muscovite can be represented by formulae I and II, respectively.

		Charge
Tetrahedral	Si_3Al	
Octahedral	$(MgFe^{++})_6$	2−
Tetrahedral	Si_3Al	
Interlayer	K_2	2+

$O_{20}(OH)_4$

I Biotite (idealised)

		Charge
Tetrahedral	Si_3Al	
Octahedral	Al_4	2−
Tetrahedral	Si_3Al	
Interlayer	K_2	2+

$O_{20}(OH)_4$

II Muscovite (idealised)

Although micas vary greatly in composition, many naturally occurring biotites and muscovites approach these idealised formulae quite closely. In high-silica muscovites there is appreciable magnesium and iron in octahedral positions so that some of the negative charge is octahedral in origin. Almost all past work shows that the potassium in trioctahedral micas, such as biotite, is more readily released on weathering than the potassium in dioctahedral micas. Serratosa & Bradley[17] compared the infra-red absorption spectra of dioctahedral and trioctahedral micas and found a difference in the disposition of O—H bonds. In trioctahedral micas there is neither scope nor evidence for the proton in O—H bonds entering into secondary association with any other oxygen. In dioctahedral micas, however, the O—H bond axis must have one or more of the orientations near the plane of the cleavage flake and there is evidence, from reduced vibration frequencies, for some hydrogen-bonding within the crystal. The view that ferrous iron in a lattice leads to ease of weathering is undoubtedly true, but the differences between the stabilities of dioctahedral and trioctahedral micas are greater than can be accounted for by ferrous iron contents. Phlogopite, the magnesium (iron-free) analogue of biotite, is only slightly less resistant than biotite to attack by hydrogen-saturated cation-exchange resin but is very much more susceptible than dioctahedral micas.[18]

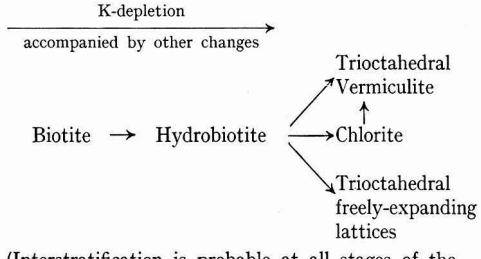
Weathering of biotite

Under conditions of free drainage, biotite weathers more easily than muscovite and there is clear evidence that the potassium in biotite is much more accessible to plants than the potassium in muscovite.[19–21] Barshad[22] demonstrated that the potassium in biotite is exchangeable, though with difficulty, to ions that make the lattice expand. The studies of Walker[23] on the weathering of biotite have removed much of the uncertainty about the changes undergone by the mineral on weathering in soils. The weathering of biotite flakes, during which potassium depletion is of primary significance, has long been known to change the colour from black through golden yellow to silver and white. Denison *et al.*[24] established that initially the weathering depends on the replacement of some interlayer potassium by water molecules, or, as is now thought, by hydronium ions, to give the mineral hydrobiotite. On weathering, changes within the silicate layers take place such as the oxidation of ferrous iron, the preferential loss of some ions and, possibly, conversion of some oxygen ions into hydroxyl groups, which decreases the net negative charge on the lattice. At the stage at which about half the potassium has been lost from the biotite, the mineral appears to become more stable, which may have caused Denison *et al.* to state that biotite and muscovite weather at very similar rates. In the early stages of weathering, however, potassium is lost from biotite much more rapidly than from muscovite. As potassium is lost from biotite and the attractive forces between sheets are decreased, a double layer of water molecules enters to give a basal spacing of 14 Å. Water is thought to spread through the crystal layer by layer, giving first a mixed layer structure in which increasing numbers of layers become of the vermiculite type. Biotite weathers in this way when leaching is active. The process of decomposition can be traced upwards through successive layers of a profile from the near-unweathered condition of the mineral at the bottom to its expanded weathering product in the surface layers. In the soils discussed by Walker,[23] little chlorite seems to form but in other soils it is an important product of weathering. In his study of rock weathering of the Malvern Hills, Stephen[25a] observed the early formation of chlorite (idealised formula III) from biotite, which may subsequently alter to vermiculite. This indicates that at least part of the interlayer potassium that is released had its place taken by the positively charged brucite or brucite-like material, although, of course, the original high negative charge on the mica layer will be decreased as ferrous iron becomes oxidised. It

			Charge
Tetrahedral	Si_3Al		
Octahedral	$(MgFe)_6$	$O_{20}(OH)_4$	2−
Tetrahedral	Si_3Al		
Brucite layer	Mg_4Al_2	$(OH)_{12}$	2+

III Chlorite (idealised)

is not possible to say how well defined the brucite component would be in soil chlorites ; under acid conditions magnesium ions would probably be lost more easily than aluminium, and Stephen[25b] proposed the existence of a range of intermediates between chlorite and vermiculite in which the brucite layer degrades through stages until isolated $Al(OH)_2^+,xH_2O$ groups are left and the weathered product is an aluminium vermiculite. Thus the weathering biotite can go through stages in which the weathering product is chloritic or the chlorite stage may be omitted and the vermiculite condition reached directly. The weathering of biotite mica under poor drainage conditions associated with basic parent material can result in the formation of a trioctahedral mineral which can be expanded beyond 14 Å by treatment with glycerol and is therefore more like a montmorillonite than a vermiculite. A picture of the weathering of biotite is represented by the sequences :



(Interstratification is probable at all stages of the weathering)

Weathering of muscovite and related dioctahedral micas

Muscovite is rather resistant to chemical weathering in soils. The first stage is believed to be the partial conversion of the muscovite to hydromuscovite by the replacement of some interlayer potassium ions by hydronium ions.[26–28] Freshly broken muscovite rapidly undergoes reaction with hydronium ions in water at room temperature, but once the easily accessible sites such as those on crystal edges have become converted to hydromuscovite, the hydronium ions penetrate into the crystal very slowly. The polar nature of the hydronium ion and the fact that it is larger (radius >1·4 Å) than the potassium ion (radius = 1·33 Å) should expand the interlayer spacing slightly, but this is probably too little to aid the penetration of hydronium ions into the lattice. The maintenance of a low potassium-ion activity on the surface of the crystal by, for example, precipitating potassium on the outside of the lattice,[29] greatly facilitates the potassium-depletion process. In the field, the products of weathering such as potassium ions must be mainly removed by leaching. As the potassium is increasingly depleted, potassium ions seem to be removed along preferred planes, eventually leading to expansion of the lattice (Jackson *et al.*[30]). Once the weathering is initiated, other changes occur within the lattice, the combined effects of which decrease the charge on the mica layer. Increasing proportions of weathered planes result in various combinations of non-expanded and expanded layers being formed in individual crystallites. Diffraction studies, internal surface measurements and K, H_2O and OH determinations all lend support to the proposed mechanism of the weathering. According to Johnson & Jeffries[31] at least some hydromuscovite in a soil will always be approaching a stage in its weathering at which the remaining potassium will be released rapidly from certain interlayer planes. The original studies of Denison *et al.*[24] showed that muscovite retains its plate-like form, although showing birefringence, down to quite low potassium contents, at least when the plates are large. However, insufficient is known about the effect of progressively

replacing potassium by hydronium ions on the tendency of muscovite particles to expand. A study using modern techniques on material similar to that examined by Denison *et al.*[24] is needed.

The term ' illite ' was originally used by Grim *et al.*[32] to cover micaceous clays in general. Although it applies equally to dioctahedral and trioctahedral lattices, most of the ensuing remarks will be confined to the alumian dioctahedral illites which occur widely both in soils and in sediments. Trioctahedral illites[33, 34] have been identified in some soils derived from basic rocks, but they are not common. The alumian illites along with their dioctahedral ferroferri analogues, the glauconites,[35] are the only true clay minerals which contain potassium as an essential constituent. In modern usage the terms ' hydrous mica ' and ' illite ' tend to be used synonymously but the former term has a wider meaning than illite and would include non-clay-like hydromicas such as hydromuscovite.

For study of illites it is very difficult to get uniformly ' pure ' materials for detailed examination. As constituents of soils and recent sediments the illites invariably occur along with other minerals but, above all, it is the interstratification with expanded non-mica layers which makes their study difficult. The expanded layers can owe their existence to any or all the following causes :

(*a*) that some layers in the mineral do not possess sufficient or suitably located charge to favour potassium entrapment ;

(*b*) that the environment in which the mineral was formed was deficient in potassium ;

(*c*) that potassium-depletion, by weathering, has occurred since the mineral was formed.

According to Jackson & Sherman[36] the term ' interstratified illite ' is unnecessary because illites are characteristically interstratified. It is now universally recognised that all illites, including reference illites such as Fithian illite [American Petroleum Research Project, No. 49 (1950)], are appreciably interstratified with expanding layers.[37] For non-degraded alumian dioctahedral illites, the total potassium contents usually fall between 1·0 and 1·5 K per $O_{20}(OH)_4$* unit of lattice and, despite the fact that they occur under widely differing conditions, they tend to possess similar properties. Each facet of the study of illites is beset with difficulties and different authorities view the illite minerals somewhat differently. Yoder[38] suggested that ' illite should be used only as a field term as Grim *et al.*[32] intended and, after identification of the various phases is made in the laboratory, the polymorph of the mica (component) should be specified and the nature of the mixed layer mineral and other phases present should be indicated '. Only in this way does it appear possible to classify illites satisfactorily and hence make progress in understanding any relationships between their constitution and their weatherability. Gross chemical analysis is of little value in elucidating the nature of illites. Chemical analysis usually shows that, on average, one-sixth of the tetrahedral sites in the lattice are occupied by aluminium ions and that there is appreciable magnesium and iron in octahedral sites, but at least half and usually more than two-thirds of the negative charge on the lattice arises from substitutions in the tetrahedral layers. Illites formed from muscovite by partial weathering probably have layers of unaltered muscovite [2·0 K per $O_{20}(OH)_4$] left interstratified with expanding layers. When illites form from muscovite by potassium-depletion and subsequent potassium-uptake by the weathered product, or by the radical alteration of minerals other than muscovite, the negative charge on the resultant clay lattice will probably be smaller than that on an unweathered muscovite. For mica structures in which the lattice charge is predominantly tetrahedral in origin, there is some evidence[39] that the total negative charge must be greater than about 1·3 equivalents per $O_{20}(OH)_4$ unit of lattice before the potassium interlayered lattice can be regarded as non-expanding. Allowing between 0·1 and 0·2 equivalents of charge for readily-exchangeable ions, the minimum potassium content could reasonably be expected to lie near 1·1 K per $O_{20}(OH)_4$ unit layer of non-expanding lattice. For illites in which the non-expanding layers are not merely unaltered muscovite, the maximum likely potassium content of the micaceous component is difficult to estimate from general considerations ; all that can be stated is that negative charges up to at least 1·8 equivalents per $O_{20}(OH)_4$ unit would appear reasonable for such illites and, after due allowance is made for some readily-exchangeable ions, such illites

* The oxygen–hydroxyl assemblage can be represented more accurately as $O_{20-y}(OH)_{4+y}$, where y attains values of at least 0·5.

might contain up to about 1·7 K per $O_{20}(OH)_4$ unit of non-expanding lattice. Viewed in this way, the statement that illites possess similar properties becomes acceptable and the range of composition of the non-expanding sheets in an illite (excluding sheets which approximate in composition to unweathered muscovite) might commonly be expected to lie within the limits

Tetrahedral	$Si_{3\cdot25-3\cdot55}$	$Al_{0\cdot75-0\cdot45}$	$O_{20}(OH)_4$
Octahedral	$(AlFe^{3+})_{3\cdot5-3\cdot9}$	$(MgFe^{2+})_{0\cdot5-0\cdot1}$	
Tetrahedral	$Si_{3\cdot25-3\cdot55}$	$Al_{0\cdot75-0\cdot45}$	
Interlayer	$K_{1\cdot0-1\cdot7}$		

IV Probable range of composition of K-interlayered sheets in illites, excluding sheets which, for one or more reasons, approximate in composition to idealised muscovite

given in formula IV. When aged under conditions suitable for low-grade metamorphism, illites of the type shown in formula IV may recrystallise into well-defined muscovite-like layers [K approaching 2·0 equivalents per $O_{20}(OH)_4$] interstratified with expanding layers of comparatively low charge. Such a change would, of course, necessitate a redistribution of aluminium within the framework of the aluminosilicate lattice. The potassium in such an illite would probably be as resistant to chemical weathering as is the potassium in muscovite.

The initial stages of the weathering of alumian dioctahedral illites should proceed similarly as with muscovite. There is little doubt that potassium depletion takes place along preferred planes as in muscovite, leading to more and more interstratification of expanded and non-expanded layers.

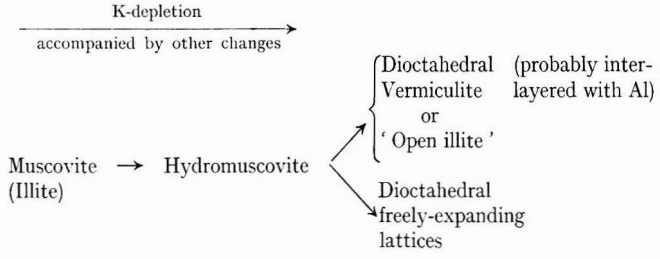
Brown[40] recognised that a material described as dioctahedral vermiculite is an important weathering product of dioctahedral alumian micas in certain soils. Numerous studies of the weathering of dioctahedral micas, many on a profile basis, have been reported.[41] Among the diagnostic criteria used for recognising the presence of vermiculites are:

(*a*) A 14 Å basal spacing which does not expand on solvation with glycerol.
(*b*) Collapse of the mineral to 10 Å on heating to 500°.
(*c*) An interlayer surface area of about 800 m.^{2} per g.
(*d*) Collapse of the mineral to 10 Å on saturation with potassium or ammonium ions.

The weathering sequence could be understood only when it was realised that the behaviour of the soil dioctahedral vermiculites can be drastically modified by the presence of interlayered sheets or islands of positively charged material, difficult to remove and behaving like the brucite layers in chlorite. The collapse of the vermiculite to 10 Å or thereabouts on potassium saturation can be prevented partly or completely by the non-exchangeable interlayer material which is thought to be mainly polymerised alumina. Brown[40] removed the interlayer material with hot KOH, after which he demonstrated the vermiculite properties of the mineral. Because the lattice is likely to be partly destroyed in KOH, milder reagents, such as normal ammonium fluoride (Rich & Obershain[41c]) and sodium citrate (Tamura[41d]), have been used to clean the mineral. The present position of the subject has been discussed by Sawhney.[42] The important weathering product needs more study, especially after removal of the interlayer contaminant. It may be unwise to draw too close an analogy between the properties of dioctahedral vermiculites and those of large flakes of trioctahedral vermiculite. How far removed the dioctahedral vermiculite is from being a freely-expanding mineral is not known. Under some weathering conditions vermiculite is replaced partly or wholly by freely-expanding lattices. The fact that dioctahedral vermiculite appears to form at the expense of micaceous minerals has been amply verified. As would be expected, small mica particles are more fully weathered than larger particles and weathering can be more intense in surface than in sub-surface soil.[43]

The term 'degrading illite'[44] has been used to describe potassium-depleted hydrous mica which fixes potassium vigorously. Van der Marel[45] used the term 'open illite' (amoorsooite) to describe a potassium-depleted hydrous mica with a basal spacing of 15–16 Å in soil derived from estuarine sedimenst. Open illite fixes potassium strongly. In time it should be possible to decide what relationships exist between dioctahedral vermiculite, degrading illite, open illite

and freely expanding lattices, all of which can appear as weathering products from dioctahedral micas. The weathering sequence of muscovite and related hydrous micas can be represented as :



In weathering transformations resulting from several processes, the true nature and relative importance of the contributory reactions are difficult to assess. The likely weathering processes concerned in the alteration of muscovite and related hydrous micas in soil have been summarised by Jackson *et al.*[30] For muscovite weathering, the fundamental importance of both potassium depletion and charge reduction in the parent lattice is widely recognised. If it is accepted that the early stages of the weathering of muscovite is a replacement of K by hydronium ions, then there remains the problem of elucidating the precise nature of the more important charge reduction process or processes. Apart from the possibility that ferrous iron oxidises, the mechanisms concerned in the charge reduction are not well understood. Weaver,[46] in a discussion on the significance of polymorphism in illites, states that most illites are 2M polymorphs and that such illites have probably inherited their structure from muscovite according to the sequence

$$\text{Muscovite 2M} \xrightarrow[\text{Charge reduction}]{\text{K-depletion}} \text{Weathered products} \xrightarrow{+K} \text{Illites 2M}$$

Because illites often contain less aluminium in tetrahedral co-ordination than do muscovites, it is a fair assumption that dealumination of tetrahedral layers must be a feature of the weathering of muscovite. When the loss of aluminium is considerable, the resulting lattice may not possess enough charge to permit the interlayer entrapment of potassium and the weathered product would be a freely-expanding montmorillinoid-type mineral. When the dealumination and associated charge reduction are relatively slight, the resultant lattice should, in a suitable environment, be able to absorb potassium and collapse to reform a 10 Å micaceous mineral.

The fact that the hydroxyl contents of micaceous lattices tend to increase on weathering is also important. It is accepted that the idealised $O_{20}(OH)_4$ assemblage of muscovite alters in the direction $O_{20-y}(OH)_{4+y}$ on weathering and that the values of y are greater for freely-expanding lattices than for vermiculites and illites. Because the increases in the hydroxyl contents are larger than can be accounted for in terms of crystal-edge effects it must be assumed that some of the hydroxyl groups occupy positions within the structure.

A formal mechanism of the dealumination of muscovite has not, so far, been suggested. It does, however, appear possible that during weathering a proportion of the tetrahedral aluminium ions in hydromuscovite interact with hydronium ions and migrate into interlayer positions where they take the place of remaining hydronium ions. Once in the interlayer spaces, the aluminium ions would readily hydrate and make the lattice expand. The lattice sites vacated by the aluminium ions either become garnetoid hydroxyl tetrahedra[47] or similar irregularities in the aluminosilicate lattice. Depending on the circumstances, a small proportion of the tetrahedral aluminium ions (in the region of one-quarter of the total in an idealised muscovite) could become highly charged interlayer ions, e.g., $Al^{2+}OH(H_2O)_5$, or more tetrahedral aluminium could be concerned in the change, resulting in less highly charged aluminium ions, e.g., $Al^{+}(OH)_2(H_2O)_4$, which occupy interlayer positions. Acid conditions would be expected to favour the former rearrangement and the product would be an aluminium-saturated vermiculite. Under less acid conditions more tetrahedral aluminium would be removed and in extreme cases the charge on the lattice would be so reduced as to result in a freely-expanding mineral. From what is known, the dealumination and charge reduction seem not to be uniform

in any particular crystallite, so that on resaturation with potassium the weathered product is transformed to an interstratified assemblage of non-expanding and expanding layers constituting a typical illite. Weathering may cause other changes than dealumination, such as the partial conversion of the garnetoid hydroxyl tetrahedra to SiO_4 tetrahedra and the exchange of interlayer aluminium ions with other ions. Thus the final weathered product may be such that its nature provides few clues about the transformations undergone by the original mineral.

As information increases on the composition of the clay and silt fractions of soils and on the weathering sequences their constituent minerals undergo, the factors that influence the ability of soils to supply potassium to crops over long periods will become better understood, as also will the reasons why potassium fixation is a problem of agricultural importance in some, but not in all, soils.

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SOME ASPECTS OF THE POTASH NUTRITION OF THE POTATO*

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The potassium requirements of potatoes are described, followed by a consideration of the relationship between potassium rate and source and the carbohydrate metabolism of the potato.

Potassium nutrition and potato quality (texture and after-cooking colour) are discussed, and the subject of potato quality is summarised with reference to the tests carried out at Levington.

Introduction

In Great Britain, the area devoted to potato growing is of the order of 700,000 acres, and to this acreage is applied annually something over 60,000 tons of potassium (K_2O). The average total rate of application of potassium in six main potato-growing areas in 1957 was 200 lb. of K_2O/acre (N.A.A.S. & Rothamsted Exp. Sta. Survey of Fertiliser Practice, Pt. II, 1957), these being areas where from one-third to three-quarters of the acreage in each area had farmyard manure applied as well as inorganic fertilisers. A good main crop of potatoes removes about four times as much potassium as does a good crop of wheat, and during its life span a potato crop may have as much as 180 lb. of K_2O/acre in the vines and tubers, and the harvested tubers may well contain 120 lb. of K_2O. These figures can be compared with the K_2O content of cereal grains of 12 lb./acre and of the straw of 36 lb./acre. Grasses take up about 30–60 lb. of K_2O.[1]

Potassium deficiency, especially when it is acute, results in reduced growth of the potato plant, and the vines or haulms are characteristically squat and bush-like in appearance. Wallace[2] described the leaves as being dull bluish-green in colour showing generally some intervenal chlorosis. The tips of the leaves develop a brown scorched appearance, and on the leaf surface a large number of brown necrotic spots usually occur which later form larger lesions. Under extreme conditions of potassium deficiency the haulms wither and collapse and the size and yield of the harvested tubers are much reduced.

Potassium is essential for various metabolic activities of the plant, especially in the synthesis of simple sugars and starch and in the translocation of carbohydrates, and it is because the potato is essentially a starch-producing crop that its requirements for potassium are high. For similar reasons, sugar beet have high potassium requirements, the average rate of potassium fertilisation in England being 220 lb. of K_2O/acre. It has been suggested that potassium affects photosynthesis and other physiological processes as a result of its influence on the chlorophyll components of the plant. Potassium produces an increase in the leaf area of the potato plant as a result of the development of a greater number of branches and leaves.[3] Leaf senescence is delayed with a resultant increase in yield of tuber starch. The yield of starch is at first increased by higher rates of potassium fertilisation but, ultimately, high rates of potassium reduce the overall starch yield. The level at which the yield of starch is reduced depends on the amount of exchangeable potassium in the soil and on the rate of potassium supplied by the fertiliser.

Potassium source and starch production

In 1926, Remy & Liesegang[4] found a reduced carbohydrate production in potato leaves of high chloride content, and later workers[5, 6] concluded that the chlorophyll content of plants was lower when plants received chloride than with sulphate nutrition. Buchner[7] examined the effect of chloride on carbohydrate metabolism and concluded that with higher chloride levels, the reducing sugar content decreased whilst the total carbohydrate content remained unchanged. Latzko[8, 9] described the physiological effects of chloride- and sulphate-containing

* Read at meeting of the Agriculture Group on 10 March, 1959.

fertilisers on the enzymic activities of plants. Chloride fertilisers reduced the hydrolytic activity of saccharase and β-glucosidase both in the leaves and in the tubers of the potato, whereas no such reduction in activity was observed with sulphate nutrition.[8] The total carbohydrate content of potato leaves increased with the formation of new products during the hours of daylight, the increase when potassium chloride was used being only half that with potassium sulphate. Latzko considered that chloride nutrition reduced the production of carbohydrates in potatoes as compared with sulphate nutrition. This reduced carbohydrate production, coupled with a diminished conversion and translocation to the tubers, was held to be directly related to the reduced starch content of potato tubers from chloride-treated plants.[9]

The decrease in starch content of tubers is, however, accompanied by an increase in both the potassium and chloride contents of the potato plant. The water content of the plant also increases with an increased rate of potassium fertilisation, as indicated by a decrease in the dry matter content of the haulms and tubers per unit fresh weight.

Terman *et al.*[10] showed that the apical halves of tubers grown with added potassium chloride were lower in dry matter and higher in potassium content than were stem-end halves. They concluded that the stem halves were higher in both dry matter and chloride, this being evidence that it is the content of potassium rather than of chloride which is the chief factor causing differences in dry matter content. The potassium content of whole tubers grown from plants fertilised with potassium chloride was slightly, but not significantly, higher than that in tubers grown with potassium sulphate. It was suggested that it was the greater solubility of potassium chloride which resulted in the absorption of more potassium, but this suggestion and one that it is the valency of the anion that controls potassium uptake does not receive sufficient support from the limited data available. If the level of exchangeable potassium is considered, with no reference to source, an increase in soil exchangeable potassium results in an increase in the potassium content of haulms and tubers.

The decrease in the starch content of potato tubers as a result of fertilisation with muriate of potash (potassium chloride) has usually been attributed solely to the chloride ion. However, at higher rates of potassium sulphate, a decrease is also obtained in the starch content of tubers, although in trials comparing potassium chloride and potassium sulphate the decrease in the starch or dry matter content of the individual tuber nearly always comes at a lower rate of potassium chloride fertilisation. Overall starch yield is then probably related to the amount of potassium taken up by the potato plant rather than solely to the effect of chloride on the metabolism of the plant. Figs. 1 and 2 illustrate the general relationship between starch yield and potassium uptake. The lower rate of application of potassium chloride required to bring about a reduction in overall starch yield is shown by a broken line in Fig. 2. The inset portion of this figure relates potassium application to tuber yield ; there is again little difference between the two sources, tubers yield following much the same trend as does starch yield in its relationship to potassium level.

