Food Chemistry 6 (1980) 1

EDITORIAL

To the organic chemist or even to many food technologists, the properties of food gels are there to be utilised, but may not, perhaps, be very well understood. The six papers assembled here were presented at a Symposium on Food Gels organised by the Food Chemistry Group of the Industrial Division of the Society of Chemical Industry at the Scientific Societies Lecture Theatre, Savile Row, London, Great Britain, on 7 June 1979, and are the third set of symposia papers to be published in this Journal.

They deal with such materials as carrageenan, alginates, pectates, polysaccharides and milk proteins. Their study is a specialised one and is concerned, to a large extent, with various physical methods of investigation. It is very clear that the inter-relationships within a manufactured foodstuff are extremely complex, for such things as heat and ionic environment can quickly change gelling properties.

It seems probable that new gelling agents are unlikely to be able to be marketed, due to the high cost of establishing their suitability, so that the emphasis must be on understanding and developing those already permitted. It is hoped that these papers will give stimulus to young food chemists, particularly, to do just that.

Fd. Chem. (6) (1980)

Food Chemistry 6 (1980) 3–14

PROTEINACEOUS GELLING SYSTEMS AND THEIR COMPLEXES WITH POLYSACCHARIDES

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ABSTRACT

Edible gels may be produced by three kinds of interaction between proteins and polysaccharides. The simplest kind is that between any protein having a nett positive charge and a sulphated or carboxylated polysaccharide. Much more specific is the formation of a gel between two macromolecules bearing the same overall charge, as, for example, the gelling of κ-carrageenan by κ-casein. Thirdly, there is the highly selective linking of polysaccharide to protein by covalent bonds. The thermostable gels made by linking alginate esters to proteins are examples here.

Recent advances in the application of all three kinds of interaction are described and assessed.

1. INTRODUCTION

Only a few polysaccharides (for example, agar, alginates, carrageenan and starches) and a few proteins (for example, gelatin, soya and whey) are used to form food gels and, despite the widespread occurrence of both types of macromolecule in a food and the importance of their mutual interactions as a key to food texture (Morris, 1973), most publications continue to deal with detailed and specific applications. This contribution attempts to outline the main general considerations that underly these interactions and to show how they can be used in gel formation. A few examples, taken from recent researches, indicate the variety of gels which may be produced.

All edible gels are three-dimensional polymer networks which hold the main, fluid phase in extremely small pores so that the shape is retained, as in a solid. The

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network itself arises from the association, at so-called junctions, of the individual polymer chains. In order that the gel should have sufficient elasticity and that it should not show syneresis, or weeping, the junctions should be formed from only a few monomers from each of its component chains, leaving the major part of each macromolecular chain with almost independent movements, as in a solution. Hill (1960) has described a highly elastic, non-synerising gel as analogous to a tightly packed collection of actively wriggling worms with each end of each worm being joined to one end of several other worms. This model would serve for the most specialised kind of protein polysaccharide gel, which arises when a reactive group, occurring infrequently on the polysaccharide, forms a covalent bond with a suitable —and also infrequent—site on the protein, and the latter is in a disorganised, or denatured, conformation rather than in the highly organised and compact form which is the native state for most proteins. Recent research in this area is summarised in Section 4.

Proteins and polysaccharides interact quite generally and non-specifically, provided they are close enough, and can create junctions which are due solely to non-covalent bonding. Ionic bonding, under suitable conditions of pH and ionic strength, is one form of such interaction, although it is restricted to charged polysaccharides. This produces the first kind of composite gel to be discussed. In such a network, the closeness of the component chains, in the junctions, ensures that the stability of the gel is enhanced by localised hydrogen bonding, where this is possible, and by co-operative but short-range inter-atomic attractive forces, which will be described as van der Waals forces. The importance of these forces is usually overlooked.

Section 3 summarises recent work on milk systems for which electrostatic interaction between the polysaccharide and protein plays an important role in assisting gel formation, although it is not involved in any of the network junctions. A detailed explanation of all the experimental observations could not be given until the precise sequence of the amino acid residues along each protein chain had been established. Such information is still restricted to a limited number of food proteins, and it seems likely that useful developments may occur when more sequences are available.

Finally, in Section 5, the subject of water-holding by edible gels is discussed, in connection with the role of free macromolecules which may be present and are not involved in network formation.