Potassium and potato quality

In the main, the quality of the cooked potato is made up of (1) the texture or degree of mealiness, (2) the colour and (3) the flavour.

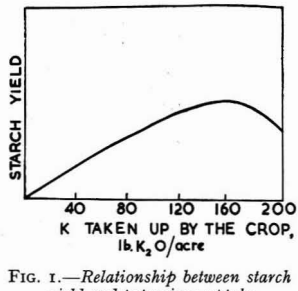

FIG. 1.—*Relationship between starch yield and potassium uptake*

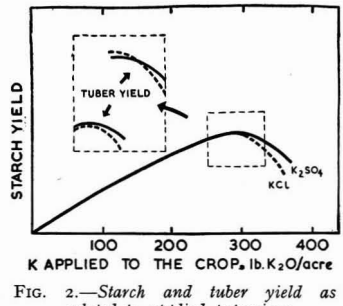

FIG. 2.—*Starch and tuber yield as related to applied potassium*

Texture

The chemical characteristics of the potato tuber associated with texture or the degree of mealiness are the contents of dry matter and starch. In the experimental results discussed later, the dry matter content and a panel assessment of texture were determined on the samples tested. Panel tests have their disadvantages, but there is not, at the present time, a convenient and accurate mechanical method for the determination of the texture of cooked potatoes.

The dry matter content, the starch content and the specific gravity of the potato tuber are directly related to one another and to the texture of the cooked potato. There is, however, no general agreement among research workers as to the degree of correlation between, say, the specific gravity and the texture rating as determined by a panel assessment. A positive correlation between texture, or mealiness, and specific gravity has been frequently demonstrated but the relationship is not constant, either within a variety or between potatoes of the same specific gravity but of different varieties. Nylund & Poivan[11] have shown that potatoes of identical specific gravity varied in mealiness depending on the date of planting and on the variety. Greenwood *et al.*[12] found that the relationship between specific gravity and mealiness varied with the locations at which the potatoes were grown.

Whatever the relationship may be between specific gravity and texture, individual tubers from sulphate of potash fertilisation (50% K_2O) will, almost without exception, have a higher dry matter content than from muriate of potash (60% K_2O) at the same rate of potassium application. Cowie[13] said that it was unlikely that it would pay farmers in the United Kingdom to use sulphate of potash rather than muriate of potash on potatoes because the practical significance of the slightly higher dry matter content was only of importance in countries where potatoes are grown for potato flour or alcohol production. However, in these days of pre-packaging and commercial preparation of cooked potatoes, the dry matter content of the potato could even yet become a criterion of commercial demand. Potatoes of high specific gravity give greater yields of crisps, and the fat absorption is less than with tubers of lower specific gravity.[14, 15] Although the improvement in crisp yield and in decreased fat absorption may be not more than, say, 5%, these advantages would be of importance to the manufacturer: it is possible that the fish and chip frier selling chips by a form of volume measure would not necessarily appreciate the advantages of an improved yield and reduced fat usage.

After-cooking colour

Although the after-cooking colour of a boiled potato and that of a fried potato are apparently two different qualities, both can be affected by potassium nutrition. Stem-end blackening, a discoloration that occurs after cooking, may be exhibited by both boiled and fried potatoes. So also may enzymic blackening which occurs as a result of exposure to air or because of bruising or other mechanical damage. Both kinds of blackening can be influenced by the level of potassium nutrition. With fried potatoes, the development of the brown colour is probably of greater importance than is blackening. The relationship between potassium source and rate and chip or crisp colour is not as yet clearly defined, but some recent work in the U.S.A. has suggested that potassium source is important.

Stem-end blackening

The most important aspect of the colour of the boiled potato is whether it will blacken as it cools after cooking. Discoloration occurs at the stem-end and decreases in intensity towards the apical end or heel of the tuber. Although the discoloration does not affect the flavour or the nutritional value of the potato, the general appearance of the cooked tuber is marred. Smith & Muneta[16] hold that stem-end blackening has had a marked effect on the general gradual decrease in potato consumption in the United States.

It cannot be said that potassium plays the dominant rôle in reducing stem-end blackening. Rieman *et al.*[17] suggested that the variety of the potato influences blackening more than does any other single factor, and Wager, in a very complete survey of the subject,[18] considered that the 'soil-locality' complex was next in importance to variety in its effect. The effect of potassium on stem-end blackening has been extensively investigated and very variable results

have been obtained. Wager[18] has said that ' given certain unknown conditions, the interaction of the potato with the soil-locality factors may result in less blackening if the potash status of the soil is improved, but the effect is small and unpredictable '. Several workers[19–22] have, however, shown that a low level of potassium, combined with a high rate of nitrogen, increases the tendency to blacken.

Blackening is said to be due to the formation of a complex between iron and *o*-dihydroxyphenols in the tuber ; the principal compounds that may be involved are chlorogenic and caffeic acids. It is thought that the ferrous ions of the potato tuber combine with the precursors of the blackening pigment. In the raw state, the natural reducing conditions and low pH tend to keep the complex in a reduced colourless form, but on keeping after cooking the ferrous iron is oxidised to ferric and a strongly coloured compound results. Juul[19] held that discoloration increased with a higher nitrogen/potassium ratio in the fertiliser and that high ratios promoted the formation of *o*-dihydroxyphenolic compounds in the tuber, the highest concentrations being at the stem ends. The ferrous iron content of the tuber was said by Juul to be independent of the nitrogen/potassium ratio, but Bolle-Jones[22] concluded that iron and potassium are interrelated in the metabolism of the potato plant, and that potassium deficiency may give rise to an increased iron content of the tuber. Cowie[23] also suggested that potassium deficiency might bring about an increased concentration of iron in the tuber. Juul stated that stem-end blackening depended on the pH of the potato tuber and that there was a linear relationship between pH and colour. Increased nitrogen/potassium ratios are correlated with a small but significant increase in pH. Potassium-deficient tubers contain much less citric acid than do those from plants with an adequate potassium supply, and Mulder[20] considered it possible that much of the iron in the tuber was normally bound by citric acid and was not then able to react with the *o*-diphenols. Recent work at the Low Temperature Research Station, Cambridge,[24, 25] has, in fact, shown that although the concentration of chlorogenic acid does decrease away from the stem-end of the tuber, there is a rise again at the heel end. This increase is not accompanied by blackening because there is a sharp rise in citric acid concentration at the heel and a corresponding drop in pH. The distribution of blackening is said to be governed by the competition between the chlorogenic acid group of phenols and citric acid for iron.

Potassium, then, is concerned in the control of the concentration of *o*-diphenolic compounds in the tuber, with citric acid content and the pH of the tuber, and its metabolism is related to that of iron. The nature of the soil and the climatic conditions during growth tend to overshadow the relationship, important as it is, between potassium rate and stem-end blackening.

Enzymic discoloration

Stem-end blackening should not be confused with the discoloration of potatoes that results either after tubers have been exposed to the air for too long before they are cooked, or after tubers have been roughly handled and bruised. The susceptibility of potassium-deficient tubers to this type of discoloration is due to the fact that such tubers have a high content of tyrosine which, as a result of cell damage, is converted to melanin, a bluish-black compound, by the enzyme tyrosinase.[18, 20, 26] Ophuis *et al.*[26] use the term ' bruise blue ' to describe the phenomenon, and Fig. 3 illustrates the influence of the potassium content of potato tubers on the susceptibility to bruise discoloration.

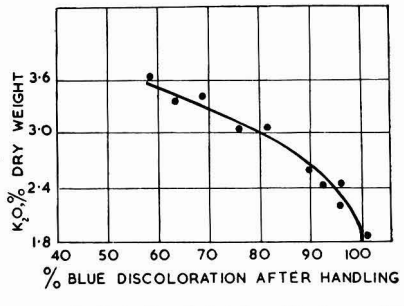

FIG. 3.—*Influence of potassium content on the susceptibility of potatoes to bruising*

Chip and crisp colour

The colour of a chip or crisp should be a pale golden brown, untinged by enzymic or stem-end blackening. The brown colour is dependent on a number of nutritional and climatic factors and is related, in the main, to the caramelisation of the reducing sugars present in the tuber. As noted earlier in the section on the relationship between potassium source and starch production, Buchner[7] observed that in chloride-treated plants the reducing sugar content was less than with sulphate. In recent years, several workers[27, 28] have suggested that chloride resulted in lighter crisp colour than did sulphate, and that increasing rates of potassium chloride tended to give rise to a lighter crisp colour. Murphy & Goven[28] have said that if crisp colour is the most important criterion for potatoes used for crisp manufacture then the use of potassium sulphate may be inadvisable. However, crisp manufacturers have always advocated the use of potassium sulphate to produce a potato with higher dry matter content with subsequently a higher recovery of the cooked product, and it is probable that the production of a better crisp or chip colour as a result of the application of potassium chloride is not truly significant.

Potato quality tests

Assessments of the texture of the cooked potato and of stem-end blackening have been made at Levington Research Station for the past 3 years, each series of experiments lasting for approximately 7 weeks. Panels of 12 people have been used for judging texture while assessment of stem-end blackening is made by laboratory staff one hour after cooking. Both qualities are given a numerical score and, in addition, the dry matter content is determined for all samples.

The samples originate from a number of field experiments designed to give information on both potato quality and yield. The experiments are carried out on many sites in Great Britain, so that a wide variety of soil types and climatic conditions can be studied in conjunction with various fertiliser practices. The experiments are planned statistically so that an adequate number of samples may be assessed in the laboratory ; from four to six, and no more than six, being cooked at a session. The potatoes are boiled for 25–35 min. without salt, and are served to a panel previously selected for reliability of assessment after a period of trial tests.

Dry matter content and texture as related to potassium source

In all eleven experiments, potassium sulphate (50% K_2O) led to a higher content of dry matter, and in four of these the increases were significant as compared with potassium chloride, i.e., muriate of potash (60% K_2O). That potassium sulphate had given rise to a significantly higher dry matter content was only confirmed by a panel assessment in one of the four relevant experiments. In another experiment potassium sulphate was judged by a panel to lead to a more floury or better-textured potato, although no significant difference was indicated by the results for dry matter.

The higher content of dry matter following the application of potassium sulphate has then not always been significantly important, and panel assessment has only differentiated between sources in two out of eleven experiments. To be confirmed by a panel assessment, the difference in dry matter content must be of the order of 1% (the dry matter content ranges from about 15 to 23%) and such a difference has only occurred in three out of eleven experiments with the two sources of potassium.

Dry matter yield and potassium level

The yield of dry matter [dry matter content × yield (ware and seed)] increases with increased rate of potassium fertilisation in very much the same manner as does total starch yield (see Fig. 2). The increase in dry matter yield up to 180 lb. of K_2O/acre is quite considerable, but beyond that the rate of increase is less marked. However, even at 250 lb. of K_2O/acre slight increases have been observed on some sites. Potassium levels above 300 lb. of K_2O/acre have not been used in the Levington experiments but the peak of the curve has certainly been reached by 300 lb. of K_2O/acre. The curves in Figs. 1 and 2 have been plotted from a combination of the results of the Levington experiments and of other workers.

After-cooking blackening

The 3-years' tests (1956 to 1958) have confirmed that high nitrogen and low potassium rates tend to give rise to stem-end blackening on sites where the 'soil–locality' complex was unsuitable. The experiments conducted from Levington were carried out as far as possible in the more suitable potato-growing areas and as a result stem-end blackening was not always significant. On three sites in 1956 and 1957, stem-end blackening was more severe with higher rates of nitrogen (up to 180 lb. of N/acre) when no potassium was applied in the basic fertiliser. In 1958 blackening was not significant in any of the relevant experiments.

Flavour

The flavour of the cooked potato would seem to be little affected by changes in fertiliser rates or types. Eighteen experiments were carried out between 1956 and 1958 in which potassium rate and source were related to taste panel assessment of boiled potatoes. Flavour assessments on samples from fertiliser trials have been made by panels of six people, these panels scoring independently of those judging texture.

Potassium rates between 100 and 300 lb. of K_2O/acre and nature of potassium source have had no effect in any of these experiments on the flavour of the boiled tuber.

Conclusions

The level, and to a smaller extent perhaps also the source, of potassium is of considerable importance in the production of a tuber of high dry matter content, and is also directly concerned with the tendency of tubers to blacken and to bruise. Dry matter content and chip and crisp colour are of great interest to the fish frier and the crisp manufacturer, especially when one considers that 20–25% of the potatoes produced for human consumption in this country are sold over the counter in fish and chip shops. That the housewife is interested in a good quality potato is shown by the report of the Potato Marketing Board, 1958,[29] which notes that 69% of housewives would readily pay more for a really good potato.

The optimum rate of potassium application for the production of good-quality potatoes will vary from soil to soil, but 200 lb. of K_2O/acre would seem to be a good average value. As a source of potassium, potassium sulphate appears to be rather more satisfactory than potassium chloride (muriate of potash) for quality production of potatoes.

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THE NITROGEN, PHOSPHORUS AND POTASSIUM REQUIREMENTS OF SUGAR CANE*

By R. F. INNES

The responses of sugar cane to N, P and K fertilisers in various sugar-producing countries are surveyed. The value of soil analysis, deficiency symptoms and tissue analyses for control of the nutrition of the plant is discussed.

The paper deals principally with the results of work carried out on annual cane in Jamaica but reference is made where appropriate to the results of work carried out in other sugar-producing countries.

The total plant food removed in millable cane at harvest fluctuates widely, depending upon variety, soil, fertiliser policy and tonnage cut. An average crop of 35 tons/acre of millable cane removes about 40 lb. of N, 50 lb. of P_2O_5 and 130 lb. of K_2O, but these figures can be greatly exceeded, especially on heavily fertilised land producing high tonnages; for example, Borden[1] records the removal of 300 lb. of nitrogen, 180 lb. of P_2O_5 and 960 lb. of K_2O by a record crop of 50 tons of dry matter produced in under two years.

Yield response of sugar cane as indicated by the results of yield experiments

Carey & Robinson[2] analysed the pre-war experiments and Hodnett[3] the post-war experiments of Jamaica and also sugar cane fertiliser experiment data from parts of the Commonwealth. Their conclusions confirm those from a more limited analysis by us of Jamaican data, i.e., the relative responses of sugar cane to nitrogen, phosphate and potash conform to a Mitscherlich relationship† and that the following constants (acres/lb.) conveniently apply:

Cane	0·006	0·010	0·010
Sugar	0·008	0·010	0·008

The nomograms in Figs. 1–3 are based upon the above constants and have the advantage of allowing optimum dressings to be deduced easily from the response to a single dressing. These relationships, whether for cane or sugar, should be considered as general guides, partly because of the error of the assumed generalised relationships and also because of the great variation in cane quality due to ecological factors: e.g., minimum temperatures are of great importance.

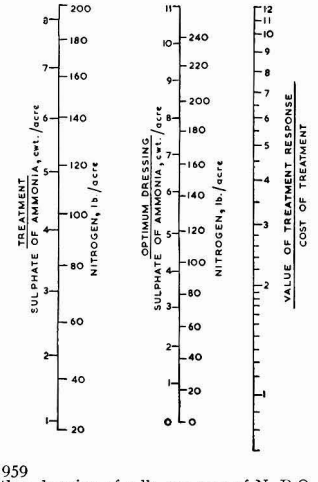


FIG. 1.—*Relationship between yield (= cane) response and optimum nitrogen dressing*

* Read at meeting of Agriculture Group, 12 May, 1959

† $y = y_0 + d(1 - 10^{-cx})$, where y = yield per acre with a dressing of x lb. per acre of N, P_2O_5 or K_2O; y_0 is the yield without fertiliser; d is the limiting response; c is the Mitscherlich constant

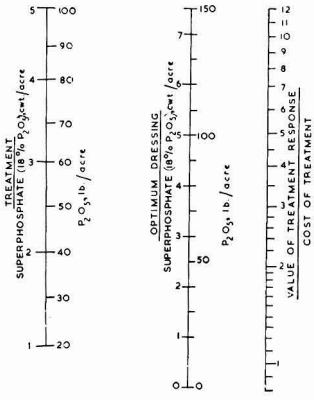


FIG. 2.—*Relationship between yield (= cane) response and optimum phosphate dressing*

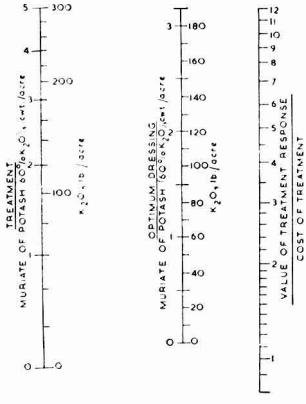


FIG. 3.—*Relationship between yield (= cane) response and optimum potash dressing*

The response of sugar cane to nitrogen

The response to a standard quantity of nitrogen varies greatly, being influenced dominantly by water supply and whether the crop is 'plants' or ratoons. The results of experiments suggest that in Jamaica the rates indicated in Table I*a* would be optimum at current prices for standing cane in the field and cost of sulphate of ammonia supplied to the soil, whereas, in actual plantation practice, the ranges indicated in Table I*b* prevail.

Table I

Dressings of sulphate of ammonia in Jamaica
(cwt./acre)

(*a*)
Optimum dressings

	Plants	Ratoons
Non-irrigated	4	5
Irrigated	5·5	7

(*b*)
Actual dressings

	Plants	Ratoons
Irrigated	3·5	4·6
Heavy rainfall 90 in.	2·5	4
Medium ,, 50–60 in.	3	3·4
Low ,, <50 in.	2	2

British Caribbean as well as British Guiana, Australian, South African and Mauritian experiences suggest that rarely are rates in excess of 6 cwt. sulphate of ammonia/acre used for annual cane and yet, in some countries, for example, in parts of Puerto Rico, over twice this quantity is applied. The practice in Hawaiian plantations is to use nitrogen levels comparable with the upper range of those used in the British Caribbean, although recent Hawaiian work[1] suggests that considerably higher rates, the equivalent of 9–13 cwt. sulphate of ammonia/acre/annum, may be required to produce maximum yields of sugar. The field implications of such dressings have not been tested on a commercial scale. The greater response of 'ratoon' cane to nitrogen compared with 'plant' cane is presumed to be due to the combined effects of the accelerated decomposition of accumulated root systems, a short fallow period, and the greater volume of well-structured soil in 'plant' cane. Hodnett's analysis[3] establishes that the residual effects of nitrogen from one crop to another are negligible.

Because of the long period taken by the crop to grow and especially in the case of 2-year cane, it is customary in many countries to split the nitrogen application; for annual cane two dressings are given, whilst for biennial cane, the number may be four or more. Provided the first dressing is made when the cane shoots are about 6 weeks old in 'plants' or soon after cutting in ratoon cane, the author knows of no experimental evidence to support the practice

of splitting the dressings. Jamaican evidence shows no benefits to be obtained and recent experiments in Hawaii[4] suggest that, even with the heavy nitrogen dressings required for 2-year cane, the benefits of splitting the dressings are in doubt.

The relative value of different sources of nitrogen

Experimental evidence[3] suggests that the response to different nitrogen carriers varies slightly, sulphate of ammonia and Nitrochalk being most effective, with ammonium nitrate, cyanamide and nitrate of soda giving somewhat smaller responses, but it is doubtful whether such comparisons can be generalised irrespective of soils.

Laboratory studies[5] with Jamaica sugar cane soils have shown that the losses of aqueous ammonia from soils injected below the surface are negligible provided the soils do not lose water; that losses increase with loss of moisture, soil lightness of texture and increasing soil alkalinity, and that ammonia injection temporarily impairs soil nitrification. Similar studies indicated that on most Jamaica clay soils with only traces or less of free calcium carbonate, aqueous ammonia injected into the soil surface is as well retained as sulphate of ammonia applied to the soil surface but only 80% as well retained on loams. Yet field experiments have shown generally, when judged by plant composition and yield, that aqueous ammonia drilled at 6 in. depth is only 70% as effective as sulphate of ammonia applied to the surface. The field results with aqueous ammonia on acid, heavy, clay soils in British Guiana have also been disappointing.[5] The difficulty of effectively sealing the injection tine-cut is partly responsible.

Aqueous ammonia is extensively used in Hawaii[6] where its application costs are helped by mounting the injection equipment on heavy crawler tractors which carry out injection simultaneous with planting, replanting, ratoon subsoiling or furrow reforming. The soil retention of the material is facilitated by the almost universally base-unsaturated nature of Hawaiian soils and their deep, friable, water-stable tilths. In Puerto Rico and Louisiana where aqueous ammonia is also widely applied, the main considerations favouring its use have been its much cheaper unit price and a fertiliser policy which was already applying nitrogen fertiliser at rates well on the flat part of the response curve.

A recent series of experiments carried out in Jamaica suggests that whereas urea may be slightly less effective than sulphate of ammonia, the difference is small and not significant.[7] In three other experiments urea with a biuret content of 2·5% gave the same yields as were given by equivalent sulphate of ammonia and urea of a low (<0·5%) biuret content. With the advent of aerial top-dressing, the use of aerially applied urea is increasing as a means of correcting temporary nitrogen shortage in standing sugar cane.

The influence of nitrogen upon crop quality

Nitrogenous dressings are universally recognised as lowering the sugar content of sugar cane. Jamaican experience suggests that on an average the adverse effects are a constant ($-0{\cdot}01 \pm 0{\cdot}004$% sugar) for each unit % change in response in cane yield—a conclusion which agrees in principle with the results published by Clements[8] for Hawaii based upon the partial linear correlation between relative response in yield and relative response in leaf nitrogen when both are influenced by nitrogen dressings.

Because of the impossibility of prejudging nitrogen dressings to weather during the growth of a crop and at the approach of harvest, a considerable resistance exists to applying nitrogen to the optimum economic limit based upon average deduction. On the whole, a dressing of 50 lb./acre of nitrogen depresses % sugar by $0{\cdot}14 \pm 0{\cdot}002$ in Jamaica and by effects of similar magnitude elsewhere, and in British Guiana also when yield responses are small.[3] By comparison, the cane response to 50 lb. of nitrogen is about 3·0 tons on non-irrigated, and 5·0 tons on irrigated, land in Jamaica.

The response of sugar cane to phosphate

The extent of phosphate-deficient soils on sugar cane plantations in Jamaica is small and the mean response to 50 lb. of P_2O_5 only 0·9 ton cane (at present prices, this is the same as an optimum economic dressing equal to 20 lb. of P_2O_5/acre) which applies for 'plants' and ratoons. On isolated areas of severe deficiency, for example, on some acid clay soils of secondary origin, the standard response is three times as great, and here the current economic dressing would

be 70 lb. of P_2O_5/acre. Commercial field applications vary between 30 lb. and 54 lb./acre. By contrast responses[3] are reported from certain Queensland and South Africa soils which would suggest that the optimum economic dressings are well in excess of 110 lb. of P_2O_5/acre, and in Hawaii dressings of up to 500 lb. of P_2O_5 are applied to 'plant' cane as ground rock phosphate on highly deficient soils.[4] More recently, as potash deficiencies have been corrected in Jamaica, there is evidence from leaf composition that the extent of phosphate-deficiency may now be increasing as a result of the increased tonnages being reaped. Because of our interest in plant composition as an index of its plant food requirements, no great attention has been paid to the experimental determination of the residual value of phosphate to date. Elsewhere, experiments show that the residual value of phosphate is very appreciable[3] and Hawaiian field practice is based upon there being a high residual value from ground rock phosphate on acid red earths.

Field studies in Hawaii where cane trash is destroyed by preharvest burning have shown the importance of placement of phosphorus into the rooting zone and that the best position is beneath the cutting at planting or directly under the old plant.[9] Elsewhere, where cane trash is not burnt and particularly in South Africa[10] and Mauritius,[11] results indicate that soluble phosphorus should be spread on the trash blanket, thereby being taken up by the plant through the surface roots feeding in the decaying mull of surface organic remains.

On phosphate-deficient soils cane juice phosphate is low and generally much below 300 mg./l., the level usually accepted as desirable for good clarification in the raw sugar process. It is customary in such cases to add triple superphosphate to the cane during the process of milling, and recent investigations in Jamaica[12] suggest that this is a very effective way of applying phosphate to the soil—by fortifying the filter mud in the factory and applying this to the soil. Dressings of nitrogen reduce juice phosphate appreciably.

In many countries[3] the effect of phosphatic dressings upon cane quality is slightly adverse; in Jamaica it is $-0{\cdot}04 \pm 0{\cdot}02\%$ cane/50 lb. of P_2O_5 applied. Because of the absence of very acid soils and the presence of traces of free calcium in most of our Jamaican sugar-cane soils, triple superphosphate is generally used.

The response of sugar cane to potash

The available potash status and potash reserves of sugar cane soils vary greatly, for example, in Puerto Rico in soils similar to Jamaican soils, Bonnet[13] shows a range of between nil and 18,200 lb. of K_2O/acre $6\frac{2}{3}$ in. in total potash content. In Jamaica, mean response curves show that the potash available to sugar cane varies between the equivalent of a negligible quantity of muriate of potash on ground water laterites to an excess of 500 lb./acre of muriate of potash on recent alluvia. The same point is made by Hodnett where he refers to the great variation in the response to a standard dressing of potash obtained between different countries and within the same country.[3] The continuation of a fertiliser policy based upon an inadequate potash supply can lead to a substantial decline in yield. In Hawaii, Humbert[14] quotes an increase of 60% in the annual production of one plantation following an increase of 200% in the potash applied annually. There can be little doubt that in the case of potash, the correct policy should be to tend to overfertilise rather than underfertilise. At present prices it costs 2·7 times as much to underfertilise with respect to the optimum dressing as it does to overfertilise by the same quantity of potash. Muriate of potash dressings to annual cane usually vary between 1 and 2 cwt./acre/annum, but from the trend of current work it is likely that these may be appreciably increased in some areas, particularly under irrigation.

The residual values of potash dressings vary. On ground water laterites quantities up to 2 cwt./acre of muriate (60% K_2O) have little residual effect. The accumulated residual effects of a phosphate or potash manurial policy are reflected in repeated plant composition surveys. In Jamaica as elsewhere[3] the effect per unit dressing of potash is greater in ratoons than in 'plants'—and is attributed to the decrease in root range from 'plants' to ratoons associated with the loss of soil structure.

The effect of potash on cane quality

In general, potash dressings improve cane quality and in Jamaica the effect amounts on the average to $+0{\cdot}06$ sugar % cane for each 50 lb. of K_2O applied and is highly significant.

Our experiments, however, show that the influence of potash upon juice or cane quality is greatest when cane yields per acre are improved most. The slope of this relationship, i.e., the regression of change in sugar % cane upon cane yield response, is $-0{\cdot}033 \pm 0{\cdot}005$ according to Hodnett. Samuels,[15] working in Puerto Rico, reports a similar effect. But since any response from applied potash tends to result in a reduction in the tissue concentration of nitrogen, the improvement in cane quality may also be associated with a reduction in cane nitrogen concentration. The Hawaiian results[16] showing that high reducing sugars and low sucrose contents are correlated with the N/K ratio of stalk tissue are in agreement with this.

The effect of interactions on yield

The incidence of significant (statistical) interactions between any pair of N, P and K is very small, and on the whole, they are of little account. Hodnett finds significant interactions but of ' no great importance in practice ' in data from some other Commonwealth sugar cane countries. More recent data quoted by Evans *et al.*[17] for British Guiana also show no significant interactions. The conclusion seems justified therefore that whatever the interactions between elements in the physiological sense in the cane tissue may be, the effects of the main elements are generally additive where yield is concerned.

Soil analysis as used for the control of sugar cane nutrition

Soil analytical techniques have been found to be of general value with sugar cane, especially when the soils are unsaturated, but the author knows of no country in which the nitrogen requirements of sugar cane are determined from soil examination. Soil analysis for the diagnosis of phosphate and potash deficiencies have probably attained the greatest accuracy in Hawaii and Mauritius on acid red earths and laterites, and in Queensland.

In Mauritius[11] with a modified Truog extractant, 23 p.p.m. of available P_2O_5 is considered the limit below which responses can be confidently expected in ratoons, whilst in ' plant ' cane crops, the figure is nearer 20 p.p.m. The correlation between yield response from a standard dressing of phosphorus and the p.p.m. of available P_2O_5 in the soil of control plots is significant but only accounts for about one-quarter of the total variance.[11] Although a value of 44 p.p.m. of P_2O_5 has been accepted in Hawaii[16] also with a modified Truog extractant, the limits have been shown to be a function of volume weight and limits above 46 p.p.m. would be expected on low-volume soils such as would be the case with stony soils or shallow soils. Queensland work[18] suggest that at below 40 p.p.m. of available P_2O_5 response can be expected with certainty, and that response magnitude is linearly related to the extent to which available P_2O_5 in the soil lies below the critical limit of 40 p.p.m. In Trinidad, the response in sugar per acre to a dressing of 50 lb./acre P_2O_5 was four times as great when available[21] phosphate in the soil was below 30 p.p.m. as when it was above this figure.[3] Hardy,[20] also using a Truog extractant, places the limit at 20–35 p.p.m. In South Africa[10] where soil analysis is also widely used, phosphatic responses are expected where the available P_2O_5 by Truog extraction is below 75 lb./acre, but not where the figure is 150 lb. or more ; no quantitative relationship is possible here between available phosphate in soil and cane response.

For Hawaiian soils[19] extracted with N-ammonium acetate, 120 p.p.m. of available K_2O would appear to be the level above which responses are unlikely and below which response is logarithmically related to available potash supply in the soil. A similar relationship is obtained in Jamaica when potash response curves are used to derive the available potash supply position in the soil. In South Africa, also with N-ammonium acetate as extractant, large responses are expected when available K_2O in the soil falls below 250 lb./acre whilst at levels of 500 lb./acre or more, responses are unlikely.[10] In Trinidad, 80–90 p.p.m. of K_2O for 0·5N-acetic acid extraction is used as a limit,[21] whilst for the same extractant and for West Indian conditions in general, Hardy[20] uses a figure around 78 p.p.m. of K_2O for sands and loams, and 115 p.p.m. for clays and silty clays. In Barbados, where much of the soil contains free lime, the limit is set at 150 p.p.m.[22]

The inadequacy in many instances of a determination of available potash in soil on its own is admitted by Kerr & von Steiglitz[18] who found it necessary to use not only available potash figures but also the level of potassium as a percentage of the total exchangeable cations. The

quantity of potash removed by sugar cane is related to the level of available potash in the soil and Ayres & Hagihara,[19] working in Hawaii, give a striking example of how with succeeding crops the decrease in available potash in soil is itself a linear function of the initial level of this plant food in the soil.

Sugar cane deficiency symptoms as a diagnostic technique

Deficiency symptoms of the major elements are quite specific and are seen in the field particularly in peasant-grown cane. They are of limited value for the control of fertiliser dressings on well-run plantations because nutrition is here generally above the deficiency symptom level, but not necessarily at a level where deficiencies have been eliminated.

Sugar cane showing nitrogen-deficiency symptoms is a uniform yellow green, the yellowing intensifying with increase in deficiency. Growth is retarded and the older leaves dry prematurely. The drying commences at the tip of the oldest leaves and progresses along the leaf margins and back along the midrib. Cane standing in the field immediately gives the impression of carrying a large quantity of trash (dead leaf). Cane stalks are thinner. On some varieties there is a tendency for the anthocyanin colour in the exposed leaf sheath to intensify. A nitrogen-deficiency promotes ripening and sugar accumulates in the stalk and sheaths.

Severe phosphorus-deficiency produces a lack of tillering and a purpling of the small leaves on young shoots ; the stalks are thinner and the internodes shortened. In some varieties the stalk is reported to taper rapidly to the growing point.[23] The leaves are narrow and a greenish-blue in colour.

Potash-deficiency symptoms show themselves on the older leaves first because of the rapid translocation of potash from these tissues. The leaves show a yellow spotting against a lighter green background, and become progressively more yellow to orange in colour and the spots more chlorotic until finally they become brown with necrotic centres. The edge and tips of the affected leaves scorch. The midribs develop a pink to red discoloration of the epidermal cells of their upper surface, a symptom which is very characteristic and ought not to be confused with a similar discoloration which develops when the midrib has been damaged by insect puncture. In the latter case, the discoloration is not confined to the upper epidermis only. When the deficiency is severe, lengths of the midrib dry out to a strawlike state, the older leaves show heavy marginal scorch, the stalk and leaves become very slender, and the internodes shorten, particularly near the growing point. In the field, the crop becomes very prone to leaf fungus attacks especially to *Helminthosporium* (eye spot) and *Cochiobolus stenospilus* (brown stripe).

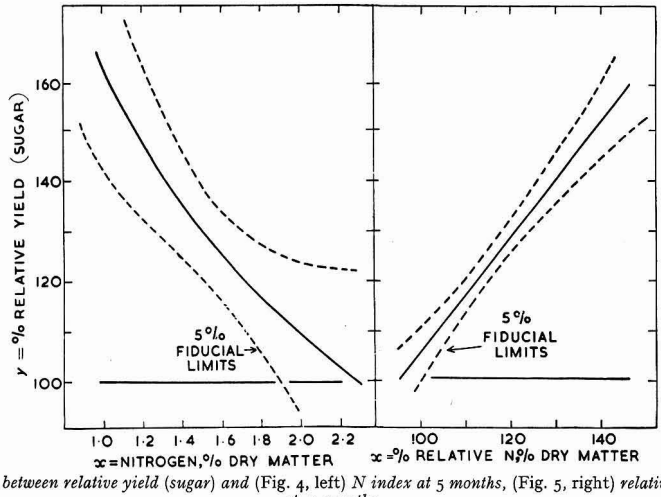
Tissue diagnostic methods

The yield responses of field experiments represent the end effects measured at harvest, of treatments imposed many months earlier. It would therefore appear desirable that fertiliser treatments should be combined with a more detailed examination of the plant in terms of the changes in its nutritional status with time so that the latter may be related to the yield responses induced. Clements[8] has stressed the importance of studying the changes in the composition of the sugar cane plant with age and today those who would argue against this must be few indeed, although there are many who would defend the study of different tissues. The advantages of tissue composition for advisory purposes need not be stressed here. The value for diagnostic purposes of tissue composition depends upon the extent to which it correlates with plant response to applied nutrient, and particularly with plant yield, so that the nature and size of the plant response may be predicted. The work has been extensively pursued in Hawaii, Mauritius, Puerto Rico, Jamaica, British Guiana and South Africa, and shows signs of becoming accepted as the means by which the nutrition of sugar cane will be conducted on a field by field basis. The most generally accepted index tissue is the first fully-developed leaf or leaves—those with the first to third dewlap or ligule exposed, and the leaf lamina is used. Sampling is either done by a punch or by taking the middle six inches or so of the lamina ; the results are expressed on a dry matter basis.

(a) *Nitrogen*

Much work has shown that the level of nitrogen in leaf lamina material falls with the age of the leaf, with increasing drought effects, and with the age of the cane.[9] The nitrogen level

is also sensitive to conditions of poor tilth and soil aeration, and temperature—leaf-nitrogen levels fall with soil temperature and increase with mean air temperature.[9] One has therefore to be particularly careful when interpreting leaf-nitrogen figures. In practice, fields are not sampled if rain has been inadequate for rapid growth during the month preceding sampling. In Puerto Rico, levels are corrected statistically for the age of cane and sheath moisture,[24] whilst in Hawaii, Clements corrects leaf nitrogen to a standard sheath moisture content. So long as cane is actively growing for a month prior to sampling, Jamaican studies show that the production of sugar per acre from the optimum economic dressing of nitrogen expressed as a percentage of the production in the absence of a nitrogenous dressing (y) is very closely related to the N % in the dry matter (x) in the leaf lamina in the absence of nitrogenous dressings and can be defined by a linear or logarithmic relationship, but the latter is the more accurate (see Fig. 4). The regression is much more significant for cane at 5 months than at 4 months. The 5% fiducial limits of the mean relationship suggest that an optimum nitrogen status would be 1·90% or higher. A better quantitative prediction of relative yield response, y, is obtained from the relative response to x. They are linearly related with very great significance when the leaf sampling is at either 5 or 4 months. The relationship is illustrated in Fig. 5 and has supplied the basis of a field method of controlling nitrogen nutrition, particularly on irrigated plantations where an adequate water supply is fairly certain. On as many fields as possible,



Relationship between relative yield (sugar) and (Fig. 4, left) *N index at 5 months,* (Fig. 5, right) *relative N index at 5 months*

Fig. 4. $y = 167/x^{0.60}$ (significant at $>1000:1$)
Fig. 5. $y = 18x - 13$ (significant at $\infty:1$)

small contiguous plots are each treated with one of three or four increasing levels of nitrogen: the dressing made to the field in the normal course of fertilising is checked and modified in the light of the nitrogen status of leaves from the treated plots relative to that of leaves from the rest of the field. The method is now also applied to sugar cane in Mauritius.[25] In other areas reliance is placed on leaf-nitrogen value at 4–5 months and upon the results of numerous factorial field experiments. The level of 1·9 N % dry matter at 5 months agrees well with Mauritius figures,[26] but is higher than the British Guiana figure.[27] Recent work in Jamaica based upon logging the growth and composition of cane on over 200 fields gives the following optimum values for ratoon cane of different ages:

Age in months at sampling	3	4	5	6	7·5	9
N % of dry matter	2·25	2·02	1·93	1·85	1·75	1·65

(b) *Phosphate and potash*

When a standard dressing of phosphate or potash is applied to sugar cane the data show that the increase in concentration of P_2O_5 and K_2O in the dry matter of the leaf lamina is related logarithmically to the concentration of these nutrients in the dry matter had no treatment been applied. The curves obtained from sampling at 4 and 5 months age are given in Figs. 6 and 7, from which it is argued that at this age, a dressing of 50 lb./acre of P_2O_5 did not influence the P_2O_5 concentration in the dry matter of the leaf lamina when this was already 0·06% or higher, whilst 120 lb. of K_2O/acre had little or no effect when its level in the dry matter already was 1·50% or more.

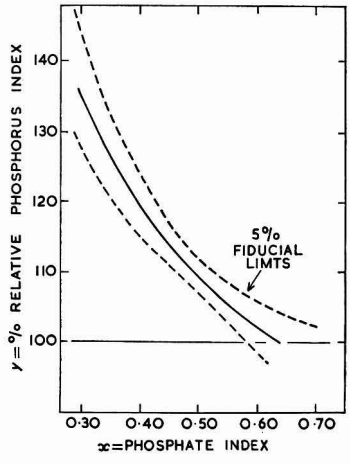


FIG. 6.—*Relationship at 4 months between relative phosphate index and phosphorus index in absence of phosphorus dressing*

$y = 84/x^{0·39}$ (significant at ∞ : 1)

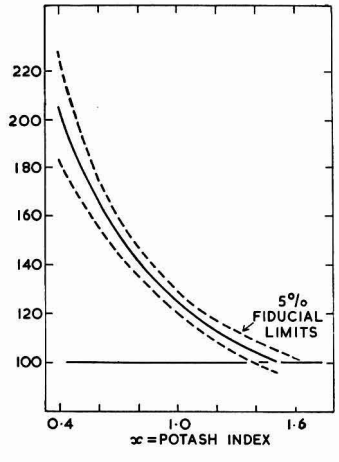


FIG. 7.—*Relationship at 5 months between relative potash index and potash index*

$y = 122/x^{0·53}$ (significant at ∞ : 1)

The relative yield (sugar) response to a standard dressing of either P_2O_5 or K_2O is also logarithmically related to the concentration of the particular plant food in the lamina dry matter in the absence of the dressing, whether the leaves are sampled at 4 or 5 months cane age. Some of the relationships are illustrated in Figs. 8 and 9. For phosphorus the relationship is more accurate at 4 months than at 5 months, but for each, when the % P_2O_5 in the dry matter stands at 0·57 or higher, the addition of 50 lb. P_2O_5 produces no response in yield. Where the average yield to be expected is 30 tons of cane/acre and at prevailing prices, 50 lb. of P_2O_5/acre would pay if the P_2O_5 in dry matter of the lamina was 0·50% or less. Mauritius workers recommend the application of phosphate up to a P_2O_5 index of 0·50%,[28] whilst in South Africa[10] it is stated that responses are common up to values of 0·45%, but unlikely beyond 0·50%. The relationships for potash are extremely accurate whether these were obtained at 4 months or at 5 months and each leads to the conclusion illustrated in Fig. 9, that no response from applied potash is obtained when the levels in the leaf lamina dry matter already stand at 1·45% K_2O or more. In Mauritius,[28] 1·40–1·50 is accepted as the limiting value below which potash responses are obtained, whilst South African workers[10] find responses small and variable between 1·20 and 1·50% and unlikely above 1·50%. At present prices and at an expected average yield of about 30 tons of cane/acre a dressing of 120 lb. of K_2O should pay when the potash content of the leaf lamina dry matter is 1·34% or less. The threshold values for phosphate and potash arrived at are also in close agreement with those quoted for British Guiana and Puerto Rico for similar tissues.[29] The relationship found to exist between the age of the plant at sampling and leaf composition in the absence of any measurable response in yield was determined from a study of the ' crop logs ' of over 200 Jamaican fields, each containing plots at two levels of each major

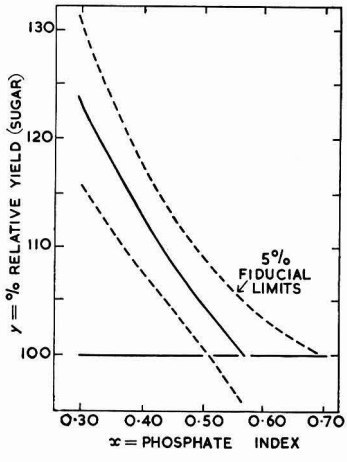


FIG. 8.—*Relationship at 4 months between relative yield (sugar) and phosphate index in the absence of phosphorus dressing*

$y = 83/x^{0.32}$ (significant at $\infty : 1$)

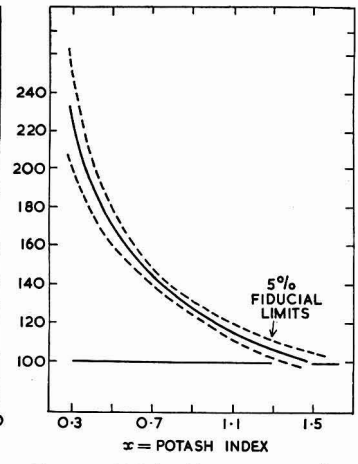


FIG. 9.—*Relationship at 5 months between % relative yield (sugar) and the potash index in the absence of potassic dressing*

$y = 118/x^{0.49}$ (significant at $\infty : 1$)

nutrient and is given below in Table II. These figures are in good general agreement with the levels used in other countries and quoted earlier.

Table II

Levels of phosphate and potash in leaf lamina dry matter above which responses in yield were not obtained from dressings of the respective plant foods

Age in months at sampling	3	4	5	6	7·5	9
P_2O_5 % of dry matter	0·51	0·47	0·45	0·44	0·42	0·40
K_2O % of dry matter	1·65	1·62	1·60	1·56	1·44	1·38

In Hawaii some notable work has been carried out on the possibility of using tissues other than the leaf lamina. Clements[30] relies upon using the leaf lamina for nitrogen status, but for the phosphate or potash status, he prefers the contents in the sheath dry matter expressed on a sugar-free basis. His critical levels are based upon a linear correlation with the total uptake of the nutrient by the whole plant. On this basis he finds it necessary to maintain the phosphate and potash contents on the sugar-free dry matter of his young index sheaths at 0·184 and 2·71%, respectively, during the period of 'rapid' elongation, otherwise production suffers.

More recently Hawaiian work[31] has centred around the distribution of the major elements in stalk tissue as distinct from leaf concentration. The ratio of total nitrogen in the 8–10 internodes to that in the basal internodes has been shown to be very sensitive to fertiliser treatment, particularly in young cane, whilst Evans[32] in British Guiana finds the basal internodes a sensitive index which he has used in conjunction with leaf analysis. Regression analyses between responses in terms of increased sugar per acre and the composition of various stalk tissues, based upon data from recent field experiments in Jamaica,[33] show that, for nitrogen, the ratios between basal stalk nitrogen and that in other stalk internodes do not correlate significantly with yield response, and that although basal internode nitrogen correlates very significantly with yield response, the composition of the internodes attached to the first and second fully-opened leaves correlates very closely and much more accurately than does the basal internodes' composition. The composition of the 1–2 internodes correlates significantly better than does leaf lamina nitrogen. The relationships are nearly twice as accurate at 4 months as at 6 months.

Jamaican data[33] have shown that stalk phosphorus is more sensitive than leaf phosphorus to phosphate dressings when phosphorus does not limit yield, but again, that the younger stalk tissues such as the 1–2 internodes are more sensitive than the basal tissues. The latest Hawaiian

data[4] suggest that at P_2O_5 levels of 0·075% in the dry matter in the 8–10 internodes gains from phosphate dressings may be expected, but none if the level exceeds 0·087–1·05%. As for phosphorus, our data[33] show that where potash is not limiting yield, potassium levels in stalk tissue are very much more sensitive than leaf potash, e.g., the 1–2 internodes are 14 times as sensitive whilst the basal internodes are 18 times, and that the stalk internodes at 6 months are more sensitive than at 4 months. Potash levels of 0·30% dry matter in the basal internodes, or 0·90 in the 1–2 internodes, are certainly at least adequate. Work by Evans and his colleagues in British Guiana[32] suggests that leaf potash values correlate better with yield responses than do the potash levels in the 8–10 internodes. Tissues whose potash content is very sensitive or which correlates closely with the potash content of the whole plant may not necessarily correlate best with yield responses. Such tissues may possess merit, however, if judged by their ability to foretell impending deficiencies as a result of a drop in their potash status consequent upon the translocation of potash to other more active tissues as threshold nutrition is reached. Evans obtains the convenience of leaf sampling and the accurate correlation of leaf composition with yield response, combined with the sensitivity of the translocation phenomenon, by studying the potash concentration in the sap expressed from the midrib of the leaf with the first visible ligule and that of the ninth leaf, counting the former as number one. Potash deficiency is shown to exist only when the potash content of the former is less than 0·78–0·84% simultaneous with a value of less than 0·36% in the latter. In well-fed fields, the potash in the midrib sap of the first leaf can amount to 1·21% K_2O whilst in very deficient fields it may be as low as 0·36–0·48%.

Major-element balance in sugar cane tissues is still little understood. Jamaican data suggest that nitrogen and phosphate levels in lamina tissue are correlated and that it may be worth correcting each to a standard level of the other. The most generally met interaction is that between N and K, especially when one or both are in limiting supply. Crop log data from Hawaii[34] suggest that above certain levels of leaf nitrogen and sheath potassium, each increases with the other, so that their ratio is approximately a constant. The value of the ratio and the levels above which it applies are influenced by soil and climatic conditions. Below the minimum potash value for the sheath, leaf nitrogen increases with decreasing sheath potash. A typical nitrogen potash balance is given by Humbert[34] from Hawaiian data. Below 2·0% K, potassium is presumed to give limited growth, and consequently nitrogen accumulates in the leaf tissues, whilst above 2·0% K in the presence of adequate soil-nitrogen, increasing the potash value brings about a simultaneous increase in nitrogen level, thus maintaining a balance. Similar phenomena are observed in field experiments in Jamaica when dressings of nitrogen fertiliser result in increased levels of potash in leaf tissue and vice versa, but in all such cases, which are not many, strongly negative NK interactions[35] in yield response are measured.

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MODE OF ACTION OF DIPYRIDYL QUATERNARY SALTS AS HERBICIDES*

By R. F. HOMER,† G. C. MEES and T. E. TOMLINSON

A specific type of herbicidal activity is shown by certain diquaternary salts of 2,2′-, 2,4′- and 4,4′-dipyridyl; the diquaternary salts of 2,3′- and 3,3′-dipyridyl are devoid of this activity. The herbicidal compounds have redox properties, giving rise to stable water-soluble free radicals by uptake of a single electron on reduction. A correlation exists between the redox potential of this reduction and the degree of herbicidal action. Evidence is presented that the compounds are normally reduced in green tissues by energy ultimately derived from light, but that as activity is also present in the dark, though at a reduced level, other reduction mechanisms are also possible.

Introduction

During the screening of a range of quaternary ammonium salts it was discovered that 1,1′-ethylene-2,2′-dipyridylium dibromide (**I**, $n = 2$) had high phytotoxic activity of a novel type.[1] The compound was extremely quick acting, leading to desiccation and top kill in a wide range of plant species. Experiments in which local application of the compound was made to small areas of whole plants led to generalised toxic effects and it was clear that the material was absorbed rapidly and translocated within the plant. Translocation was amply confirmed by the use of material labelled with ^{14}C, radioactivity rapidly becoming widespread in the aerial parts of the plant. The tracer experiments indicated also that very little material was translocated down to the roots; this was in agreement with the observation that although quite dramatic top kill of perennials was obtained, rapid regeneration followed. Applications of the herbicide to the soil produced no effect on plants, a phenomenon found to be due to the adsorption of the highly polar herbicide on to the soil by an ion exchange mechanism. It is, however, clear that uptake is possible through the roots since addition of the compound to a culture solution led to rapid death of plants growing therein.[2] There is obvious utility in the field of crop desiccation and rapid non-selective weed destruction for a compound having rapid action and no residual effect. This has led to the examination during the last four years of a range of quaternary salts derived from related heterocyclic systems, and particularly from the various possible isomeric dipyridyls, in an attempt to determine the chemical requirements for the appearance of this type of phytotoxic activity, and to elucidate if possible the mode of action of these compounds.