2. NON-SPECIFIC IONIC INTERACTIONS

The general electrostatic attraction of negatively charged polysaccharides for positively charged proteins has been a subject of considerable interest for a very long time. The attraction is characterised by a sensitivity to pH—since the development

of charge on each component depends on the pH of the surrounding medium--and to ionic strength. The process is usually reversible for these variables. Interaction is strongest at low ionic strengths and the attraction becomes minimal as the ionic strength, compounded from added electrolytes, macro-ions and counter-ions, is raised. Thus, a precipitate can form at low ionic strength, and at a pH below the isoelectric point of the protein, whilst it will dissolve on adding electrolytes. Bungenberg de Jong (1949) gave a comprehensive appraisal of early work, with particular emphasis on coacervation, which can give a very stiff gel. More recently, Imeson *et al.* (1977, 1978) have shown how these interactions can be utilised to recover food proteins from effluents, etc. through suitable adjustments of the most important variables, i.e. the concentrations and mixing ratios of the macroions, the concentrations and charges of low molecular weight ions, pH, the charge densities of the polyelectrolytes and the spacing and stereochemistry of the charged groups on the polymer backbones. Similar considerations apply when these interactions are used for fractionating a mixture of proteins, for clarification by solubilising proteins, for turbidimetric procedures in enzyme assays or for synergistic thickening by soluble complexes with proteins. The last of these uses indicates that the interaction can cause some change to the native structure of a protein, presumably a partial denaturation or unfolding of the polypeptide chains. The optimal use of positive ions (for example, Ca^{++}) to link two negatively charged polyions, such as polysaccharide with protein above its isoelectric point (e.g. Chakraborty & Randolph, 1972), also requires attention to be given to these same variables. In all these applications the polysaccharides have carried ionisable carboxyl groups (as in alginates, gum arabic, pectates and carboxymethylcelluloses) or, for the practical pH range of interest, fully ionised sulphate groups (e.g. carrageenans).

The formation of useful gels through these interactions is not so readily achieved as precipitation, although Muchin *et al.* (1976) and Tschumak *et al.* (1976) have reported that alginate and pectate, respectively can form high melting point gels with gelatin. These gels form only over a narrow region of pH and for restricted ratios of the two components. The procedure involves several stages--a precipitate or coacervate is prepared, separated (by centrifugation), disorganised (by heating) and then cooled to give a gel. The strength of the gel increases during storage—say a week at room temperature—and, over the same period, the melting temperature rises (e.g. from 30 °C, which is typical for a pure gelatin gel, to 80 °). The freshly formed gel, unlike the aged gel, is broken down in concentrated urea. The suggestion is that the new gel is essentially a hydrogen-bonded gelatin network entrapping ionically held complexes of gelatin and polyuronide. In time, however, the dynamic nature of the bonds holding together the original network enables new electrostatic bonding to form with adjacent complexes. In this way a more heat-stable gel forms, but it becomes more easily disrupted by added salts. The raising of the melting point, in the absence of salt, through dynamic transfer, is very dependent on the overall balance between the polysaccharide and protein in the coacervate and this, in turn,

depends quite sharply on pH and on the molecular ratio before coacervation or precipitation.

Similar interactions between milk proteins and anionic polysaccharides have sometimes been claimed, but the situation is complicated by the inevitable presence of Ca^{++} and there may also be subtle and specific association, as described in the next section.

3. SPECIFIC IONIC INTERACTIONS

In chocolate milk desserts, for example, the sedimentation of the cocoa particles is prevented when a weak gel network is present. Such a network forms when kappa-carrageenan is added, even though the polysaccharide concentration is at least ten times lower than that expected for gel formation by the polysaccharide alone. Formation of the network thus depends on interactions between milk proteins and the carrageenan. Experiments using model systems (i.e. purified carrageenans and milk proteins) have shown that the interaction is electrostatic in nature and that it is specific (Snoeren, 1976).