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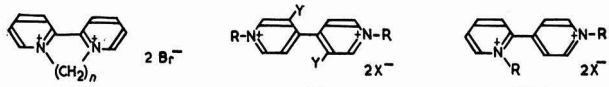
Experimental and discussion

Certain trends in activity were soon apparent, and it rapidly became clear that in the 2,2′-dipyridyl series of compounds activity was present only in a small group of compounds (**I**) where the quaternising group formed a bridge between the nitrogen atoms, and then only when $n = 2$ or 3, activity being much reduced in the latter case. 2,2′-Dipyridyl dimethiodide was



(I) (II) (III)

inactive as was the tetramethylene compound (**I**, $n = 4$). On the other hand it was found that in a series of diquaternary salts from 4,4′-dipyridyl (**II**) high phytotoxic activity of a similar character to that shown by the active 2,2′- compounds was obtained. The activity was present for a very wide range of quaternising groups R provided that Y was hydrogen.[3] When, however, the 3,3′- positions were substituted, for example if Y = CH_3, activity was lost. In all these compounds the nature of the anion was without effect on activity, Cl^-, Br^-, I^-, $CH_3SO_4^-$, SO_4^{2-} etc., all being equally effective on a molar basis.

Since, despite the different spatial dispositions of the nitrogen atoms and quite unrelated shape of the molecules, these two series of compounds both displayed similar herbicidal activity, a common factor involving a property of the molecules as a whole was sought in order to try and find a common explanation of their activity. The first factor which appeared to be relevant was that of coplanarity of the two pyridine rings, i.e., flatness of the molecule. By analogy with the extensively studied diphenyl system[4] it is clear that in all 4,4′- compounds of type (**II**, Y = H), the two pyridine nuclei are coplanar or can assume a coplanar configuration without hindrance. With the aid of models it could be seen that 2,2′- compounds of type (**I**, $n = 2$) were virtually flat molecules, while when $n = 3$ the amount of twisting of the molecule was such that coplanarity should not, it appeared, be impossible under suitable conditions. The inactive compounds, 2,2′-dipyridyl dimethiodide, 1,1′-tetramethylene-2,2′-dipyridylium dibromide (**I**, $n = 4$) and 1,3,1′,3′-tetramethyl-4,4′-dipyridylium di-iodide (**II**, R = Y = CH_3), due to steric hindrance, could not readily, if at all, adopt a planar configuration. This deduction from models is supported by the shift in the intensity and position of the ultra-violet absorption maximum (Table I), which is related to the amount of ' end-to-end ' resonance possible, and is thus a measure of the ease with which the rings may become coplanar.[5]

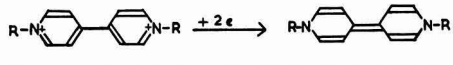
Table I

Ultra-violet spectra of bridged quaternary salts in water

Compound	$\lambda_{max.}$	ϵ (molar)
Pyridine methiodide	258	4770
I, $n = 2$	308–311	19,000
I, $n = 3$	287	15,600
I, $n = 4$	275	15,000
2,2′-Dipyridyl dimethiodide	270	13,600

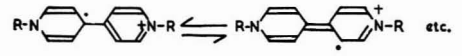
An initial hypothesis was therefore made that, for phytotoxic activity to be present, a molecule was required which was, or which could become, coplanar. That this was not the only requirement for activity was, however, shown by the lack of phytotoxicity in two other series of coplanar quaternary salts, those derived from 2,3′- and 3,3′-dipyridyl. A further link between the 2,2′- and 4,4′- isomers was therefore sought which would exclude these inactive isomers.

It has long been known that the behaviour of 4,4′-dipyridyl diquaternary salts on reduction is complex, the reaction taking place in two steps by way of an intensely coloured intermediate



regarded by earlier workers as a ' quinhydrone '. Michaelis & Hill[6] showed in 1933, however, that the first step in this reduction involved the addition of one electron to the quaternary salt

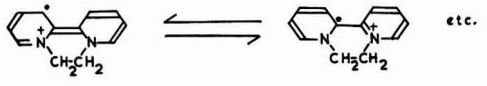
to form a stable free radical. This can be written in 18 possible resonance forms not involving charge separation, the odd electron being able to occupy any one of the nuclear positions. **IV** and **V** are typical examples. It should be noticed that the resonance forms include ones (e.g., **V**)



(IV) (V)

having a double bond between the rings. The radical, being still a quaternary salt, is water-soluble. The redox potential of this reduction to the radical varies with the nature of R, and Michaelis & Hill examined four salts with values between −446 mV (R = CH_3) and −359 mV (R = benzyl). These compounds have been subsequently used as redox indicators under the name of ' viologens ', and were, until the present work, unique among known organic redox compounds in that the reduction in aqueous solution involved only electron transfer, not hydrogen uptake.

It appeared that 1,1′-ethylene-2,2′-dipyridylium dibromide should behave similarly and form a radical on reduction.[7] As with the 4,4′- salts, 18 canonical forms could be written for the radical, and again the odd electron could be considered to be at any position in the pyridine nuclei. Two typical forms are shown at **VI** and **VII**; as before a number of the forms involve



(VI) (VII)

an inter-ring double bond. Treatment of 1,1′-ethylene-2,2′-dipyridylium salts with zinc dust was, in fact, found to give an intensely green solution, and by titration with dithionite in the absence of oxygen the redox potential of the reaction was found to be −349 mV—of the same order as that of the viologens. From the shape of the redox curve, it was clear that the reaction being studied involved one electron only and that true free radicals were being formed.

It is now apparent that the requirement of coplanarity already deduced is a necessary condition for the stability of the free radical, since such stability depends essentially on delocalisation of the odd electron over the whole molecule or, in other words, on conjugation between the pyridine nuclei of the radical. The greater is the number of possible canonical forms, the more stable the radical. Forms such as **V** and **VI**, requiring the presence of a double bond between the rings, can only exist if the radical is planar. It follows, therefore, that in a non-planar molecule the number of possible resonance forms is decreased and the radical becomes less stable.

A further requirement for radical stability is that the two nitrogen atoms should occupy suitable relative positions in the molecule. Only in the cases where these atoms are *pp′*, *op′* or *oo′* with respect to the inter-ring bond, is it possible to write canonical forms for the radical in which the odd electron can be considered as being located in any position. With the nitrogen atoms otherwise disposed, the electron is excluded from certain positions and complete delocalisation is prevented. The inactive 2,3′- and 3,3′-dipyridyl quaternary salts are examples of this class of compound. These isomers cannot be reduced to radicals at potentials obtainable potentiometrically in aqueous solution.

It seemed likely that phytotoxicity and reduction were related, and the hypothesis was therefore considered that herbicidal activity depended on the ability of the active compounds to form free radicals by uptake of one electron. This theory predicted that phytotoxicity should be found in the diquaternary salts of 2,4′-dipyridyl (**III**) where the above-mentioned favourable *op′* disposition of the nitrogen atoms is present. 2,4′-Dipyridyl dimethiodide[8] (**III**, R = CH_3), already noted by Krumholtz[9] to give a purple colour on reduction with zinc dust, was synthesised and found to be active. The activity was, however, at a reduced level, probably due to steric hindrance between the *N*-methyl group of the 2-linked ring and the *o′*-hydrogen atom of the 4-linked ring. This hindrance is further suggested by the much lower redox potential (−640 ± 40 mV) of this compound, which reflects the greater energy required to force the

molecule into a flat configuration. On similar steric grounds the lower activity of the trimethylene-2,2′-dipyridyl compound (**I**, $n = 3$) is explained, this compound having a redox potential of −548 mV, 200 mV lower than (**I**, $n = 2$).

The identity of requirements for phytotoxic activity and for reducibility to a radical suggested that a correlation should be sought between these factors. The redox potentials of a considerable number of compounds have been measured and a close relationship has been found between the value of the redox potential and degree of herbicidal activity. Table II indicates the range of redox potentials of a representative series of compounds as measured in aqueous solution using dithionite as reducing agent. It should be noted that as the reduction does not involve hydrogen the values are independent of pH. The majority of the titrations were carried out in alkaline buffer solutions of pH 10 which enabled the lower potentials to be measured without exceeding the hydrogen potential.

Table II

Redox potentials of dipyridyl diquaternary salts

	Redox potential, mV
(a) *4,4′-Dipyridylium salts*	
1,1′-dimethyl (iodide)[a]	−446 ± 2
1,1′-di-*n*-propyl (iodide)	−438 ± 2
1,1′-di-*n*-hexyl (bromide)	−439 ± 2
1,1′di(ethoxycarbonylmethyl) (chloride)	−422 ± 2
1,1′-di-β-hydroxyethyl (bromide)	−408 ± 5
1,1′-di-β-ethoxyethyl (iodide)	−386 ± 2
1,1′-dibenzyl (chloride)[b]	−350 ± 10
(b) *2,2′-Dipyridylium salts*	
1,1′-ethylene (bromide)	−349 ± 3
1,1′-trimethylene (bromide)	−548 ± 3
1,1′-ethylene-5,5′-dimethyl (bromide)	−479 ± 3
1,1′-ethylene-4,4′-dimethyl (bromide)	−487 ± 3
(c) *2,4′-Dipyridylium salts*	
1,1′-dimethyl (iodide)	−640 ± 40

(*a*) Michaelis & Hill[6] give −446 ± 2 mV
(*b*) Michaelis & Hill[6] give −359 ± 2 mV

Sign convention: the more difficult the reduction the more negative is the redox potential.

If it is postulated that these compounds are active because they give rise, within the plants, to toxic radicals, which are formed by reduction of the applied herbicide by a system in the plant functioning at a fixed reducing potential, it is clear that a greater proportion of radical will be formed in the plant from an easily reduced compound than from one more difficult to reduce present at the same molar concentration. Hence, assuming that the radicals from different compounds are of equal toxicity, and that differences in the absorption of applied solutions of different compounds are negligible, the more negative the redox potential of the compound the less phytotoxic it should be. In order to demonstrate this relationship, one leaf of each of a series of plants was dipped into a solution of the herbicide, and the least concentration of herbicide which just killed the plant (the threshold concentration) was determined. As expected, it was in fact found, within the range examined, that the lower the redox potential the higher was the threshold concentration, i.e., the less toxic was the compound. The relationship was investigated for several plant species, and Table III gives the results obtained with a selection of compounds on mustard and tomato plants. The threshold concentration (T) is seen to rise rapidly with compounds of increasingly negative redox potential.

A further interesting relationship is also shown in this table. It is quite easy to calculate from the relationship

$$E = E_0 + \frac{\mathbf{R}T}{F} \log \frac{[\mathrm{Ox}]}{[\mathrm{Red}]}$$

the proportion of reduced form present in a redox system of potential E_0 for any applied potential E, and if a value of −380 mV is assumed as an applied potential, then the concentration of radical produced (C, Table III) by applying this potential to each of the widely varying threshold solutions is roughly constant. While the applied threshold concentrations vary 350-fold, the derived radical concentration varies only by a factor of three. Hence a reducing system in the plant functioning at −380 mV would produce at the threshold roughly the same concentration of radical from each of the equally toxic applied solutions. This is certainly a very suggestive result pointing strongly to a common free-radical toxic mechanism in all cases. The redox potentials[10] of the TPN ⇌ TPNH and DPN ⇌ DPNH enzyme systems, themselves pyridine quaternary salts, vary from −316 mV at pH 7 to −378 mV at pH 9, close to the value of E which gives the most constant derived radical concentrations.

The fact that such a striking relationship is obtained despite the assumptions made regarding the toxicity of the radicals and the absorption of the herbicide is also suggestive of the validity of these assumptions.

Table III

Threshold concentration $\times 10^{-5}$M (T) *and derived radical concentration at* E = −380 *mV* (C) *for a series of dipyridyl quaternary salts*

Compound	E_0, mV	Mustard		Tomato	
		T	C	T	C
1,1′-Ethylene-2,2′-dipyridylium dibromide (**I**, n = 2)	−349	1·5	1·16	1·1	0·85
1,1′-Di-β-hydroxyethyl-4,4′-dipyridylium dibromide (**II**, R = $-CH_2 \cdot CH_2 \cdot OH$)	−408	10	2·54	3·5	0·89
1,1′-Di-β-carboxyethyl-4,4′-dipyridylium dichloride (**II**, R = $CH_2 \cdot CH_2 \cdot CO_2H$)	−431	—	—	9·0	1·11
1,1′-Dimethyl-4,4′-dipyridylium di-iodide (**II**, R = CH_3)	−446	30	2·21	7·5	0·55
1,1′-Ethylene-5,5′-dimethyl-2,2′-dipyridylium dibromide	−479	70	1·53	30	0·66
1,1′-Ethylene-4,4′-dimethyl-2,2′-dipyridylium dibromide	−487	100	1·62	—	—
1,1′-Trimethylene-2,2′-dipyridylium dibromide (**I**, n = 3)	−548	500	0·79	300	0·48

While the above relationships have been deduced on the assumption that the radical is toxic *per se* (and there is no direct evidence for this), the foregoing assumption is not the only one which would give rise to the relationship found. It could, for instance, be argued that it is the mechanism by which the radical is produced from the quaternary salt which is toxic, or that the reduction could give rise to toxic reaction products formed by oxidation of the reductant. The essential feature of any assumption is that the amount of toxicant liberated, or the degree of metabolic disorganisation caused, should be proportional to the concentration of radical eventually produced.

The compounds chosen to illustrate these relationships all have relatively compact molecules, and it should be remarked that the above-obtained constant radical concentration does not hold for all compounds, in particular 4,4′- compounds having long alkyl quaternising groups are anomalous. Also, in some other plant species, perhaps due to absorption differences, such good results are not obtained.

Considering now the reducing systems which may be involved in this reaction, the first crucial observation is that in the dark the action of 1,1′-ethylene-2,2′-dipyridylium dibromide is, on most plant species, much slower than in the light. Whereas, for example, in the light a treated broad bean plant is killed in a matter of hours, in the dark an identically treated plant will continue to live almost unaffected for several days. That the compound is absorbed and translocated in the dark is shown by the extremely rapid death of the treated plant when it is brought into the light. This is observed even when the source of the toxicant, e.g., a dipped leaf, is removed before the plant is illuminated. It is clear that in the dark the plant accumulates a super-lethal concentration of herbicide which is activated by subsequent illumination. Death of broad bean tissues is accompanied by development of a black coloration, and it has been shown that broad bean leaf discs floating in a solution of the herbicide blacken at a rate

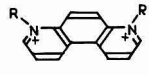
proportional to the intensity of the incident light up to a value of at least 10,000 lux. Moreover, only green tissue is affected rapidly in the light, and etiolated shoots, which fail to develop chlorophyll in the light after treatment with 1,1′-ethylene-2,2′-dipyridylium dibromide, are killed only slowly.

These observations suggest that the herbicide can be reduced in green tissues by energy ultimately derived from light, and because chlorophyll is needed for a rapid response by the plant, it is probable that the reducing system may be in part similar to that normally operating during photosynthesis. The chemical reaction which leads to radical formation cannot at present be identified, but it is possible that the radicals are produced by interaction with the quaternary salt of one of the naturally occurring radicals connected with photosynthesis.

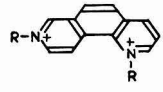
It is interesting in this connexion that Commoner *et al.*[11] have shown that the naturally occurring free-radical concentration, as revealed by electron spin resonance, is, in barley, much greater in the light than in the dark. More recently, it has been shown[12] that the electron spin resonance spectrum of a naturally occurring chlorophyll/lipoprotein complex indicates the presence of an unpaired electron, and that the intensity of the spectrum increases in the light.

Although light is essential for the rapid kill of green tissue, the compound is eventually, but usually much more slowly, lethal in the dark. The mechanism which leads to kill in the dark is not so apparent but there is some evidence that the radicals may interfere with respiration.[13]

Further experiments have shown that the presence of oxygen is essential for the functioning of the material in the light as it has been found that in the total absence of oxygen the response of broad bean leaf discs is completely inhibited. The interpretation of this result is somewhat obscure, but one possibility is that not only is reduction to a radical necessary, but that a re-oxidation is also involved. There is some supporting evidence for this idea in that a number of phenanthroline compounds, e.g., **VIII** and **IX**, are inactive as herbicides.



(VIII)



(IX)

These compounds give rise to free radicals on reduction but reoxidation of the radical does not reform the starting material, and the redox titration curve does not follow the theoretical path. This lack of reversibility is believed to be due in these cases to relative instability of the radical. Irreversible behaviour on reduction has also been found in a number of diquaternary salts derived from other diheterocyclic systems. All these irreversibly reduced compounds, though many produced radicals at least transiently, were found to be inactive as herbicides. In some cases the irreversibility appeared to be due to instability of the radical; in other cases the original quaternary salt was unstable in aqueous solution. The stability and reversibility required for activity appear to be difficult to achieve, and so far have been found in the quaternary salts of the dipyridyls discussed and a few closely related compounds only.

Conclusions

The relationship shown between redox properties and phytotoxic activity in a series of quaternary salts derived from 2,2′-, 2,4′- and 4,4′-dipyridyl leads to the conclusion that these compounds are active by virtue of their redox properties. These compounds appear to be reduced in the plant by processes connected with photosynthesis and respiration. For compounds to show this type of activity they must be capable of being reversibly reduced in the appropriate potential range to free radicals which are stable in aqueous solution. These conditions appear to be uniquely met by the dipyridyl diquaternary salts.

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DETERMINATION OF MOISTURE CONTENT IN CEREALS. II.*—Errors in the Determination by Oven Drying of Known Changes in Moisture Content

By T. A. OXLEY† and S. W. PIXTON

Water added to a soft wheat, whether as vapour (humid atmosphere) or liquid, was accurately assessed by the oven method (heating 4 h. at 113°), but water added to a hard wheat by either method was over-assessed proportionately to the amount added. The over-assessment amounted to 1% when the moisture content was increased from 9% to 25%, on the dry weight basis.

The amount of water removed from both varieties of wheat by drying in a warm air current was over-assessed. That from the hard wheat was over-assessed proportionately to the amount removed (but only to the extent of about 0·25% on drying from 25% to 9% on the dry weight basis), whilst the over-assessment was inversely proportional in the case of the soft wheat.

It is concluded generally that the ability of an oven-drying method to measure known quantities of water in wheat is related to the type of wheat. This is consistent with results obtained in the first paper in this series.

Introduction

It is generally accepted that the results obtained in any determination of water content in hygroscopic materials are, to some extent, arbitrary, since the forces involved in adsorption of parts of the water, i.e., parts of ' available ' water, are not greatly different from those involved in the looser chemical compounds. Hence, whatever method is used for the removal of the water (or measurement of its activity *in situ*) must tend to confuse adsorbed water with the chemically combined water, so that the line between the two is inevitably somewhat blurred.

It is, therefore, customary to define moisture content in terms of the method used for its determination. Obviously, however, a measure of the closeness with which the results obtained by a particular method approach the ideal can be obtained by adding known quantities of water to an already hydrated material (or removing known quantities by drying) and comparing the calculated levels of moisture with those obtained by use of a method under test. Since the calculation assumes that the ' zero ' level of moisture content, i.e., oven-dried material, accurately reflects the true limit of available moisture, it is reasonable to suppose that any differences between observed and calculated results indicate that the method under test does not assess the available water accurately. That is, in the case of an oven method, the oven-dried material either still contains some available water, or has lost some water molecules which were produced by decomposition.

The present paper records the results of such a test applied to a well-established oven method of moisture determination using a hard and a soft variety of wheat.

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Materials and methods

The varieties of wheat chosen for this series were, (1) high grade Canadian wheat, protein 13·0%, grown in Saskatchewan, and (2) Cappelle, a soft variety of English wheat, with a protein content of 8·9% (% protein is calculated on a 13·5% moisture basis).

The oven method employed for the determination of moisture content was the standard method used in this laboratory, namely, 113°, 4 h., in a Gallenkamp ventilated oven. All samples for moisture determination were ground using a Regent Maskin burr-type grinder set to give a fairly coarse grind, as defined in the previous paper in this series.[1] The amount of ground material used per tin was 0·2–0·3 g./sq. cm. Each moisture determination was replicated twelve times (spread over three or six days) and the mean value calculated. Throughout the experiment the drying oven was never heavily loaded, all the samples being confined to one shelf in order to avoid an inter-shelf variation which had been detected in earlier experiments.

Experimental

Stock samples, each of 2500 g., of both varieties of wheat, were drawn and put into sealed containers after thorough mixing, screening and scouring to remove foreign seeds, unthreshed wheat, chaff and straw, loose beeswing and dust. They were kept at room temperature for 24 h. before being sampled for moisture to obtain their moisture content 'as received'.

In order that the moisture content changes desired should be over as wide a range as possible, it was necessary to start from 'initial' levels of moisture content considerably different from those of the grain as received. The original stocks of wheat were therefore conditioned by addition of water or by drying, as described below for each of the experiments.

In Experiment I the moisture content of the stock of each variety was raised to 22% approximately (28·2% dry weight basis), by addition of water, and the wheat allowed to equilibrate for 21 days at 2–4° before re-determination of the moisture content. This figure then became the 'initial' moisture content for the experiment.

Eight 1-l. flasks, fitted with ground-glass stoppers or rubber bungs, were weighed, four flasks to be used for each variety of wheat. Approximately 300 g. of wheat were put into each flask which was then sealed and re-weighed. Each of the four samples of Canadian and four of Cappelle wheat was then dried to four different moisture levels, approximately 20, 16, 12 and 8% (25, 19·5, 13·6 and 8·7% on dry weight basis).

The amount of water to be removed from these samples in order to reach these levels was calculated from the initial moisture content. The wheat in each flask was dried on a laboratory tray dryer, until approximately the required weight had been lost, and then returned to the flask which was re-sealed. The flasks containing the prepared samples were allowed to equilibrate for 14 days at approximately 3°, after which they were re-weighed, and the final moisture content calculated. This is referred to as the 'calculated' moisture content. The moisture content of each sample was then determined by the method under test, by the procedure described above, and the mean of the twelve determinations for each moisture level (the 'observed' moisture) compared with the 'calculated' moisture content.

In Experiment II, the stock sample was dried down to an initial moisture content of approximately 7% (7·5% dry weight basis), and after an equilibration period at 2–3° the four flasks for each variety of wheat were prepared from this to different moisture levels, approximately the same as in Expt. I, by the addition of measured quantities of water. After a period of equilibration at 2–3°, the increase in weight was measured and the final moisture content calculated. Samples were again drawn, as in Expt. I, for moisture determination and the observed and calculated results compared.

In Expt. I the change in moisture content was effected in the vapour phase (by drying), whereas in Expt. II the change was effected in the liquid phase. It was thought that this difference in technique might, to some extent, invalidate comparison between the experiments. It was also feared that grains initially wetted in Expt. II might take up more than the average proportion of water in spite of the care that was taken to mix the grain thoroughly immediately after dampening. For these reasons Expt. III was devised in which water uptake occurred in the vapour phase. Except for this the plan was very similar to Expt. II.

A vertical section of the apparatus used for the exposure of wheat to a humid atmosphere is shown in Fig. 1. The wheat for dampening was put on to a circular, 14-mesh wire sieve, which was supported over water in a crystallising dish 220 mm. diam., 69 mm. deep and the whole enclosed in a polythene bag. A polythene tube, $\frac{1}{4}$ in. diam., leading to a small suction

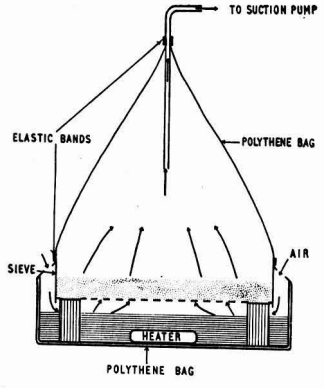


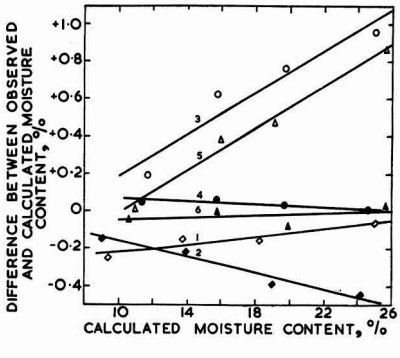
FIG. 1.—*Vertical section of the apparatus used for exposure of wheat to a humid atmosphere*

pump was inserted through the mouth of the bag, which was then sealed. The polythene bag itself was held tightly around the sieve by an elastic band, and air holes were made through it between the top of the crystallising dish and the sieve to ensure that air must pass over the water, so raising its relative humidity, before passing up through the wheat. It was found necessary, when conditioning to the 16% and 20% moisture levels, to raise the temperature of the water to 45–50° by means of a small electric heater. As an example of the rate of moisture pickup, Canadian wheat at 7% moisture (approx.) required about 3 h. to reach the 8% level, and 86 h. to reach the 20% level, using heat in the latter case. When the desired weight increase had been approximately achieved, the samples were returned to the flasks which were re-sealed and, after a period of 21 days at 2–3°, weighed to determine the exact weight of water taken up.

It might be expected that wheat conditioned by addition of liquid water would exhibit a wider range of moisture content between individual grains than that conditioned by water vapour, and, hence, a bigger difference between the moisture content of sub-samples. Some support was given to this hypothesis by the fact that results with Canadian wheat from Expt. II showed a wider spread than those from Expt. I and III. This was investigated by determination of the moisture content of single grains, in lots of 40, from the conditioned samples of Expt. II and III, using the method and apparatus described by Oxley.[2]

FIG. 2.—*Summary of all the data obtained from Expt. I, II and III. The straight-line regression of closest fit for each variety, in each experiment, is shown. (All results expressed on a dry weight basis)*

Key to Fig. 2 Line No.	Symbol	Experiment No.	Wheat variety	Regression coefficient
1	◇	1	Canadian	+0·0110
2	◆	1	Cappelle	−0·0220
3	○	2	Canadian	+0·0554
4	●	2	Cappelle	−0·0038
5	△	3	Canadian	+0·0565
6	▲	3	Cappelle	+0·0029



All the foregoing moisture content data are quoted on the conventional wet weight basis, but since changes in weight are not directly proportional to changes in moisture content expressed on this basis, it was found more convenient for the purpose of presentation of results, and for statistical treatment, to use the dry weight basis. Except for the results of the single-grain moisture determinations, therefore, all data which follow are presented on the dry weight basis.

Results and discussion

Fig. 2 gives a summary of all the data obtained. The differences between observed and calculated results (always expressed as observed minus calculated) are plotted against the calculated values. The straight line regression of closest fit for each variety in each experiment was calculated by the method of least squares, and is shown in Fig. 2, the coefficients being given in the legend.

Expt. I (drying)

It will be seen that, since all the differences are negative, the observed results for the two varieties of wheat were consistently below the calculated figure, which means that the method does not extract as much water as was calculated to be available.

There is a difference between the behaviour of the two varieties of wheat. The difference between the observed and calculated moisture content for the Canadian wheat apparently decreases with increase in moisture content (i.e., decreased drying) but the slope of the line is not quite significant statistically at the 5% level. In the case of the Cappelle wheat the difference between the observed and calculated results increases with increase in moisture content and the slope of the line is significant at the 2% level.

Expt. II (dampening with liquid water)

The observed results for both varieties were all above the calculated results, i.e., the method extracted more water than was calculated to be available.

Again there is a difference between the behaviour of the two varieties. For Canadian wheat the difference between the observed and calculated results increases with increase in moisture content, and the slope of the line is statistically significant at the 5% level.

Although all the observed results are slightly above the calculated figure for Cappelle wheat, the slope of the line is not significant.

Expt. III (dampening with humid air)

This treatment produced a very similar pattern to that of Expt. II. There was a difference between the behaviour of the two varieties of wheat. The slope of the line for Canadian wheat is almost the same as in Expt. II, the regression coefficients being +0·0565 and +0·0554 respectively. In this experiment the line fitted the points much more closely so that the slope is significant at the 1% level. The observed results are all above the calculated figure and, as

Table I

Summary of the data obtained from the determination of the moisture content of single grains from the prepared samples of Expt. II and III

(Moisture contents expressed on a wet weight basis)

Wheat variety	Moisture level	Experiment II — Water added as liquid		Experiment III — Water added as vapour	
		Mean %	Standard deviation	Mean %	Standard deviation
Canadian	1	10·57	0·44	10·07	0·43
	2	13·95	0·26	14·43	0·42
	3	16·85	0·47	16·09	0·55
	4	20·24	0·19	20·51	0·30
Cappelle	1	9·80	0·32	8·91	0·42
	2	13·43	0·31	12·95	0·36
	3	16·10	0·24	15·32	0·37
	4	19·37	0·56	19·47	0·28

in Expt. II, increase with increasing moisture content, but the differences are approximately 0·2% less.

None of the results for Cappelle wheat differs significantly from zero.

Moisture content of single grains

Table I gives the mean moisture contents obtained by determining the moisture content of 40 individual grains taken from each of the samples prepared for the Expt. II and III, together with the standard deviations for these samples. It will be seen at once that the data provide no evidence for the hypothesis that the addition of water as a liquid gives any more uneven distribution of moisture between grains than does the addition of water in vapour form.

Conclusions

Three facts are evident from Fig. 2:

(1) There is a great difference between the behaviour of the two types of wheat.

(2) Judged by results from the addition of water, and with Cappelle wheat, the present oven method would be assessed as excellent, since at all levels of moisture content it correctly estimates the amount of water added. But if Canadian wheat were used, the same method would be adjudged faulty, since added water is consistently over-estimated.

(3) The results obtained from the removal of water, by drying, showed that the method did not correctly estimate the amount of water removed, particularly where Cappelle wheat was used, the amount being consistently over-estimated, i.e., the amount of water remaining in the grain after drying is under-estimated.

(4) As far as the evidence from the determination of the moisture content of single grains goes, it is not possible to establish any connexion between the closeness of fit of the data to the regression lines and the range of moisture content between individual grains. Also, there is no evidence that the addition of water in liquid form gives any more uneven distribution of moisture between grains than does the addition of water in vapour form.

Although no general explanation for the results is offered, it is suggested that in Canadian wheat the addition of water tends to free some proportion of the 'bound' water, but there is no such effect in Cappelle wheat. Drying, on the other hand, appears to increase the degree of binding of the water in the Canadian wheat. The behaviour of the Cappelle wheat in drying is anomalous, since the degree of increase in binding appears to be greatest when drying is least. No explanation is offered for this phenomenon.

It is concluded, generally, that the ability of an oven-drying method to measure known quantities of water is related to the type of wheat. This is consistent with results reported in the first paper in this series.[1]

Acknowledgments

The authors are grateful to Miss J. N. Brown for assistance with the determination of the moisture content of single grains of wheat, and to Mr. R. W. Howe for advice on the statistical treatment of the results obtained from all the experiments.

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EXAMINATION OF WHEAT GLUTEN BY PARTIAL SOLUBILITY METHODS. I.—Partition by Organic Solvent

By P. MEREDITH, H. G. SAMMONS and A. C. FRAZER

Gluten from defatted flour was purified by dispersion in acid and precipitation, and partitioned in a methanol–chloroform mixture. Similar partition of washed gluten and of flour gave results suggesting that a complex is split on treatment of gluten with dilute acid.

Amino-acid analyses of the fractions suggest that the properties of the more soluble fraction may be explained by a relative lack of ionisable groups. Comparison with the amino-acid composition of barley proteins may give an indication of the structural components responsible for the characteristic properties of wheat gluten.

Introduction

Little is known of the essential differences between strong and weak flours and one may ask if the physical properties of dough and gluten can be accounted for in terms of the chemical groupings of the molecules, or of the spatial arrangement of the constituents. Little fundamental is known of the effects of salt concentration and pH, on dough properties or of what part is played by lipid in the dough structure. There has been no theoretical basis for the many fractionations of gluten protein which have been carried out in the past and it has not yet been decided if gluten is an individual compound, a system of many components, or a few interacting components. The present investigation was planned with these problems in mind. There is an extensive literature but the present position is adequately surveyed by three recent reviews.[1]

A purified preparation of gluten protein has been fractionated using two different solvent systems, and the fractions obtained subjected both to physical examination and to chemical analysis in the hope of explaining the markedly contrasted physical properties in terms of the amino-acid composition of the proteins. The effects of oxidation on the properties of these fractions have been examined. Part I deals with the first fractionation and the amino-acid analyses of the fractions, whilst Part II deals with fractionation by dilute acid and the physical examination of the fractions. Conclusions will be presented at the end of Part II (following paper).

Experimental

Materials and methods

Flour.—This was commercially milled from the following grist: 50% No. 2 Manitobas; 10% No. 3 Manitobas; 10% Plate; 5% Australian; 25% English. It was straight run flour of 75% extraction, was free from added flours or any other admixtures or treatments and was vacuum-packed in 7-lb. tins.

Analytical methods.—Total nitrogen was determined by a Kjeldahl digestion method based on that of Chibnall *et al.*,[2] using samples which were in equilibrium with the air. Results were calculated to dry, ash-free basis.

Moisture was determined by drying for 24 h. at 110° under vacuum over P_2O_5.

Ash was determined by ignition in a silica crucible to constant weight. Those samples containing sodium formate gave a fusible residue which merged into the body of the crucible. A correction has therefore to be applied to such samples, the factor 2·19 ($H{\cdot}COONa/\frac{1}{2}Na_2O$) being used.

Total phosphorus was determined by the method of Allen.[3]

Amino-acid assay—Acid hydrolysis of protein samples was carried out under the dilute conditions described by Dustin *et al.*[4] and in an atmosphere of oxygen-free, ammonia-free nitrogen to prevent undue oxidation occurring. Refluxing in redistilled, constant-boiling HCl was carried out under atmospheric pressure for 24 h.

Chromatographic separation of amino-acids in the hydrolysates was carried out according to the method first described by Moore & Stein[5] and later by Schram *et al.*[6] The ninhydrin reaction was carried out on the eluted fractions by the method of Moore & Stein[7] with only minor technical modifications.

A control analysis of a sample of crystalline bovine plasma albumin was compared with the published data of Moore & Stein.[8] Statistical analysis gave the regression line $y = 1{\cdot}018x - 0{\cdot}01$ (x = control analysis; y = data of Moore & Stein, both as mmoles of amino-acid/100 g. dry, ash-free material; 99% confidence limit of slope of line = $\pm 11{\cdot}8\%$). N recovery was 100·4%.

Tryptophan analyses were carried out by a colorimetric method based on the Voisenet–Rhode reaction with *p*-dimethylaminobenzaldehyde in acid solution.

Extraction of lipids from flour

In a preliminary experiment 200-g. samples of flour were extracted by percolation with 500 ml. solvent in each case. The extracts were taken to dryness in a Craig rotary evaporator at 40° and re-extracted with light petroleum (b.p. 40–60°). The yields of dry lipid at constant weight are given in Table I, each result being the mean of at least two determinations.

Table I

Yields of lipid extracted from flour by various solvent systems

Solvent	Extract as % of dry flour weight	Extract as % of ether plus ethanol–ether extract
Benzene	1·13	72·5
Carbon tetrachloride	1·05	67·6
Chloroform	1·33	85·5
Light petroleum, 40–60°	1·01	65·1
Ethanol	1·54	99·1
Ether after ethanol	0·04	2·8
Total ethanol plus ether	1·58	101·9
Ether	1·13	72·5
Ethanol–ether (3 : 1) after ether	0·42	27·5
Total ether plus ethanol–ether	1·55	100·0

The main extractions have been carried out by percolating 2 l. of dry ether, 2 l. of ethanol–ether (3 : 1) and 1 l. of ether successively through 1·5 kg. of flour. The defatted flour was dried under vacuum and in air; the analysis then showed: moisture 11·4% of dry weight; ash 0·39% of dry weight; N 2·38% of dry, ash-free material.

Preparation of glutens

For dissolution and precipitation of gluten the methods of Cunningham *et al.*[9] have been followed. Defatted flour was extracted with distilled water. The residue was homogenised (Waring Blendor type) with 0·01N-formic acid and the mixture stirred for a further 30 min. The mixture was allowed to settle and the liquid decanted, the deposit then being washed with more 0·01N-formic acid. The combined acid extracts were passed through the 'Sharples' supercentrifuge to remove residual starch. A 5M-sodium formate solution was added, with mechanical stirring, to give a final concentration of 0·25M. The coagulated gluten was collected from the stirrer, the remaining suspension was passed through the supercentrifuge and the clear effluent discarded. Coherent gluten was peeled from the inside of the rotor. The two lots of gluten were combined, weighed and freeze-dried.

The wet weight yields of six successive batches, each from 250 g. of flour, were 59, 60, 59, 58, 57 and 57 g. The analysis of four pooled batches of the precipitated gluten was: moisture 9·0% of dry weight; corrected ash 2·30% of dry weight; N 17·57% on dry, (corrected) ash-free basis; P 0·038% of dry weight. The average yield of precipitated gluten was 78·4 g. (dry weight)/1000 g. (14·1% moisture) of flour.

For comparison, a sample of gluten was hand-washed from the defatted flour and freeze-dried. The yield was 101·6 g. (dry weight)/1000 g. of flour and the gluten had the analysis: moisture 10·1% of dry weight; ash 0·49% of dry weight; N 16·65% on dry, ash-free basis; P 0·18% of dry weight.

The weight yield of precipitated gluten compared with the weight yield of hand-washed gluten is 77·2%, but perhaps the fairest statement of recovery from the precipitation procedure is to compare the yield with that of hand-washed gluten reduced to the same N content. On this basis the yield is 81·4%.

Partition of gluten by methanol–chloroform mixture; fractions A and B

Wet gluten, prepared from 250 g. of flour by the formic acid–sodium formate method, was homogenised into 1·5 l. of 1 : 2 methanol–chloroform mixture and boiled under reflux overnight. The mixture was filtered while still warm through a sintered glass plate of No. 3 porosity. The insoluble portion was refluxed with a further 1·5 l. of solvent and again filtered. The combined extracts (fraction A) were evaporated in a Craig rotary evaporator at 40°. The residue (fraction B) was dried by passing a current of dry air through it under reduced pressure. Both fractions were further dried under high vacuum, then equilibrated with the air.

Yields were 8·1 g. of fraction A and 12·1 g. of fraction B, equivalent to 37·3% and 62·7% of the recovered material. Other experiments, on gluten derived from another bread flour, gave yields of 41·0% and 41·1% for fraction A. In the latter case the extraction was shown to be virtually complete since the first extraction yielded 40·2% and the second extraction produced only a further 0·9%. Analyses of fractions A and B were respectively: moisture 12·4 and 9·5% of dry weight; ash 4·99 (corrected) and 0·33% of dry weight; N 15·8 and 17·35% on dry, (corrected) ash-free basis; P 0·018 and 0·039% of dry weight.

Elution of flour with methanol–chloroform; fraction C; the complex nature of gluten

Before commencing further studies on fraction A of gluten an attempt was made to simplify the method of preparation in order that larger quantities should be available.

Methanol–chloroform mixture was passed through the column containing the defatted flour, immediately after the defatting process, the column being externally heated so that extraction was carried out near the boiling point of the solvent. The first 3 l. of solvent extracted 30 g. of protein. The next 3·6 l. extracted 12 g. and the next 1·5 l. extracted 1 g. Extraction was thus virtually complete with a total of 43 g. of protein (fraction C) extracted from 2 kg. of flour (only about 17% yield of the gluten-protein).

A sample of defatted flour was washed by hand for gluten, the wet product being homogenised with methanol–chloroform and refluxed overnight. The yield of soluble material was only 14% of the protein, this being increased to a total of 18% by further treatments. Thus we have the situation that methanol–chloroform extracts 37% of the gluten-protein from reprecipitated gluten but only 17% from flour or 18% from hand-washed gluten.

This suggested that it is the acid purification treatment which is necessary to produce 37% solubility in the organic solvent, as in the preparation of fraction A. Further experiments suggested that treatment with dilute acid is the factor responsible for the foregoing effects but unequivocal proof of the point could not be obtained because of technical difficulties.

These experiments have suggested that gluten proteins exist as a complex which is split by the dilute acid treatment used in the purification process so that the component proteins are more easily separated.

Preparation of gliadin

A sample of gliadin was prepared, for comparison with the other fractions, by a method due to Van de Kamer.[10] Flour which had not been defatted was stirred with 10% sodium chloride solution, centrifuged and decanted. The residue was stirred with 55% ethanol and centrifuged. The solution was evaporated and the recovered gliadin washed with water to remove salt. The product was finally freeze-dried. The yield of gliadin was 47 g./1000 g. of flour. The analysis was moisture 10·7% of dry weight; ash 2·92% of dry weight; N 16·69% of dry, ash-free material; P 0·040% of dry weight.

General properties of fractions

Fraction A formed a very sticky gum with water, but fraction B, on the other hand, was not easily mixed with water and showed no glutinous properties. A mixture of the two fractions ground together in equivalent proportions and wetted gave a gluten-like structure after working in the fingers for a few seconds.

Fractions A and C were completely and easily soluble in 0·01N-formic acid, after a brief period for solvation to occur. Fraction B, however, required considerably longer to solvate and then did not dissolve but gave a suspension of gel particles apparently corresponding to

the particles into which the solid had been ground. The suspension did not further disperse, even after keeping for a week, and could be centrifuged down. This observation is the basis of the fractionation in dilute formic acid to be described in Part II. When the suspension of fraction B was homogenised, an opalescent colloidal solution resulted which resisted centrifuging and was stable for at least a week. Thus the gel structure could be broken mechanically.

Oxidised specimens of fractions A and B were prepared by the performic acid techniques of Sanger[11] and Blackburn & Lowther.[12] The diluted reaction mixtures were concentrated by freeze-drying, thus giving complete recoveries. Before oxidation a difference in properties was noticeable when the fractions were mixed with 90% formic acid. Fraction A formed a clear, mobile solution, as expected, but fraction B formed a thick gel which was difficult to handle. In both reaction mixtures a transient pink colour occurred during the first 2 min. after addition of hydrogen peroxide. Between 6 and 7 min. after the addition of the peroxide, the gel suddenly liquefied completely, the resulting solution being just as mobile as that given by fraction A.

The oxidised fraction A, on being mixed with water, no longer gave a slimy gum as did the original material but formed a coherent rubbery mass, very similar to normal gluten. The gummy properties had largely disappeared and this observation could account for the lack of coherence in over-oxidised glutens. The oxidised fraction A was insoluble in methanol–chloroform, whether water was present or not. The oxidised fraction B behaved with water very similarly to the unoxidised material, being insoluble and incoherent. Oxidised fraction B was insoluble in methanol–chloroform as expected. The outstanding property of the oxidised fraction B was, of course, the formation of a mobile solution in 90% formic acid instead of a gel.

Amino-acid analyses

Complete analyses of fractions A and B were carried out in replicate, the mean results and ranges being given in Table II. Recoveries of N were 100·5% and 107·8% and the weight recoveries 88·2% and 107·6% respectively. When classified according to type of group, the

Table II

Amino-acid analyses of fractions A and B of gluten

	Amino-acid/100 g. dry, ash-free material					
	Millimoles				Wt. in g.	
	Fraction A		Fraction B		Fraction	Fraction
	Mean	Spread	Mean	Spread	A	B
Alanine	22·0	3·3	36·0	7·4	1·96	3·21
Ammonia	294·8	2·1	281·5	1·5	5·00	4·78
Arginine	13·6	5·6	19·8	10·8	2·35	3·44
Aspartic acid	21·5	5·6	30·2	3·7	2·86	4·02
Cystine/2	17·6	11·3	20·9	16·7	2·11	2·51
Glutamic acid	277·6	1·8	327·7	4·1	40·8	48·1
Glycine	21·7	2·9	77·0	1·0	1·63	5·78
Histidine	10·5	21·5	11·8	1·7	1·63	1·83
Isoleucine	30·5	0·1	35·2	4·8	4·00	4·61
Leucine	50·8	2·2	65·6	5·0	6·66	8·60
Lysine	4·3	8·3	9·4	2·0	0·63	1·38
Methionine	10·6	1·5	11·9	6·0	1·58	1·78
Phenylalanine	39·3	0·1	38·9	3·3	6·48	6·42
Proline	149·5	0·4	106·7	10·2	17·2	12·3
Serine	39·8	3·7	63·4	7·8	4·17	6·64
Threonine	17·2	7·4	29·4	6·3	2·05	3·50
Tryptophan	1·4		2·8		0·29	0·57
Tyrosine	12·5	6·5	29·9	3·0	2·26	5·41
Valine	31·1	6·2	41·8	6·6	3·64	4·88
Total	—	—	—	—	107·30	129·76

Columns 1 and 3 give the means of 3 or 4 analyses.
Columns 2 and 4 give the spread of results as % of mean.

proportions present in fractions A and B respectively were: no group (glycine) 2·8 and 8·0%; ionic groups 4·3 and 12·3%; polar, not ionic 51·0 and 45·9%; non-polar 41·9 and 33·8%. The apparent partial specific volume of fraction A, calculated from the amino-acid analysis, was 0·723, in good agreement with later pycnometric determinations.

Only partial analyses of gliadin and fraction C were carried out without replication (Table

III). Statistical analyses were made comparing each of these with the mean data for fraction A. For gliadin the regression equation obtained was

$$y = 1{\cdot}003x - 6{\cdot}03 \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

(x = gliadin; y = fraction A; both in mmoles/100 g.; 99% confidence limit of slope of line = $\pm 5{\cdot}8\%$). This implies a similarity in composition of the two fractions, but the values for gliadin are a small, constant amount higher than those of fraction A. For fraction C the regression equation was

$$y = 1{\cdot}017x - 1{\cdot}31 \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

(x = fraction C; y = fraction A; both in mmoles/100 g.; 99% confidence limit of slope of line = $\pm 23{\cdot}6\%$). In view of this large scatter it appeared, therefore, that the two fractions were not identical.

Table III

Amino-acid analyses of gliadin and of fraction C

	Mmoles amino-acid/100 g. dry, ash-free material				
	Gliadin	Fraction C		Gliadin	Fraction C
Alanine	30·8	26·0	Leucine	62·6	49·3
Ammonia	296·3		Lysine	4·7	
Arginine	13·8		Methionine	11·5	10·1
Aspartic acid	25·7	23·4	Phenylalanine	46·9	39·4
Cystine/2	20·3	21·8	Proline		
Glutamic acid			Serine	52·3	40·5
Glycine	38·4	25·0	Threonine	21·8	19·0
Histidine	10·3		Tryptophan		
Isoleucine	38·1	29·2	Tyrosine	21·3	10·6
			Valine	37·3	30·6

Discussion

Exposure of gluten-protein to alcohol, in the presence of water, is alleged to 'denature' the glutenin.[13, 14] In the defatting process, therefore, a preliminary washing of the flour with dry ether was used to extract most of the water before the main extraction with alcohol–ether mixture. The residual alcohol was also washed out with ether, before the flour was air-dried. Gluten may be hand-washed from this defatted flour in the normal way and is only very slightly less coherent than gluten washed from the whole flour.

There is some loss of protein during the preliminary washing with water to remove excess salts since the gluten-proteins are more soluble the less salt is present in solution, and even under precipitation conditions there is still a considerable solubility. Some protein is not completely dispersed and is thrown down when the dispersion is centrifuged. This has been noted previously.[13] There will also have been some loss due to mechanical entrainment in the starch residue.

Since the recovery of gluten from the formic acid solution and sodium formate precipitation processes is about 80%, the recovered material is representative of the majority of the dough structural protein freed from contaminants. Certainly the recovered protein appears to retain all of those properties (elasticity, plasticity, hydration, stickiness) usually considered characteristic of gluten.

The attempt to obtain fraction A by direct extraction of flour with methanol–chloroform led to consideration of the parts played by prior acid treatment and by homogenising in fractionation processes, these being the principal processes by which direct extraction differed from preparation from purified gluten. Although it proved technically impossible to provide direct proof that the acid treatment was the important factor, the indirect evidence was reasonably conclusive. This leads to the concept that part or all of the gluten-protein exists as a complex which is irreversibly split by the acid treatment. The idea that acid treatment causes dissociation of large molecules into smaller units has been suggested already for several seed and nut proteins, including amandin and excelsin.[15] Cunningham *et al.*[9] have proposed a theory of polymer cross-linkages occurring through the interaction of amide groups. The mixture of components behaves mechanically the same as the undissociated complex since material precipitated from acid dispersion retains most of the characteristic properties of gluten.

The extraction of gluten with methanol–chloroform was originally carried out in an attempt to remove any remaining lipid and the bound phosphorus. The apparently clear-cut fractionation which was found, however, warranted further investigation since the two fractions had very different physical character, neither of them resembling gluten but capable of combining to form a gluten. This partial solubility in a less polar solvent is similar to the extraction of gliadin from gluten by 70% alcohol. Furthermore, the physical properties of the two fractions, A and B, were very similar to those of gliadin and glutenin. The properties of these have been described[16] as follows : ' In water gliadin readily swells and forms a sticky mass which can easily be pulled out into threads, whereas glutenin in water gives a flaky deposit showing few signs of cohesion.' It has also been shown that gluten may be partially solubilised in aqueous mixtures of many other organic solvents.[17]

De Deken & Mortier[18] suggested that the solubility of prolamine in alcohol could be accounted for by the low content of ionisable groups in the protein or by lipoprotein structure. Such a structure for gliadin has been suggested by previous authors.[19, 20] The amino-acid analyses of fractions A and B provide material for possible explanations of several of the properties of these proteins.

It is a reasonable assumption that all of the ammonia determined has been derived from the hydrolysis of glutamine, isoglutamine, asparagine and possibly isoasparagine. Under the dilute hydrolysis conditions used, complete recovery of the hydroxyamino-acids (the most important source of additional ammonia on heating protein with acid) is obtained.[4] Osborne & Nolan[21] in 1920 showed that the ammonia found on hydrolysis of gliadin comes from an easily hydrolysed group. Damodaran *et al.*[22] in 1932 isolated glutamine from an enzymic digest of gliadin. Sullivan & Payne[23] in 1951 found both glutamine and asparagine in aqueous extracts of wheat flour. Kovács *et al.*[24] in 1955 concluded that isoglutaminyl residues are also present in gliadin. Thus the circumstantial evidence for the assumption is quite strong.