Since the interaction involves milk at its natural pH, the proteins are above their isoelectric points and, like the carrageenans, carry nett negative charges. Nevertheless, kappa-casein, unlike the other major caseins (α_{s_1} and β) can displace the positively charged dye, methylene blue, from carrageenan. The released dye then causes an increased absorption of light at a suitable wavelength (665 nm). Furthermore, when mixtures of caseins and carrageenan, previously heated to disperse aggregates, are subjected to ultracentrifugation, aggregates are spun down only with kappa-casein. In both types of assay, ionic strength is an important variable, as would be expected for electrostatic interactions. Only very low concentrations of the carrageenan are needed, yet the intensity of light scattered at 45° from the mixtures shows a sharp increase when plotted as a function of temperature. The plots indicate that complex formation takes place and that it has a more abrupt nature, similar to the sol–gel transition, than is shown when more concentrated solutions of the pure carrageenan are studied. The temperature for the abrupt change in intensity for the mixture is close to that for the change with the pure polysaccharide.

These results are in keeping with the view that a gel forms in these model systems through a two-stage process. In the first stage, despite the identity of the nett charge on each type of macromolecule, an electrostatic attraction occurs and involves only a part of each molecule. Subsequently, as the second stage, the remaining, free parts of each carrageenan chain undergo normal network formation via the coil $\rightleftharpoons$ double helix transition proposed by Rees (1969, 1972). The second stage can only be effective if the molecular weight is high enough, and Snoeren (1976) suggests that a minimum value of 10^5 is required. The specificity of the interaction resides in the first

stage and is understood in terms of the amino-acid sequences for the three major caseins. These primary structures have been given by Mercier *et al.* (1973) and are included in the review on micellar aspects of casein by Schmidt & Payens (1976). All caseins have an unusually high proportion of apolar residues and are abundant in proline, which prevents the formation of ordered structures such as helices. Indeed, in solution an isolated casein molecule is best described as a random coil. It is the distribution of the polar residues, however, which lies at the heart of the electrostatic interaction with carrageenans, and Snoeren (1976) has pointed out that kappa-casein contains an extensive and nett positively charged region between residues 20 and 115. (There are 13 +ve charges and 3 −ve at pH 5.) No such accumulation of nett positive charge occurs with α_{s_1} or β casein, where the positive and negative charges are nearly evenly distributed along the whole chains in near-neutral solutions. Thus, a section of the kappa-casein chain can be held ionically by part of a carrageenan chain. It is considered unlikely that the whole section from residue 20 to residue 115 is involved. The dependence on ionic strength favours, via Debye Huckel type calculations, a very much shorter active site. Additionally, the interaction is considerably reduced by raising the pH from 5 to 7, i.e. over the range of pH where histidine residues lose their positive charge. All three histidines in kappa-casein are close together (residues 98, 100 and 102) so it is not surprising that the short active site for electrostatic attraction is suggested in the region where six positive charges (three histidine, one arginine and two lysine) and no negative charges can accumulate.

This first stage—electrostatic attraction—is common to all types of carrageenans, yet practical experience shows that λ-carrageenan cannot stabilise dairy products. The reason is that, unlike kappa or iota carrageenan, the λ-type chains cannot form a gel so that, with it, stage two is impossible.

In milk, part of the kappa-casein lies on the surface of the voluminous micelles (Schmidt & Payens, 1976), and is thus available for interaction with carrageenans. The adsorption of part of a carrageenan molecule by a micelle leaves most of the polysaccharide free in solution, in the form of loops or tails (Fig. 1).

This is now believed to be a normal consequence of macromolecular adsorption. The free loops, or tails, then associate as in the formation of a pure carrageenan gel provided that the ionic conditions and the temperature are appropriate. The networks form only in the spaces between micelles, and not throughout the whole volume of the system. This may account for the low overall concentration of polysaccharide that is required. Pedersen (private communication) has drawn attention to the importance of potassium ions, from the natural milk salts, in enhancing the gelation of kappa-carrageenan, i.e. in aiding stage two. This effect, although not understood in molecular terms, is readily accommodated in Fig. 1.