In fraction A the sum of glutamic acid and aspartic acid almost equals the ammonia found, leading to the conclusion that mainly amide groups are present and few free carboxyl groups. Fractions A and B have similar amide contents but fraction B contains additionally a considerable proportion of free carboxyl groups, to the extent of about one for every four amide groups. It seemed possible that fraction B might be an artefact, having been partially deamidated by the acid treatment used in the gluten purification. However, the difference in concentrations of other amino-acids shows the presence of at least two distinct proteins, even if neither had free carboxyl groups. Furthermore neither fraction alone has the properties of gluten although the reconstituted mixture has. This is good evidence that the separated fraction is not an artefact.

The lysine content of fraction B is about double that of fraction A whilst the content of arginine and histidine is also higher in fraction B than in fraction A. Indeed the content of basic amino-acids in fraction A is remarkably low compared with the majority of proteins. The virtual absence of free carboxyl groups and the low content of basic groups combine to make fraction A almost non-ionic in character. This is in agreement with the electrometric titration studies of Léonis & Van Buggenhoudt[25] who found that gliadin contained only 27 ionisable groups per molecule of weight 41,000 and concluded that the presence of few ionisable groups is one of the characteristics of prolamines. Fraction A contains 18 ionisable groups per molecule of weight 49,000, while fraction B has 39 ionisable groups per molecule of weight 36,000. This difference in molecules which are otherwise low in ionic groups compared with other proteins must surely account partly for the difference in properties between fractions A and B. The obvious conclusion is that the very low ionic character of fraction A accounts for the solubility in the methanol–chloroform mixture and provides explanation of what was, at first sight, an unexpected phenomenon. Explanation of the solubility by a lipid complex mechanism has not, of course, been excluded but other evidence makes the possibility unlikely.

This theory of the low ionic character of fraction A was capable of experimental verification since oxidation by performic acid should produce ionisable sulphonic acid groups from the S–S and S–H groups of the molecules. In fact, the oxidised material was found to be completely insoluble in the methanol–chloroform mixture. The almost non-ionic character of fraction A is thus of fundamental importance in determining its properties.

The difference in carboxyl content of the two fractions should lead to a difference in

isoelectric point and electrophoretic mobility. Fraction A has 28·5 moles of basic groups/10^5 g. and 4·3 moles of acidic groups/10^5 g. and is thus predominantly basic, although only weakly so. The respective values for fraction B are 41·2 moles of basic groups and 76·4 moles of acidic groups/10^5 g. and it is therefore predominantly acidic and much more ionic in character. Whole gluten is approximately equally charged, the pH of maximum precipitation determined in early purification experiments being 6·1–6·2 while the isoelectric points of the ' neutral ' amino-acids are at pH 6·0–6·1. It is to be expected that the isoelectric point of fraction A would be of the order of pH 7 and that of fraction B pH 5. This agrees with the observations of Schwert *et al.*[26] that gliadin contains two fractions with isoelectric points at pH 5 and 7.

In accounting for the difference in physical properties of fractions A and B on hydration, the ionic groups are probably of primary importance in the formation of salt bridges, both inter- and intra-molecular, whereas the polar, non-ionic groups can at the most form only the weaker hydrogen bonds. As has been suggested for gelatin,[27] intermolecular bonds may prevent solution on hydration and confine the swelling of the gel to a definite limit. In the hydrated state fraction A has very little possibility of forming salt linkages by reason of its relative lack of ionisable groups, and is therefore semi-soluble. Fraction B on the other hand can form salt bridges between carboxyl and basic groups of adjacent molecules and therefore remains coherent and insoluble. It is possible that salt linkages might be formed between two or more types of protein molecule in the intact gluten.

Fraction A has 55% and fraction B 58% of polar groups and this may account for the marked hydrophilic character of these proteins. Not only the polar groups but also the peptide bonds are to be considered points of attachment for water molecules, since it has been shown[28] that peptides synthesised from non-hygroscopic amino-acids are themselves hygroscopic. The hydrophilic character is well seen in solubility studies carried out on fraction C.[29] The strong affinity of the protein for aqueous media leads to the formation of two liquid phases rather than liquid and solid phases. The hydrated structure of the gel is probably due in part to the polar composition of the protein molecules, but it is also probable that the three-dimensional network structure which will be postulated in Part II would entrain a large quantity of solvent without actual physical attachment.

The amino-acid concentrations of fractions A and B showed marked grouping into certain levels, investigation of which showed that for fraction A only one set of small whole numbers would satisfy the relationship. The minimal molecular weight calculated from these ratios was 49,100. Although physical examination has shown this fraction to be multicomponent, the marked grouping still suggests that a series of closely related proteins may be involved, having a partial structure in common. Similar arguments for the analysis of fraction B gave the minimal molecular weight 36,400 which may represent the size of the individual polypeptide chains. For both fractions A and B the minimal molecular weight corresponds to one residue of tryptophan. Such discrete concentration levels shared by several amino-acids may also be seen for example in the amino-acid analyses of insulins.

The only molecular weight determinations available for comparison are for gliadin, viz., 34,500 ;[30] 25,000–28,000 ;[31] 40,000–75,000 ;[32] 38,000 ;[33] >35,000 ;[34] 42,000.[35] The minimal molecular weights deduced from the present analyses are thus of the same order as those derived from existing physical data.

The outstanding point of the fundamental analyses of fractions A and B is the low N content of fraction A. In view of Osborne's figures of about 17·6% for the total N content of gliadin, the 15·8% N found for fraction A seemed abnormally low. However, the endosperm protein isolated by Hess[36] had a protein factor of 6·9, corresponding to 14·5% of N, compared with protein factor 5·9 (16·9% N) for gluten-protein in the same work. The N recovery of 100·4% for the control amino-acid analysis and of 100·5% for fraction A of gluten must be considered highly satisfactory, but the 88% weight recovery in the amino-acid analysis of fraction A is far short of the required 100%.

The low N content and the low weight recovery of the amino-acid analysis of fraction A could both indicate the presence of about 10% of some non-nitrogenous addendum. The P content of the material is too low for any appreciable proportion of phosphatide to be present and several attempts to find fatty acids in hydrolysates were negative. Since all the N has

been accounted for as amino-acids, any lipid present must have all of its N as one of the amino-acids assayed or be N-free. There is only 0·32 atom of P present per 49,000 molecular weight; thus either the P is a non-structural contaminant or it is only a minor structural element in the protein. The possibility has been noted that phosphate linked to cysteine may occur in gluten.[1]

Considering other possibilities for an addendum in fraction A, nucleic acid may be excluded by the low P content, the complete N recovery and the normal partial specific volume. If indeed an addendum is present, it could be polysaccharide in nature, interaction of this type having been demonstrated by Udy.[37] The apparent pentosan content (determined by HCl distillation) is only 0·7%, which accounts for little of the deficit. The acid hydrolysate, however, when tested with alkaline ferricyanide, had reducing power equivalent to 10·8% glucose. Negligible reduction was given by the unhydrolysed material and by a hydrolysate of an aqueous extract of the material. (Formate does not reduce ferricyanide under these conditions.) The presence of a bound polysaccharide moiety may thus be presumed.

In view of the satisfactory N recoveries for the other amino-acid analyses, that of 107·8% for fraction B of gluten is rather disturbing, being much higher than the mean spread of results would lead us to expect. A second triplicate analysis of fraction B for total N confirmed the first result. The 107·6% weight recovery for fraction B almost exactly corresponds to the 107·8% N recovery and indicates that the analyses may contain a fundamental error of weighing or moisture determination.

Apart from the difference in content of free carboxyl groups, fractions A and B differ considerably in several amino-acids as can be seen from Table II. The electrometric titration studies of Léonis & Van Buggenhoudt,[25] quoted earlier, showed gliadin to contain 8 free carboxyl, 5 imidazole, 2 ε-amino, 7 phenolic and 5 guanidino groups per 41,000 molecular weight. These values compare well with the present analysis of fraction A which shows 2 free carboxyl, 6 imidazole, 2 ε-amino, 7 phenolic and 8 guanidino groups per 49,000 molecular weight.

These differences in amino-acid analyses of fractions A and B mean little in themselves but, in comparison with data for fractions of the same type from barley, interesting identities and differences can be seen. The data of Folkes & Yemm[38] for the alcohol-separated fractions hordein and hordenin of barley have been recalculated to mM per 100 g. protein and are presented in Table IV. The prolamin hordein is very similar in composition to fraction A but hordenin

Table IV

Amino-acid analyses of hordein and hordenin of barley, recalculated from the data of Folkes & Yemm[38]
(mmoles amino-acid/100 g. protein)

	Hordein	Hordenin		Hordein	Hordenin
Alanine	26	75	Leucine	55	66
Ammonia	273	131	Lysine	5	27
Arginine	18	34	Methionine	9	12
Aspartic acid	14	53	Phenylalanine	43	31
Cystine/2	18	10	Proline	181	75
Glutamic acid	276	133	Serine	38	48
Glycine	20	59	Threonine	22	35
Histidine	9	16	Tryptophan	4	6
Isoleucine	43	40	Tyrosine	19	22
			Valine	42	56

and fraction B differ considerably. As in the case of wheat, in the more soluble fraction of barley most of the carboxyl groups are amidated whereas some are free in the insoluble fraction. The contents of glutamic acid, ammonia and proline are lower in hordenin than in fraction B, whereas the contents of lysine and alanine are much higher. In other respects the analyses are similar. Calculation of the regression line for a plot of the amino-acid composition of fraction A of wheat against that of hordein of barley gave the equation

$$y = 1{\cdot}02x - 3{\cdot}4 \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

(x = barley; y = wheat; 99% confidence limit of slope of line = ±8·3%). Similar calculation for fraction B of wheat and hordenin of barley gave the regression line

$$y = 2{\cdot}25x - 43{\cdot}5 \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

(x = barley; y = wheat; 99% confidence limit of slope of line = ±30·3%). In the first

case the slope of the line is 1·0 within experimental error and it passes close to the origin. The scatter of the points about the curve is small. All these factors support the conclusion that fraction A and hordein have similar amino-acid composition. The regression line for fraction B against hordenin amino-acid compositions has slope 2·25 and does not pass through the origin. The points are widely scattered about the line. Thus fraction B and hordenin differ in amino-acid composition.

It would appear, then, that the differences in properties between wheat and barley protein may be correlated with the difference in glutamine, proline, lysine and alanine contents of the less soluble fractions. This comparison gives a lead to the structural components of the protein responsible for those characteristic properties which make wheat flour superior in the production of leavened products.

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EXAMINATION OF WHEAT GLUTEN BY PARTIAL SOLUBILITY METHODS. II.*—Partition by Dilute Formic Acid

By P. MEREDITH, H. G. SAMMONS and A. C. FRAZER

When gluten is treated with dilute acid, swelling of the gelatinous component occurs with concomitant diffusion from the gel of the more soluble proteins. This process is independent of the partition by organic solvent described in Part I and the two processes may be applied consecutively in either order with substantially the same result. The resulting three fractions have been examined by viscosimetric and electrophoretic methods. The possible structure of gluten in terms of these fractions is discussed.

Introduction

In the preceding paper it has been suggested that gluten-protein is mainly or entirely a single complex which can, nevertheless, be easily split by dilute acid, and probably by alcohol and other reagents. Some, or more probably all, fractions obtainable may thus be artefacts derived from a gluten super-molecule as a result of the acid treatment. However, they are real proteins, albeit degradation products, and the investigation of their structure and properties must be a proper stage in the investigation of gluten itself.

In view of the gelatinous nature of fraction B, an attempt has been made to prepare the gel protein directly by treatment of lipid-free gluten with dilute formic acid. This paper describes the properties of the fractions obtained and further physical examination of all the fractions.

Experimental

Materials and methods

Details of original flour, methods of defatting and purification and general analytical methods as in Part I.

Electrophoresis

Analyses were carried out at 0·5° in a Tiselius apparatus incorporating a Philpot–Svensson optical system. The cells were 0·75 cm.² cross section.

Glycine–HCl buffer containing 0·008M-sodium chloride was made according to Mills,[1] and sodium acetate–HCl buffer according to Walpole, quoted by Mills.

Generally, 1% suspensions of protein in buffer were made and dialysed against a large volume of buffer for at least 2 days. The solution was then centrifuged.

Determination of intrinsic viscosity and axial ratio

Viscosities have been determined in a capillary viscometer (Grade No. 0) at about 20·2°±0·02°, all normal precautions being taken. Densities of the solutions were determined with a pycnometer equilibrated in the same water-bath. Protein solutions were dialysed against buffer for at least 48 h. and centrifuged. Successive dilutions were made with a constant pipette and Grade A volumetric flasks. Concentration of protein was determined by drying a 10-ml. weighed sample.

Intrinsic viscosity was found by the formula,

$$[\eta] = \lim_{c \to 0} 1/c(\eta/\eta_0 - 1)$$

where c is the concentration of solute in g./l., and,

$$[\gamma] = [\eta] \times 1000/\bar{V}$$

where $\bar{V}$ is the apparent partial specific volume.

The axial ratio was found, for the assumed prolate ellipsoidal model, from the table of Mehl, Oncley & Simha.[2]

Partial specific volume was calculated from the concentration and pycnometric determinations.

* Part I: preceding paper

X-ray diffraction studies

These were carried out using the microfocus cameras described by Finean.[3] Both low-angle and wide-angle diffraction patterns were examined. No orientation was found in any of the preparations examined.

Partition of gluten by dilute formic acid: fractions D and E

About 3 g. of freeze-dried, powdered gluten from defatted flour were gently agitated with 100 ml. of 0·01N-formic acid. After 4 h. the mixture was centrifuged and the supernatant liquid poured off, the volume being noted. A further 100 ml. of formic acid was added to the gel and the mixture was again kept with occasional shaking for a further 17 h. This process of centrifuging and resuspending was carried out several times, up to 95 h. A second experiment was carried out similarly, but the processes of centrifuging and resuspending were carried out at more frequent intervals. The volumes occupied by the gel phase were calculated and aliquots of the extracts and the final gel were analysed for total nitrogen.

The results are plotted in Fig. 1 in three different ways to show the changes occurring during the extractions. Three concurrent processes could be distinguished. Firstly, the gel was swelling, which was a function of the number of elutions rather than of time, as shown

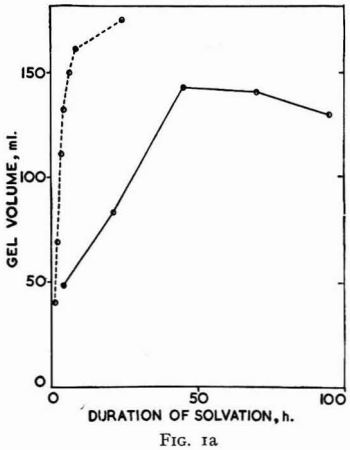


FIG. 1a

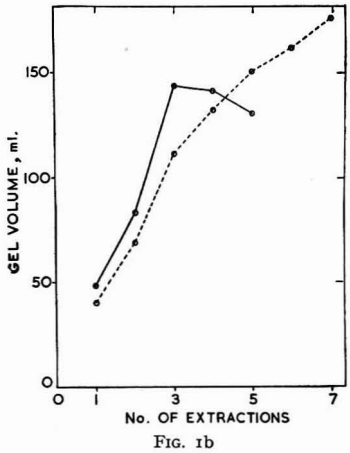


FIG. 1b

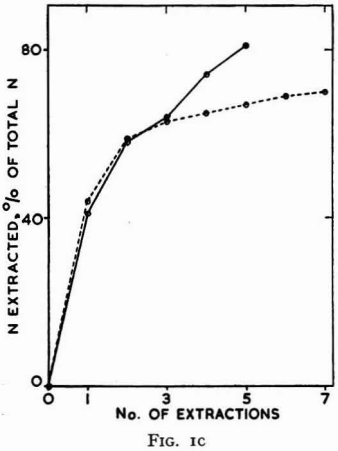


FIG. 1c

FIGS. 1a–c.—*Partition of gluten by dilute formic acid*

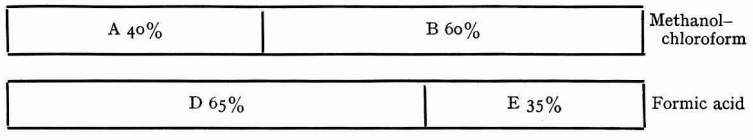
——— First Exp. - - - - - - - Second Exp.

by the graphs of gel volume. Secondly, there was breakdown of gel structure, much slower and a function of time rather than of the number of elutions and therefore probably due to the proteinases known to be present in low concentration in gluten which had not been heated.[4] The effect is seen in all three curves and accounts for an increase in the amount of N solubilised after the third elution in the first experiment. Thirdly, there was an elution of protein into the acid in a soluble form. Almost all of the N determined was precipitable by trichloroacetic acid. The proportion of the total gluten N extracted approached 65–70% asymptotically, with a further increase due to the breakdown of the gel.

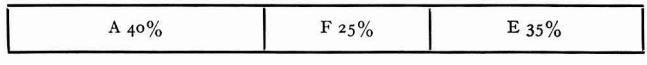
The protein extracted by dilute formic acid from gluten without breakdown of the gel due to extended or repeated treatments has been termed fraction D. The gel has been termed fraction E.

Refractionations of fractions B and D: fraction F

The fractionations by methanol–chloroform and by dilute formic acid may be shown diagrammatically thus



There is obviously an overlap and the material common to both fractions B and D has been termed fraction F.



If the two fractionation processes were completely independent and not due to reactions other than simple solution of components, the more complex fraction in each case should be susceptible to refractionation by the other process, i.e., fraction B on treatment with dilute formic acid should be split 25/35 into fractions F and E ; fraction D on treatment with methanol–chloroform should be split 40/25 into fractions A and F. These possibilities were experimentally tested.

Fraction B (200 mg.) was extracted three times with 10 ml. of 0·01N-formic acid to give fraction F. The remaining gel (fraction E) was dissolved in H_2SO_4 and total N was determined in all fractions. The gel contained 67·4% and the total extracts 32·6% of the recovered N, but 14·2% of the N taken was not recovered due to mechanical difficulties in handling the mucous gel on a small scale. The 32·6% extracted approaches the 41·7% required and, considering the losses, is compatible with the idea of the two types of fractionation being independent.

Fraction D (2·4 g.) was refluxed with 200 ml. of methanol–chloroform and filtered. The residue was again homogenised briefly with solvent, refluxed and filtered. The two extracts (fraction A) contained 184·5 mg. and 25·4 mg., and the residue (fraction F) 173·2 mg. of N. Thus 55% of the total N had dissolved in the organic solvent and this figure would probably be increased a little on further extraction. It is a fair approximation to the expected figure of 61% and also is compatible with the two types of fractionation being independent.

Electrophoretic analyses

The main problem in this work is the incompatibility of sufficient protein solubility and sufficient salt concentration in the buffer to reduce ionic interactions to an acceptable level. A compromise has to be made in all cases.

Analyses were made for fraction A in both acetate (Fig. 2a) and glycine (Fig. 2b) buffer systems and in both cases heavy precipitation occurred in the descending limb. The patterns obtained with the two buffers were not identical but were very similar in number and arrangement of components. They agree with those of Mills[1] for gliadin in similar buffers. It is probable that there was not sufficient salt present to overcome completely the electrostatic effects.

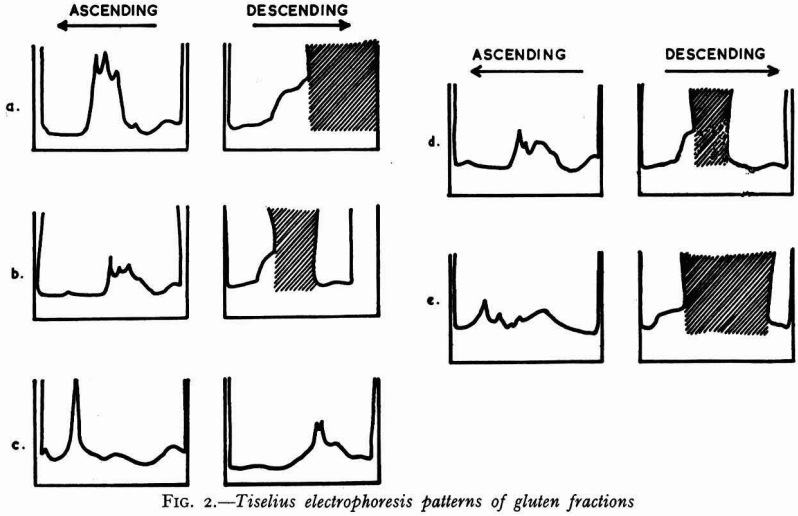


FIG. 2.—*Tiselius electrophoresis patterns of gluten fractions*

a. Fraction A 0·61% in acetate buffer pH 2·3; 150 min. at 4·9 V/cm.
b. Fraction A 0·90% in glycine buffer pH 2·8; 80 min. at 5·6 V/cm.
c. Fraction F 0·72% in glycine buffer pH 2·8; 76 min. at 8·3 V/cm.
d. Fraction C 0·93% in glycine buffer pH 2·9; 65 min. at 8·3 V/cm.
e. The same after 134 min. at 8·3 V/cm.

The shaded areas represent precipitation occurring in the descending limb due to change in ionic atmosphere during the separation.

Fraction F was almost insoluble in the acetate buffer and hence was only examined in the glycine one. In this case (Fig. 2c) no precipitation occurred in either limb, but the ascending and descending patterns were not identical, due presumably to insufficient salt in the buffer. The calculated mobilities (Table I) were quite different for the two limbs.

Attempts were made to examine the oxidised states of fractions A and E but both proved to be too insoluble in buffers containing any reasonable amount of salt.

On examination of fraction C in glycine buffer (Fig. 2*d* and *e*) at least one further major component was found. Otherwise, the pattern showed certain peaks similar to those of fraction A in both mobility and proportion (Table I).

Viscosity measurements

Intrinsic viscosity was determined for those solutions examined in the Tiselius electrophoresis apparatus and also for solutions of fractions A and F in 0·01N-formic acid. The results for intrinsic viscosity, axial ratio and apparent partial specific volume are given in Table II.

Table I

Calculated results from Tiselius electrophoresis analyses

The analyses (a) to (e) correspond to Fig. 2

Mobilities ($\times 10^5$)

Peak	A_1	A	B	Others			
From ascending patterns							
(a)	9·8			4·7	4·1	3·0	2·1
(b)	10·4	6·2	5·6	3·6	2·9		
(c)	9·6			7·1	5·6	4·0	
(d), (e)	10·8	6·2	5·7	5·1	4·9	3·9	
From descending pattern							
(c)				5·9	4·8	4·6	

Proportions (area %), from ascending patterns

Peak	A_1	A	B	Others			
(a)	6			28	34	25	6
(b)	4	24	18	38	16		
(c)	3			73	4	19	
(d), (e)	5	24	10	4	7	50	

Table II

Results derived from viscosity measuremenls

	Intrinsic viscosity	Axial ratio	Partial specific volume
Fraction A in glycine–HCl	9·9	7·8	0·725
Fraction F in glycine–HCl	13·7	10·0	0·717
Fraction A in 0·01N-formic acid	20·5	(13·2)	0·726
Fraction F in 0·01N-formic acid	107·9	(37·5)	0·721
Fraction A in sodium acetate–HC	7·9	6·5	0·764
Fraction C in sodium acetate-HCl	11·4	9·0	0·733

An electroviscous effect is seen in the comparison of the intrinsic viscosities in glycine buffer and in formic acid solutions. The increase of viscosity in salt-free solution is small for fraction A but is very large for fraction F.

Precipitation studies

Sodium formate was added to a solution of fraction B prepared by homogenisation in dilute formic acid, and also to a solution of fraction C. Immediately after the addition, the proportions of protein precipitated were, respectively, 91% and 85%, raised to 94% after keeping the precipitation mixture from fraction B for 4½ days and to 86% after 2½ days in the case of fraction C. The precipitability, which is appreciably lower for fraction B than for fraction C, does not significantly alter within a few days.

Interfacial attraction of fraction A

Purification of a methanol–chloroform solution of fraction A was attempted by Folch's technique[5] of keeping the solution in contact with a large volume of water until all of the methanol has diffused from the mixture. Most of the material separated as solid from the interface, although 55·4 mg. out of 544 mg. originally in solution went into the water phase. No material remained in the chloroform phase.

Washing of a methanol–chloroform solution of fraction A with dilute formic acid was then attempted. The solution containing 176·6 mg. of N in 180 ml. was washed three times, with about one litre of 0·01N-formic acid each time. After the first washing the chloroform layer cleared but there was a heavy interfacial precipitate. On subsequent washings the chloroform layer did not clear but there was less precipitate. The final chloroform layer was an emulsion which was broken with great difficulty and incompletely by centrifuging, the resulting compact interfacial emulsion being separated from the almost clear chloroform layer. This chloroform layer, on evaporation, gave 1–2 mg. of material. The interfacial emulsion was dried in a vacuum desiccator, to yield a white residue which dissolved easily and completely in dilute formic acid.

The three acid washings contained 58·6%, 24·8% and 12·0% of the N, the interfacial emulsion contained 4·4% and the chloroform solution 0·1%. The protein thus has a strong affinity for the chloroform phase and is only removed from the interface with difficulty, although in the absence of chloroform it is easily soluble in the dilute acid.

Dispersion in urea and salicylate

The dispersion of fraction E in dilute formic acid, in 30% urea solution and in 12% sodium salicylate solution was investigated, the solubility being judged visually. Similar suspensions were oxidised by the addition of 1/5 volume of 30% hydrogen peroxide, and a further set reduced by the addition of 1/25 volume of thioglycollic acid, giving a final concentration of 5% w/v. Oxidation and reduction were allowed to proceed for 2 days at room temperature.

The original gel swelled in dilute formic acid without dispersion, while in solutions of urea and sodium salicylate the swelling was less marked and a large proportion of the protein went into solution (about 2/3 for salicylate). The oxidised gel dissolved in dilute formic acid except for a slight deposit, while in urea solution it dissolved completely to a clear solution. In salicylate solution, the undispersed gel did not appear to be affected by oxidation and remained insoluble. The reduced gel behaved in all three solvents exactly as did the oxidised gel.

Discussion

The gelatinous nature of the hydrated state of fraction B led to attempts to prepare this gel directly from gluten by extraction of the soluble protein with dilute acid. About 65% of the protein dispersed in the acid under conditions where the gel did not break down due to enzyme action or to lack of salt. The amount of protein extracted was thus greater than the 37% corresponding to fraction A which was expected. The gel, fraction E, was thus only a part of fraction B. It appeared to depend for its stability on the presence of traces of soluble materials, presumably salts, since continued washing with acid caused expansion of the gel eventually to the point of mechanical instability and fragmentation.

The key to the structure of the gel is the observation that although it is stable to strong acid (90% formic acid) it is easily destroyed and rendered soluble by oxidation. The most plausible explanation is that the gel is of the three-dimensional network type[6] with cross-linkages between the polypeptide chains provided by cystine bridges and by ionic attractions (salt-linkages). It is the latter which break on washing out the salts whilst the former are broken by oxidation. A sulphur-linked structure for protein gels has been postulated by Huggins *et al.*[7] and this type of structure further allows a speculative theory of the plastic properties of gluten since, in a system linked by S–S bridges but containing a few free S–H groups to act as 'free radicals', the linkages are potentially mobile and can break and rejoin in other arrangements.

The network is presumed to be random since a brief examination of this fraction by *X*-ray diffraction failed to show any orientation. The work of Traub *et al.*[8] and of Bloksma & Isings[9] has shown that an ordered state may be produced in gluten proteins under orientating stress, and that the disorder increases with time during relaxation. Broeckhuysen & Broeckhuysen[10] have concluded from studies of the solubility of gluten in alkaline aqueous media that a three-dimensional structure exists, but postulate a semi-ordered arrangement of networks in superimposed sheets. Further *X*-ray diffraction studies are necessary to elucidate this point.

Interesting from the point of view of gel breakdown is the solubilising action of urea and sodium salicylate solutions. Some mechanical action is required to make dispersions of gluten in either of these reagents. It has been shown above that the gel is partially but not completely dispersed in the two solutions. The mechanism of dispersal in urea solution may be envisaged as the reverse of the gel formation promoted by urea in many soluble proteins and discussed by Huggins *et al.*[7] In the present case there is a change from interchain bonding to intrachain bonding. Dispersion by sodium salicylate solution presumably has a similar mechanism with the additional complication of complex formation.[11] However, the non-mechanical dispersion process is not complete in either of these reagents and, in the case of salicylate dispersions, McCalla & Gralén[12] showed that gluten was only partly in molecular dispersion and, indeed, some was not dispersed at all, about 18% of the total protein being centrifuged out in their clarification process.

The intermediate fraction, F, has not the clear-cut properties of fractions A and E. Since it could be obtained by application of the two fractionation systems in either order, it is felt that this is a definite fraction just as much as are A and E. It is not, however, possible to explain easily its properties in terms of molecular architecture. It has, presumably, a sufficiently high content of ionisable groups to make it insoluble in the methanol–chloroform solvent, whilst it lacks the definite structure of the gel. It has been considered that this protein may be a 'soluble' form of the gel protein, similar polypeptide chains existing in a soluble form without cross-linking. This point has not yet been investigated.

Electrophoretic examination of fraction F in glycine buffer showed four components and the pattern only compares with that of fraction A in the presence of the small, fast-running component. The pattern is, however, asymmetric and the mobilities cannot be relied on for identification. Viscosity studies showed fraction F to have apparently a higher axial ratio than fraction A, although there is the possibility of false results due to electrostatic interaction here. This type of interference would be of greater importance for fraction F in view of the considerable electroviscous effect demonstrated.

The amino-acid analyses presented in Part I showed that both fraction C and gliadin, while very similar to, were not identical with, fraction A in composition. No physical examina-

tion of the gliadin preparation was carried out, but electrophoretic patterns for gliadin in the same systems as used here have been published by Mills.[1] Fraction A shows five components corresponding to the components A_1, A, B, C and D described by Mills for gliadin. Of these components, only A, B and C are present in quantity, their relative proportions, calculated from the areas under the curves, being shown in Table I. Fraction C shows a pattern similar to that of fraction A with the addition of a large slow-moving component. The resemblance between the two fractions in components A_1, A and B is seen in Table I. The slow-moving component is about 50% of the whole and possibly represents undissociated complex.

The calculated axial ratios for fractions A and C are lower than those reported by most workers for gliadin and other gluten preparations. Using purified gliadin, Mehl *et al.*[2] found axial ratios of 10·5–11·1. Barmore,[13] using acetic acid dispersions, found axial ratios of about 15 for the most soluble fractions and up to 41 for whole gluten, the deduced value for glutenin being 47. Working with salicylate dispersions, Colvin & McCalla[14] by viscosity found axial ratios of 16·3 to 17·1. By consideration of the electrical dipolemoment of gliadin, Entrikin[15] found axial ratio about 8.

The difference in axial ratios obtained may be attributed to the different character of the preparations used. All previous work has been carried out either on gliadin (alcohol-soluble) preparations, or on dispersions of gluten obtained in various solvents by mechanical treatment such as would break the gel structure. All these preparations therefore contained more or less of the gel protein which does not go into molecular dispersion and will greatly affect the results. In the case of alcohol extracts, Kuhlmann[16] has shown that some protein fractions are carried into dispersion by the presence of others already in solution. He infers that micelles of varying length are present, the more insoluble proteins being the longer ones. The present preparations of the more soluble proteins (fractions A and F) entirely exclude the gel fraction and are more probably molecularly dispersed, although no data have been examined on which this point could be decided.

The viscosity due to solute molecules may be considered as the sum of two factors, the volume occupied by the solute and interactions between molecules. The latter may be due to asymmetry, solvation, hydrogen bonding or charge attractions. In the absence of salts to suppress electrical interactions the viscosity of protein solutions is considerably increased (the electroviscous effect). The decrease in viscosity in the presence of salts is associated with a decrease in the double-layer (zeta) potential of the molecules. The electroviscous effect is shown in the present results to be relatively small for fraction A, since solution in acid rather than in salt-containing glycine buffer produces an increase of intrinsic viscosity only from 9·9 to 20·5. The comparable increase for fraction F is from 13·7 to 107·9. It is clear that it is mainly the ionisable groups of a macromolecule that are responsible for the electroviscous effect.[17]

The electroviscous effect is seen repeatedly in early work on the viscosities of dispersions of flour proteins in acid solutions. It seems from the present work that the viscosity of unheated (i.e., with the starch not gelatinised) acid extracts and dispersions of flour will depend both on the salt content of the medium and on the proportion of ionisable groups in the protein molecule.

Lüers & Ostwald[18] showed that addition of lactic acid markedly increased the viscosity of aqueous wheat flour suspensions, particularly for flours of low extraction rate. Lüers also showed that acids and bases produced maximal viscosities in solutions of gliadin. Gortner & Sharp[19] plotted viscosity against pH for gluten dispersions and for flour suspensions, obtaining maxima at pH 3·0 and 11·0. It is possible that all these effects are due to the production of the charged state of the protein molecules at pH remote from the isoelectric point in the absence of sufficient salt to suppress the electroviscous effect, as suggested by Edsall.[20] Indeed, Johnson & Herrington[21] showed that previous leaching with water led to acidulated flour suspensions of even higher viscosity than without previous leaching. Barmore[13] was aware of the possible dangers in his study of the viscosities of aqueous alcohol extracts of glutens and used solutions containing 0·05–0·075M-sodium chloride to minimise the electroviscous effect. Udy[17] in his work on the viscosity of dispersions of gluten proteins in dilute acetic acid and in sodium salicylate was also aware of the possibility of this electroviscous effect and discusses

it in some detail. McCalla & Gralén[12] found electrostatic difficulties with dispersions in dilute acetic acid and noted that addition of salts to prevent these caused insolubility.

The general conclusion, therefore, to be drawn from the viscosity studies is that the molecules are asymmetric, but to a lesser degree than has been found by some previous workers, due possibly to the use of different preparative methods. Fraction A, with axial ratio of about 7, shows only a small electroviscous effect in agreement with the small number of ionisable groups shown by analysis and by its solubility in non-polar solvents. Fraction F, with axial ratio about 10, exhibits, however, a considerable electroviscous effect in accordance with its probably much higher content of ionisable groups.

The low ionic character of fraction A is reflected in other observations. When partitioned between chloroform and water by Folch's washing technique, the protein remained almost entirely at the interface although the solubility in distilled water is quite high. On being partitioned between chloroform and dilute formic acid, in which the protein should have been freely soluble, it still showed a strong affinity for the chloroform phase and was only removed from the interface by repeated washings.

The partial specific volume has been calculated for fraction A from the amino-acid analysis. The value obtained, 0·723, is in good agreement with existing figures for gliadin and also with the present pycnometric determinations (Table II). The values for fractions A and F are entirely normal for proteins. McCalla & Gralén[12] obtained a value of 0·73 for gluten proteins in acetic acid and alcohol solutions. Krejci & Svedberg,[22] for solutions of gliadin in dilute HCl, found values about 0·72–0·73, these varying slightly with concentration. More exact measurements for gliadin in aqueous alcohol were made by the dilatometric method by Foster & French,[23] the value $0{\cdot}724 \pm 0{\cdot}004$ being obtained. Undoubtedly the case of gluten-protein dispersed in sodium salicylate solution is quite distinct since McCalla & Gralén[12] found the partial specific volume to be 0·700 in 8% and 0·690 in 12% sodium salicylate solution. McCalla[11] later showed that the gluten–salicylate complex behaves abnormally in several respects.

Conclusions

The influence of prior acid treatment on the extractability of certain fractions showed the complex nature of the gluten-protein. The fractions derived from the dissociated complex are, however, more suitable for examination.

The gelatinous nature of fraction E and the dissolution of the gel on oxidation suggested that this protein has a three-dimensional network structure. Since examination by *X*-ray diffraction failed to show any orientation, the molecular network of the gel is presumed to be random. The main linkages between polypeptide chains are probably covalent S–S linkages. These are possibly mobile by an S–H ' free radical ' mechanism and such mobility could explain the plasticity of gluten. These intermolecular bonds will prevent solution on hydration, unless mechanically broken, and confine the swelling to a definite limit. The chains could be additionally linked by ionic linkages between charged groups and the degree of this additional linkage will depend on the pH and salt content of the medium. From the consideration of wheat and barley proteins the unique properties of wheat dough appear to be due in some part to the structure of the gel protein and, in particular, to its content of glutamine, proline, lysine and alanine.

The most soluble fraction, A, has very little ionic character and this probably explains its solubility in relatively non-polar solvents. All the fractions have a high content of polar groups and this may account for their strong hydrophilic character. None of the fractions isolated is electrophoretically homogeneous.

Since the entire gluten is a complex, it is logical to assume that the gel protein provides the structural framework of the complex. The other protein sub-molecules must then be attached to this skeleton although the type of linkage involved in the complex formation is not known. To this complex water is attracted, due to the polar groups of the polypeptide chains and by mechanical entrainment. The degree of this mechanical entrainment of solvent should depend on the salt content of the ambient solvent since this regulates the degree of ionic linking of chains. Varying strengths of ionic linkage can be envisaged depending on the degree of protection afforded by adjacent groupings in the polypeptide chain. The absence of individual

molecules probably partly accounts for the insolubility of gluten in water and saline solutions. Oxidative improving agents may modify the structure of the complex by rupture of some of the sulphur cross-linkages and may further modify the properties by the production of additional ionic groups from ruptured sulphur linkages.

The protein complex structure postulated may be formed in doughing since the work of Hess has shown that two distinct types of protein occur in the wheat grain and that these may be separated. It will be shown in a later paper that some lipid is bound to the protein complex in gluten, the mechanism of binding possibly bearing some similarity to the binding of the low-ionic protein in the structure. Again, this binding may only occur on doughing.

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ORGANIC ACID METABOLISM IN CIDER AND PERRY FERMENTATIONS. II.*—Non-volatile Organic Acids of Cider-apple Juices and Sulphited Ciders

By G. C. WHITING and R. A. COGGINS

The non-volatile organic acids of cider-apple juices and of ciders from sulphited fermentations have been separated using chromatographic methods, and identified. Several juice acids not previously detected were identified, namely mucic, benzoic, gluconic and 2-methyl-2,3-dihydroxybutyric acid. During fermentation malic and gluconic acids often increased considerably in amount, appreciable quantities of succinic and lactic acids being formed together with small amounts of 2-methyl-, 3-methyl-, 2-ethyl- and 3-ethyl-dihydroxybutyric acids, fumaric, α-hydroxyglutaric and perhaps 2-ketogluconic acids; pectic degradation gave rise to appreciable quantities of mono-, di- and tri-galacturonic acids.

* Part I: *J. Sci. Fd Agric.*, 1956, **7**, 31

Introduction

The non-volatile organic acids detected in juices and ciders by Phillips *et al.*[1] included several unidentified and some tentatively identified acids. The present work describes the separation of these substances by a combination of ion-exchange resin adsorption, silica gel chromatography and paper chromatography, and their subsequent identification. Keto-acids, separated by a different technique, will be described in a later paper. Addition of sulphur dioxide to juices in order to suppress the growth of bacteria[2] is now widely practised in the cider industry and the work described is confined to sulphited fermentations.

Experimental

Extraction of juices

Carefully selected apples free from injury and microbiological attack were milled and pressed by the normal cider-making technique in stainless steel apparatus.

Fermentation of sulphited ciders

Sulphur dioxide (150 p.p.m.) was added to the juice in a 5-gallon glass jar immediately after it had been pressed and the jar then closed by means of a cotton-wool plug. After the initial frothing the cotton-wool plug was replaced by a rubber bung fitted with an air-lock. Fermentation proceeded at 15° until all fermentable sugar had disappeared.

Total and titratable acidity of juices and ciders

These were determined by the method of Burroughs[3] on samples before and after passage through a column of Amberlite resin IR-120 (H^+ form).

Chromatographic separation of the acids

(i) *Ion-exchange resin treatment.*—The freshly-pressed juice or cider was passed through a column of Amberlite resin IR-120 (H^+ form), 150 g. of resin for 5 l. of juice or cider. The eluate was then passed through a column of Amberlite resin IRA-400 (acetate form) of such a size that a little more than half the capacity of the resin was used. The column was then well washed with water (approximately 50 l.). The acids were eluted first with acetic acid, the normality of which was increased in steps from 0·5N to 6N, then with 6N- and finally 8N-formic acid. Fractions of 100 ml. were collected and stored at −17°. Each fraction was subjected to paper chromatographic examination as described below.

(ii) *Silica gel column partition chromatography.*—Appropriate fractions from (i) were bulked, concentrated in a rotary evaporator under reduced pressure at a temperature not exceeding 40°, and finally dried in a vacuum desiccator over phosphoric oxide and sodium hydroxide. The residue was dissolved in a little water, mixed with silica gel and added to the top of a previously-prepared silica gel column.

The silica gel was prepared from water-glass as previously described.[4] To make up the column, silica gel was thoroughly mixed with distilled water (1 g. required 1·3 ml. of water) so that it still appeared dry, and was then made into a slurry with water-equilibrated chloroform. The slurry was poured into a glass tube fitted at the lower end with a sintered disc and glass tap. After allowing the gel to settle, the acids to be separated were added. They were separated by elution first with 5%, then with water-equilibrated mixtures containing 10, 15, 20, 25, 30, 35, 50 and 60% of t-butanol in chloroform. An aliquot of the fractions of the major acids and the whole fractions of the minor acids were titrated. Very hydrophilic acids were eluted with t-butanol containing 10% water, and water. A 10-g. silica gel column sufficed for the separation of 15 mequiv. while a 75-g. column separated 150 mequiv. of acid. Fractions of 25 or 50 ml. were collected and each was examined by paper chromatographic means.

(iii) *Paper chromatography.*—The fractions from the above separations were examined by the descending paper chromatographic technique using the following solvents:

Solvent A. Benzyl alcohol, 3 ml.; isopropyl alcohol, 1 ml.; t-butyl alcohol, 1 ml.; water, 1 ml.; formic acid, 2%.[5]
Solvent B. n-Propyl alcohol, 7 ml.; conc. aqueous ammonia, 3 ml.[6]
Solvent C. Phenol, 3 g.; water, 1 ml.; formic acid, 1%.[5]

One other solvent used for particular separations was :

Solvent D. Ethyl acetate, 2 ml. ; glacial acetic acid, 1 ml. ; water, 2 ml. (upper phase).[7]

Whatman No. 1 chromatographic paper was used with solvents A–C and Whatman No. 54 paper with solvent D.

Quantitative determination of the acids in juices and ciders

The main acids in juices and ciders were quantitatively determined by the method previously described.[8] The preliminary concentration of ciders was however done in a vacuum desiccator over calcium chloride.

Separation of acids in a cider

Cider (6 l.) from the apple variety Court Royal was treated as described in (i). The composition of the eluting acid and of the fractions eluted from the anion-exchange column were as shown in Fig. 1. The fractionation of the corresponding juice showed a simpler picture.

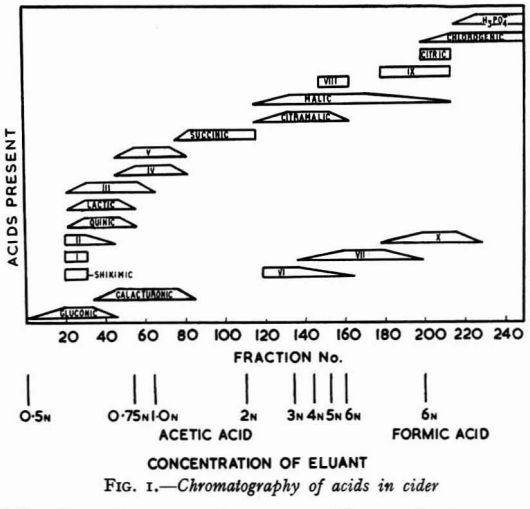

FIG. 1.—*Chromatography of acids in cider*

The cider acid fractions after paper chromatographic examination were bulked as follows :

Fractions 1–18. These fractions contained gluconic acid only, which was precipitated as the calcium salt, after removal of acetic acid *in vacuo*.

Subsequent bulked fractions were treated as described in (ii) and the acids were separated on a silica gel column as follows :

Fractions 19–29. Quinic, lactic and shikimic acids together with unknown acids I, II and III were separated ; gluconic and galacturonic acids eluted with water. Acid I decomposed on re-running on a small silica gel column.

Fractions 30–45. Quinic, lactic and unknown acids II and III were separated ; gluconic and galacturonic acids eluted with water.

Fractions 46–55. Quinic, lactic and unknown acids III, IV and V were separated ; galacturonic acid eluted with water.

Fractions 56–65. After concentration and removal of acetic acid *in vacuo*, barium hydroxide solution was added until the solution was neutral to phenol red and two volumes of ethanol were added. After keeping at 0° overnight the precipitate of barium galacturonate was filtered off on a sinter and the filtrate passed through a column of Amberlite IR-120 (H^+ form). The eluate was concentrated and unknown acids III, IV and V were separated on a silica gel column. Acid IV was lost by volatilisation.

Fractions 66–74. Acids IV and V were separated (silica gel column) and galacturonic acid was eluted with water.

Fractions 75–81. Succinic acid was separated from acids IV and V ; galacturonic acid eluted with water.

Fractions 82–116. These fractions contained succinic acid only. The fractions were taken to dryness, acetone added and succinic acid allowed to crystallise.

Fractions 117–134. Malic and citramalic acids were separated; unknown acid VI eluted with water.
Fractions 135–148. As fractions 117–134; unknown acids VI and VII eluted with water.
Fractions 149–164. Malic and citramalic acids were separated from unknown acid VIII; unknown acids VI and VII eluted with water.
Fractions 165–180. Malic acid was separated from acid VII; the latter eluted with water.
Fractions 181–199. Unknown acid IX was filtered off from the concentrated solution after it had been cooled. Malic acid was separated from unknown acids VII and X on a silica gel column. The last two acids were eluted with water.
Fractions 200–216. Acid IX was filtered off after cooling; malic, citric, *p*-coumarylquinic and chlorogenic acids were separated from acid X on a silica gel column; the last acid was eluted with water.
Fractions 217–249. These fractions contained *p*-coumarylquinic, chlorogenic and phosphoric acids.

Further separation of the acids from silica gel column separations

The fractions from the silica gel column corresponding to the acid peaks were evaporated under reduced pressure, dissolved in water and where the whole fraction had been titrated, passed through a column of Amberlite IR-120 (H^+ form) resin to remove sodium ion and indicator. Paper chromatographic examination using solvents A, B and C showed whether a given peak was due to one or more acids. Unknown acids III and V appeared in one peak and separated only in solvent B. They were therefore separated with this solvent and eluted from the paper with water. Each acid was re-run on a small silica gel column in order to separate it from indicator and impurities.

Alternative method of separation of acids in a cider

Cider (8 l.) from the apple variety Dabinett was passed through a cation-exchange resin column and an anion-exchange column (IRA-400 acetate form) as previously described and the acids fractionally eluted with 0·1N-HCl. The fractions were stored at $-17°$ until required. An unknown acid IX was filtered off from some of the malic-acid-containing fractions after thawing. The malic-acid-containing fractions were bulked, concentrated as in (ii) and run on a large silica gel column; the quinic and succinic acid-containing fractions were similarly treated. Malic, citramalic, quinic and succinic acids were each crystallised from their respective combined peak fractions. The mother liquors were combined with the remaining fractions from the resin column and after evaporation were added to a 50-g. silica gel column. The same four acids were again crystallised from their respective peak fractions and the mother liquors combined with the remaining fractions. The minor acids were then added to a 25-g. silica gel column and eluted with 500 ml. of each of the t-butanol–chloroform mixtures, 10, 15, 20 and 25%. Fractions of 50 ml. were collected. The fractions on paper chromatographic examination were found to contain the following acids:

fractions 11—15 lactic and unknown acids XI and XII.
fractions 16—20 lactic acid and unknown acids XIII and XIV.
fractions 21—25 succinic acid and unknown acids XIII and XIV.
fractions 29—36 α-ketoglutaric acid.
fractions 37—42 unknown acid XV.

Acid XI crystallised from fractions 11–15 on evaporation. Mild hydrolysis of the residue converted acid XII into acid XV which was then easily separated from lactic acid and acid XI on a small silica gel column.

Acids XIII and XIV were only separable on a paper chromatogram using solvent B. These acids were eluted from a paper chromatogram and re-run on a silica gel column to remove impurities and indicator.

Identification of the separated acids

Except where otherwise stated the acids were tentatively identified by comparing their R_F values in solvents A, B and C with those of authentic specimens run on the same chromatogram. The R_F values and the peak fraction in the small-scale silica gel column separation[4] are shown in Table I.

[All melting points given below are uncorrected. The main acids of apple juices have been fully identified[9, 10] and observations reported are those which appear to differ from previous work.]

Table I

R_F values and peak fractions on small silica gel column

	Acid	Peak fraction	Solvent A	Solvent B	Solvent C
	Malic	43	0·47	0·07	0·45
	Quinic	85	0·22	0·20	0·50
	Succinic	18	0·75	0·10	0·64
	Lactic	18	0·70	0·39	0·72
	Citramalic	28	0·61	0·11	0·61
	Citric	55	0·39	0·02	0·32
	Shikimic	67	0·42	0·17	0·58
VIII	Benzoic	5	0·96	0·62	—
III	2-Methyl-DHB*	29	0·61	0·41	0·72
V	3-Methyl-DHB	29	0·61	0·49	0·72
XIII	2-Ethyl-DHB	17	0·74	0·48	0·82
XIV	3-Ethyl-DHB	17	0·74	0·62	0·79
II		43	0·47	0·07	0·45
IV		5	0·84	0·58	0·83
VI	2-Ketogluconic	W	0·08	—	—
	Gluconic	W	0·13	—	—
	Galacturonic	W	0·08	—	0·25
IX	Mucic	—	0–0·02	0–0·02	—
XI	Fumaric	9	0·79	0·11	0·63
XII	α-Hydroxyglutaric lactone	9	0·73	0·25	0·79
XV	α-Hydroxyglutaric acid	36	0·57	0·11	0·60

W denotes elution with water
* DHB = 2,3-dihydroxybutyric

L-*Malic acid* was recrystallised from equal volumes of ethyl acetate and acetone with addition of light petroleum (b.p. 60–80°).[11] The m.p. 101° agreed with the value reported by Hulme[9] which was higher than previously published figures (lit.[12] gives m.p. 100°). The rotation in the presence of ammonium molybdate (determined as described by Hulme) was $[\alpha]_D^{22}$ +605°; Hulme[9] obtained $[\alpha]_D^{20}$ +569°. This method, however, does not give the maximum rotation for any particular concentration of malic acid. The method of Krebs & Eggleston[13] gave $[\alpha]_D^{20}$ +723°; these authors obtained $[\alpha]_D^{18}$ +716°. Other reported values are those of Dakin[14] $[\alpha]_D$ +695°, and Auerbach & Krüger[15] who recorded a molecular rotation of +1020° which gives $[\alpha]_D$ +759°.

D-*Citramalic acid* was recrystallised from ethyl acetate–light petroleum and finally from ether, m.p. 109–109·5° (Hulme,[10] m.p. 109–111°). The optical rotation like that of malic acid varies with the concentration: the value obtained was $[\alpha]_D^{20}$ −23° (*c* 9·1 in water). Hulme[10] reported $[\alpha]_D$ −30° (concentration not given); Marckwald & Axelrod[16] obtained $[\alpha]_D^{14}$ +25° (*c* 7·89 in water) for D-citramalic acid.

A much increased rotation was obtained on addition of ammonium molybdate. The method of Krebs & Eggleston[13] gave $[\alpha]_D^{20}$ −796° which on addition of citrate became −1290°; $[\alpha]_D^{18}$ −748°, or −1335° in presence of citrate.[13]

l-*Quinic acid* was crystallised from hot ethyl acetate and finally from ethanol as flat hexagonal prisms, m.p. 170–171°. Hulme[9] reported m.p. 159–160°, but values up to 184° have been reported.[17]

Shikimic acid was crystallised from ethanol and finally from glacial acetic acid. The m.p. 183–184° showed no depression on admixture with an authentic specimen.

Citric acid was crystallised from ethyl acetate–light petroleum. The m.p. 150–151° showed no depression on admixture with an authentic specimen.

Succinic acid was crystallised from acetone. The m.p. was 182·5–183·5° and admixture with an authentic specimen showed no depression.

Gluconic acid obtained as the calcium salt was recrystallised from water. The free acid gave a positive reaction with the silver nitrate reagent as described by Trevelyan *et al.*[18] but did not react with aniline hydrogen phthalate[19] or with *p*-anisidine hydrochloride.[20] The optical rotation of the calcium salt was determined in the presence of ammonium molybdate by the method of Vintilesco *et al.*[21]; a 0·89% solution in a 5-cm. tube gave α = +0·998°; the formula of the above workers $\alpha = \frac{226 \cdot 67lc}{100}$ gave α = +1·009°.

Acid I decomposed on a silica gel column and was lost.

Acid II had R_F values in solvents A, B and C identical with those of malic acid but its early elution from the resin column and the test with lead acetate[22] showed that it was not a dibasic acid. Ferric chloride solution gave a weak yellow colour, silver nitrate gave a white spot, but tests for phosphate, periodate oxidation and keto group were negative. There was insufficient material for further examination.

Acid III which occurred in all the ciders examined was identified as 2-methyl-2,3-dihydroxybutyric acid.[23]

Acid IV volatilised and was lost.

Acid V occurred in all the ciders examined and was finally obtained as a syrup and characterised as 3-methyl-2,3-dihydroxybutyric acid.[24]

Acid VI had similar R_F values to those of galacturonic acid in solvents A, C and D. Aniline hydrogen phthalate[19] gave a red colour while *p*-anisidine hydrochloride[20] gave a violet-red colour similar to that given by 2-ketogluconic acid.[25] Acid VI may therefore be 2-ketogluconic acid.

Acid VII was purified by precipitation with strontium chloride in 60% aqueous ethanol solution. The precipitate was taken up in water and passed through a column of Amberlite resin IR-120 (H^+ form) and the free acid treated with lead nitrate solution. The precipitate containing acid X was filtered off and the filtrate containing acid VII was again passed through a second column of the resin and re-precipitated with strontium chloride. The acid was purified by repeating the precipitations several times. It had the same $R_{\text{galacturonic acid}}$ value (0·66) in solvent D as given by Demain & Phaff[7] for digalacturonic acid, and gave reactions with silver nitrate reagent,[21] aniline hydrogen phthalate[19] (brown colour) and *p*-anisidine hydrochloride[20] (brownish-red colour). These observations and the properties of the strontium and lead salts[26] identified acid VII as digalacturonic acid.

Acid VIII was purified by sublimation and recrystallised from water. It was identified as benzoic acid by R_F 0·62 in solvent B; a pale-brown or buff colour with ferric chloride solution; m.p. 120·5–121·5° and mixed m.p. with an authentic specimen 121–122°.

Acid IX was purified by dissolving in a little NaOH solution and precipitating with 2N-HCl at 0°. The acid had a very low R_F in solvents A and B, but did not react with either aniline hydrogen phthalate[19] or *p*-anisidine hydrochloride[20] but reacted with Trevelyan's silver nitrate reagent.[18] The material dried over phosphoric oxide *in vacuo* at room temperature had m.p. 211–212° (decomp.); admixture with an authentic specimen of mucic acid showed no depression of the melting point. Acid IX was therefore identified as mucic acid.

Acid X was precipitated together with acid VII with strontium chloride and the lead precipitate obtained in the separation above was again converted to the free acid on the Amberlite resin. After repeating the precipitations several times the free acid was taken to dryness *in vacuo*. Acid X gave the same reactions with Trevelyan's silver nitrate reagent, aniline hydrogen phthalate and *p*-anisidine hydrochloride as acid VII and had $R_{\text{galacturonic acid}}$ value in solvent D of 0·46, identical with Demain & Phaff's value for trigalacturonic acid.[7] An electrometric titration gave an equivalent weight of 192·7 (calc. for trigalacturonic acid monohydrate, 188). The molecular weight (hypoiodite method)[7] was 575 (calc. 564), ratio of COOH/CHO was 2·98/1,[7] and $[\alpha]_D^{20}$ +193° (*c* 2·16 in water). When the acid was heated in a melting-point tube it showed colour changes from 134° upwards (pale cream), reddish-brown at 142° but there was no true melting point. Phaff & Luh[26] observed decomposition at 143° and a deep salmon colour at 145–155°. Acid X was therefore identified as trigalacturonic acid.

Acid XI sublimed when heated. It was dissolved in ethanol and exposed to ultra-violet light for 1½ h., when the resulting solution examined on a paper chromatogram run in n-pentanol–5N-formic acid showed two acid spots of R_F 0·44 and 0·77, identical with the R_F values of maleic and fumaric acids respectively run on the same chromatogram. Acid XI was identified as fumaric acid.

Acids XII and XV were readily inter-convertible. Mild hydrolysis of XII gave XV and XV readily lactonised to give XII. On a paper chromatogram the free acid spot reacted strongly with ferric chloride solution (presence of α-hydroxy acid) and with bromocresol green and lead acetate reagent[22] (presence of dibasic acid) while the lactone spot reacted feebly with these reagents. The R_F values in solvents A, B and C and peak fractions on a silica gel column were identical with those of α-hydroxyglutaric acid and its lactone. Acids XII and XV were identified respectively as α-hydroxyglutaric lactone and α-hydroxyglutaric acid.

Acids XIII and XIV have been identified as 2-ethyl- and 3-ethyl-2,3-dihydroxybutyric acids, respectively.[24]

Identical methods of separation and identification showed the presence in juice of malic, quinic, citramalic, shikimic, citric, benzoic, mucic, galacturonic, gluconic and 2-methyl-2,3-dihydroxybutyric acids.

Quantitative amounts of acids in juices and ciders

Table II shows the ranges of amounts of acids in juices from mature cider-apples, the differences being mainly due to variety. Acids present only in trace amounts were benzoic (0·01 mequiv./100 ml.), mucic (0·01 mequiv./100 ml.), gluconic, galacturonic and 2-methyl-2,3-dihydroxybutyric acid (tentatively identified in juices by R_F values only as there was insufficient for characterisation).

With the exception of malic, gluconic, galacturonic and 2-methyl-2,3-dihydroxybutyric acids which increased, the above acids remained unchanged during sulphited fermentations; the acids formed, together with the amounts, are shown in Table III.

Discussion

The acids previously reported in apple fruits have been reviewed by Pollard[27] and Hulme.[28] Acids identified in cider-apple juices additional to those listed were mucic acid (found also in

Table II

Range of concentrations of acids in cider-apple juices

Acid	Content in juice (mequiv./100 ml.)
Malic	1·5–15·0
Quinic	0·25–1·25
Citramalic	Trace–0·65
Chlorogenic	Trace–0·85
p-Coumarylquinic	Trace–0·15
Shikimic	Trace–0·08
Citric	0·03–0·16

Table III

Acids formed during fermentation (in mequiv./100 ml.)

	Acid	Quantity
	Succinic	0·20
	Lactic	0·19
III	2-Methyl-2,3-dihydroxybutyric	0·04
V	3-Methyl-2,3-dihydroxybutyric	0·01
XIII	2-Ethyl-2,3-dihydroxybutyric	0·004
XIV	3-Ethyl-2,3-dihydroxybutyric	0·002
VII	Digalacturonic	0·08 (approx.)
X	Trigalacturonic	0·08 (approx.)
I		0·01
II		0·01
XII, XV	α-Hydroxyglutaric acid and lactone	0·05
VI	(2-ketogluconic), XI (fumaric), gluconic and galacturonic acids—not determined	

pears and peaches[29]), benzoic (found previously in berries of *Vaccinium macrocarpum*[30] and *V. myrtillis*[31]), gluconic acid (said to occur in trace amounts in sound apples[32]) and 2-methyl-2,3-dihydroxybutyric acid (tentatively identified). Tanner & Rentschler[33] identified glycollic acid in Swiss cider-apples by paper chromatography, but this acid has not been detected in English cider-apples. Both lactic and succinic acids have been found, sometimes in appreciable amounts, in apple juices,[27] but in the present work, using carefully-picked fruit, neither acid was detected.

During sulphited fermentations, no decrease in the amount of any acid present in the original juice has been observed. Malic acid may however increase considerably in amount, by more than half the original content in some fermentations. Formation of malic acid was first observed by Dakin[14] and has also been shown to occur in perry fermentations.[34] Fumaric acid found in a few fermentations might be expected from the action of fumarase on the malic acid formed. The formation of gluconic acid may have resulted from the fact that a small proportion of sugar was attacked, not via the Embden–Meyerhof scheme but perhaps by the hexose monophosphate pathway.

The dihydroxy-acids and α-hydroxyglutaric acid formed probably arose by the further metabolism of keto-acids and these will be discussed in a subsequent paper.

The presence of comparatively large amounts of mono-, di- and tri-galacturonic acids in ciders indicated pectin degradation during fermentation. The work of Pollard & Kieser[35] showed that pectin degradation in cider fermentations was brought about by the action of a yeast polygalacturonase after demethylation by a methylesterase present in the fruit.

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CHROMATOGRAPHIC ANALYSES OF THE FREE AMINO-ACIDS, ORGANIC ACIDS AND SUGARS IN WHEAT PLANT EXTRACTS

By BYRON S. MILLER and T. SWAIN

Plants of three hard red winter wheat varieties differing in their resistance to attack by hessian fly (*Mayetiola destructor* Say) were compared at the fourth leaf stage for their content of constituents extractable by 80% ethanol. Eleven amino-acids, five organic acids and two inorganic acids were identified. Seven sugars were separated, two of which were unidentified polysaccharides. The main difference was that the susceptible variety (Tenmarq) contained allulose, or allulose in combination with some other component. This compound was present to a lesser extent in the semi-resistant variety (Ponca) and not present in the plants of the resistant variety (C.I. 12855). Sorbitol also was found in Tenmarq but not in the other varieties.

Introduction

Excellent reviews on the resistance of plants to insects have been published by Painter[1] and Martin.[2] One possible explanation for resistance is that nutritional factors may determine the reaction of insects to the host plant. Fraenkel[3] concluded that, although most plants contain abundant food for insects, specificity may depend on the presence of one or more particular substances. Such substances may be members of any one of several classes of compounds. Auclair *et al.*[4] found, for example, that three varieties of peas (*Pisum sativum* L.) which were susceptible to the pea aphid (*Acyrthosiphon pisum* Harr.) generally contained a higher concentration of free and total amino-acids than three resistant varieties.

Several workers[5–8] have determined qualitatively the free amino-acids present in various parts or in the whole wheat plant representing one variety. Similarly, several workers[9–13] have investigated the organic acids present in wheat plants and others have investigated the carbohydrates.[10, 11, 13–20] Hassebrauk & Kaul[10, 11] and Nakagawa[16] seem to have been the only workers who investigated different varieties. Hassebrauk & Kaul[10] found that the variety, Chinese 166, which was resistant to yellow rust contained a lower percentage of organic acids than the susceptible variety, Michigan Amber ; also[11] that the variety, Michigan Amber, which is susceptible to brown rust contained less polyphenols and ascorbic acid but more sugar than

Triticum monococcum var. Hornemanni. Nakagawa[16] found that wheat plants carrying the gene for resistance to *Gibberella sauburetti* contained relatively high amounts of fructose.

The object of the present work was to determine if any qualitative and proximate quantitative differences exist in the 80% ethanol-soluble amino-acid, organic acid and sugar composition of three wheat varieties which differed in their susceptibility to attack by hessian fly (*Mayetiola destructor* Say).

Experimental

Materials

Three hard red winter wheat varieties, Tenmarq, Ponca and C.I. 12855, which were highly susceptible, semi-resistant and immune, respectively, to attack by hessian fly were used. Tenmarq also is susceptible to leaf rusts but the other varieties are resistant to several races. All varieties were imported from the United States and grown in seed boxes or flats in the open air during September 1958.

The plants were lifted when they reached the fourth leaf stage and separated into leaf and stem fractions. This stage of growth corresponds to that normally used for testing varieties for resistance to hessian fly.

Authentic allulose was prepared by epimerisation of glucose and purified by paper chromatography.[21] All other reference substances were commercial samples.

Extraction

The fresh leaves (~250 g.) and stems (~100 g.) were extracted separately. The tissue was cut into $\frac{1}{4}$–$\frac{1}{2}$ in. lengths, boiled in 80% ethanol (5 ml./g. fresh weight) for 3 min., filtered and re-extracted with 80% ethanol (3 ml./g. fresh weight). The residue (15-g. portions) from the second extraction was macerated in a Waring Blendor with 100 ml. of 80% ethanol, filtered, washed twice with a small quantity of solvent and discarded. All extracts were combined and stored at 0°. After 24 h. the cloudy extract was filtered through Celite, and chlorophyll and related pigments removed by shaking the extract twice with $\frac{1}{4}$ volumes of light petroleum. The ethanol–water soluble components were concentrated to about 2 ml./g. of fresh weight *in vacuo* and stored at 0° under toluene.

Analytical techniques

Nitrogen was determined by a micro-Kjeldahl method with copper and selenium catalysts. Total carbohydrate was determined by means of orcinol[22] and citric acid as in the method of Taylor[23] and free amino-acids by the ninhydrin procedure.[24]

Separation and purification

The extracts were separated into amino-acid, organic acid and sugar fractions as described by Cruickshank[25] except that Dowex 50-X5 resin was used instead of Zeo-Karb 215 and the amino-acids were eluted with 0·5N instead of 0·1N aqueous ammonia. The solutions were concentrated to suitable (5–10 ml.) volumes and stored under toluene at 0°.

The individual compounds in each fraction also were separated by the technique of Cruickshank[25] with certain minor changes. The sugars were chromatographed in n-butanol–acetic acid–water (6 : 1 : 2, v/v) for 72 h.

Identification

The purified solutions and authentic known compounds were chromatographed as described by Cruickshank.[25] Paper electrophoresis also was employed to identify both amino-acids[25] and sugars.[26] For the electrophoresis of the sugars, borax (0·1M) was used as the buffer and the apparatus was run for 1·5 h. at 450 V and 10 mA. The methods of chromatography including solvents, the use of markers and spray reagents were those discussed in Lederer & Lederer.[27] The resolution of organic acids was aided greatly by employing the two-dimensional technique of Howe[28] on $5\frac{1}{4}$ in. square papers. The solvents employed were: n-propanol–2N-aqueous ammonia (70 : 30, v/v) and n-propanol–sulphur dioxide-saturated water (70 : 30, v/v).

Results

The results of quantitative analyses of the concentrated crude extracts are shown in Table I, and of qualitative analyses for amino-acids, organic acids and sugars in the wheat plant extracts in Tables II–IV.

Discussion and conclusions

Table I indicates that there were no significant differences in the total nitrogen, free amino-acid, carbohydrate or citric acid present in the plant tissue from the different varieties.

Table I

Summary of analytical data on 80% ethanol extracts of wheat plant tissue representing varieties that are susceptible, semi-resistant, and immune to attack by hessian fly

Variety	Part of plant analysed	Dry matter, %	Total nitrogen	Total free amino-acid	Total carbohydrate	Citric acid
				mg./100 g. of fresh weight		
Tenmarq	stem	14·4	64	61	1050	79
Ponca	stem	17·5	43	79	1070	87
C.I. 12855	stem	13·3	60	68	1010	69
Tenmarq	leaves	12·4	44	87	1070	70
Ponca	leaves	13·5	39	78	1010	87
C.I. 12855	leaves	12·0	47	68	1010	74

Eleven ninhydrin-reacting compounds were identified (Table II). Arginine and histidine also were faintly observable on two-dimensional chromatograms obtained by running the extract on Whatman No. 1 paper first with water-saturated phenol in the presence of ammonia and second with n-butanol–acetic acid–water (6 : 1 : 2, v/v). No qualitative or quantitative differences were observed in the chromatograms representing the three varieties.

Table II

Amino-acids in 80% ethanol extracts of Tenmarq wheat plant leaves and identified by paper chromatography

Amino-acids*	Butanol–acetic acid–water (6 : 1 : 2, v/v) R_F value of acids		Water-saturated phenol R_F value of acids		Paper electrophoresis, 5N-acetic acid $R_{proline}$ values of acids	
	in wheat plant	authentic	in wheat plant	authentic	in wheat plant	authentic
Alanine	0·15	0·16	0·58	0·58	1·28	1·28
Aspartic acid	0·06	0·06	0·15	0·15	0·87	0·87
γ-Aminobutyric acid	0·28	0·29	0·75	0·75	1·94	1·94
Glutamic acid	0·10	0·11	0·24	0·25	0·97	0·97
Glycine	0·07	0·08	0·42	0·43	1·31	1·30
Leucine	0·44	0·44	0·82	0·83	1·11	1·11
Phenylalanine	0·40	0·40	0·82	0·82	1·01	1·01
Serine	0·07	0·07	0·38	0·39	1·07	1·08
Threonine	0·12	0·12	0·50	0·50	1·03	1·03
Tyrosine	0·33	0·33	0·61	0·61	0·74	0·74
Valine	0·38	0·38	0·74	0·74	1·29	1·29

* Arginine and histidine were detected on two-dimensional chromatograms but were not isolated from the amino-acid fraction

Five organic acids and two inorganic acids were identified in the Tenmarq wheat extract (Table III). A trace of lactic acid was also found but this may have been formed from sugar in the resin columns.[30] The *trans*-aconitic acid which was identified, was probably present in the original extract as *cis*-aconitic acid. No qualitative or quantitative difference in the organic acid composition of the three varieties was observed.

The presence of seven sugars in Tenmarq wheat plant extract was demonstrated, but two were unidentified (Table IV). Sucrose formed the bulk of the sugars present. This corroborates the work of Ward[20] and Roberts.[18] One of the unknown sugars (No. 1, Table IV) yielded only

Table III

Organic acids in 80% ethanol extracts of Tenmarq wheat plant leaves and identified by paper chromatography

Acid	Propanol–2N ammonia (70 : 30, v/v) R_F values of acids		Propanol–SO_2-saturated water (70 : 30 v/v) R_F values of acids		Butanol–formic acid–water (6 : 1 : 1, v/v) R_F values of acids	
	in wheat plant	authentic	in wheat plant	authentic	in wheat plant	authentic
Citric	0·05	0·05	0·68	0·69	0·34	0·34
Hydrochloric	0·38	0·38	0·41	0·42	0·07	0·07
Malic	0·16	0·16	0·71	0·71	0·44	0·44
Quinic*	0·25	0·25	0·51	0·51	0·14	0·14
Succinic	0·20	0·20	0·80	0·80	0·71	0·71
Sulphuric	0·13	0·13	0·29	0·30	0·02	0·02
trans-Aconitic**	0·07	0·07	0·83	0·83	0·76	0·76

* Confirmed by piperazine tests[29] ** Confirmed by spraying with $KMnO_4$

Table IV

Sugars in 80% ethanol extracts of Tenmarq wheat plant leaves identified by paper chromatography and electrophoresis

Sugar	Butanol–acetic acid–water (4 : 1 : 5, v/v) R_F values of sugars		Ethyl acetate–pyridine–water (2 : 1 : 2, v/v) R_G values of sugars		Butanol–ethyl alcohol–water (5 : 1 : 4, v/v) R_F values of sugars		Paper electrophoresis (0·1M-borax) R_G values of sugars	
	in wheat plant	authentic	in wheat plant	authentic	in wheat plant	authentic	in wheat plant	authentic
Glucose	0·11	0·11	1·00	1·00	0·10	0·11	1·00	1·00
Fructose	0·15	0·15	1·14	1·14	0·14	0·14	0·96	0·96
Sucrose	0·04	0·04	0·88	0·89	0·07	0·07	0·75	0·75
Raffinose	0·02	0·02	0·57	0·57	0·02	0·02	0·74	0·74
Allulose	0·19	0·19	1·40	1·40	0·20	0·20	0·91	0·91
Unknown I	0·01	—	0·36	—	0·00	—	0·95	—
Unknown II	0·04	—	0·64	—	0·03	—	0·75	—

fructose on hydrolysis, the other yielded both glucose and fructose. Similar unidentified high-molecular-weight carbohydrates were found in wheat by Bradfield & Flood,[14] and Lopatecki *et al.*[15] The latter authors also found glucose, fructose and sucrose at all stages of growth from early shot blade to maturity. Stems and leaves contained the same carbohydrates. Bradfield & Flood[14] also found sorbitol in plants of Squarehead Master Wheat. In the present work sorbitol was found in the Tenmarq extract but not in that from the other two varieties.

Allulose (psicose) was found in the extracts of both the stems and leaves of Tenmarq wheat plants and to a lesser extent in Ponca extracts which had passed through the ion-exchange resin columns. It did not occur, however, in the fractions from the resistant variety, C.I. 12855. No allulose was detected by chromatography of the original Tenmarq extracts which had not been concentrated or passed through the resin columns. This is not surprising, however, because the detection of any single compound in the crude extract is difficult. Allulose was not present in solutions of pure glucose, fructose, sucrose or sorbitol which were passed through the resin columns and concentrated in a manner similar to the way sugar fractions from the crude extracts were treated, so it was concluded that allulose was not produced from these substances. It is possible, however, that the allulose was released by hydrolysis of a parent compound during the processing of the wheat extracts. If this is so, then this parent compound is present in Tenmarq and to a lesser extent in Ponca wheat but is not in C.I. 12855 plants. The sugar was indistinguishable from authentic allulose in four different chromatography solvents and by paper electrophoresis and gave all the typical colour reactions of a ketose with various spray reagents.[27]

Allulose has been found previously in the unfermented residues of molasses derived from both sugar cane[31] and beets[32] where it could be an artefact formed at low hydrogen-ion concentrations caused by the lime used in the manufacturing process. There does not appear to be any other reference on the natural occurrence of this sugar.

It is impossible to say whether the presence of allulose or its precursor is related in any way to the susceptibility of certain wheat plants to attack by hessian fly. It may, however, be indicative of a peculiar physical structure of the cell wall[33] which may be the real basis for susceptibility of the young wheat plant to attack by the parasite, and this warrants further study. Similarly, it is impossible to say whether sorbitol (or possibly other polyhydric alcohols) is related to the susceptibility of wheat to rusts. This aspect of the work also appears to be worthy of further investigation.

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