It used to be thought that the networks in carrageenan/milk products arose from the formation of ionic calcium bridges between the negatively charged macromolecules. No doubt this does occur, but it cannot be of major importance or

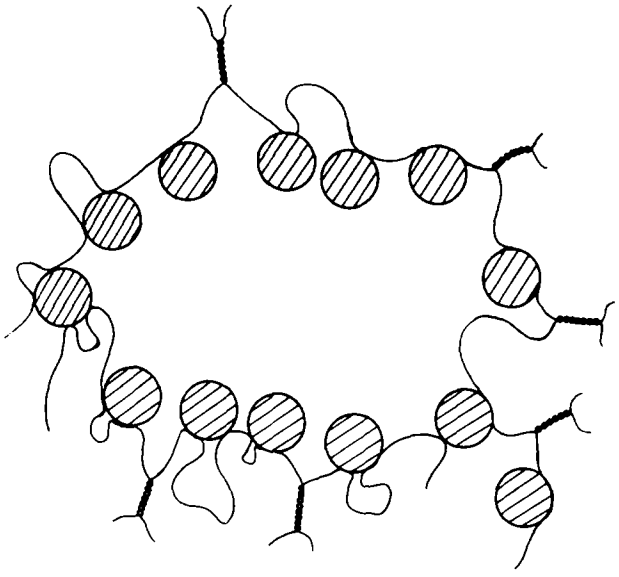

Fig. 1. Part of the gel formed from kappa or iota carrageenan and milk (after Snoeren, 1976).

all carrageenans would act as stabilisers. Polyuronides, on the other hand (alginates, pectates), form networks entirely by divalent metal ions holding together the polysaccharide chains at the junctions, and seem unable to stabilise dairy products.

4. COVALENT BONDING

The main advantage of a system held by covalent bonds, and not by secondary forces, is that it is more thermo-stable. It no longer truly melts on heating, but loses its gel-like character only through disruption of chemical bonds. The bonds forming the polymer chains may well be less stable than the crosslinks, so that 'melting' is then really due to degradation, or hydrolysis and subsequent cooling does not lead to setting to a gel again.

Only a very limited number of crossbonds needs to be introduced to create a suitable gel. If, for example, no additional crossbonding by secondary forces is present, the gel will behave like a simple rubber-like solid and the extent of bonding may be calculated from an elastic modulus, measured when the deformation is very slight (Treloar, 1975; Flory, 1977). The shear modulus, G, is probably the easiest to measure for a gel. A value of G of 3×10^4 dynes cm^{-2} (i.e. 3×10^3 N m^{-2}) is quite reasonable for a gel at room temperature and corresponds to a 'molecular weight between crosslinks', M_c, of $1{\cdot}6 \times 10^4$ when the usual expression $M_c = RT_w/G$, is applied for a 2% gel. (M_c is the weight of a worm in Hill's analogue (Section 1).) R

and T have the usual meanings and w is the weight of polymer forming unit volume of gel, which must be isotropic. (The concentration, w, is in g ml^{-1} if G is in dynes cm^{-2}, and in kg per m^3 when G is in N m^{-2}.) The extent of bonding is then deduced from M_c via the residue 'molecular' weight of the component monomers. Thus, if the example referred to a protein having an average residue weight of 114, M_c corresponds to 140 amino acids in the chain and only one of these is involved in crosslinking. When the crosslinking is by a second macromolecule the resulting gel still behaves as an elastic solid but M_c, calculated from the shear modulus, gives the average separation between cross bonds for all the polymer molecules (e.g. protein plus carbohydrate) which form the network, and is of rather restricted usefulness. It should be possible, however, to analyse the situation by studying gels made from a series of mixtures of the two component macromolecules, provided that their mole-fractions in the networks can be determined. This would be essential, since it cannot be presumed that the gel is created from all molecules present initially, whatever the composition. Ultracentrifugation can help here, by squeezing out the non-gelling component(s). Despite the lack of quantitative information at present, however, it is clear that only a few crossbonds are needed between polysaccharides and proteins to create thermostable gels.

The only polysaccharides studied so far, in this connection, are the esters of pectin and of alginate and although pectic esters can be made to crosslink readily with small molecules (such as 1:6 diamine hexane) there has been no success in making gels with proteins (Wilson, 1978). Alginate esters, however, present no such difficulty and their use for hardening photographic gelatin gels—i.e. to prevent melting and to control swelling during the developing processes—was patented in 1964 (British Patent 962,483). The crosslinking reaction proceeds smoothly when aqueous solutions of the ester and protein are mixed, provided that the pH of the system is optimal: above pH 10 the rate is uncontrollably rapid and non-uniform and lumpy products form, whilst below pH 8 there appears to be no reaction at all. The most likely site for reaction, on the protein, is the epsilon amino group of lysine. This, in the uncharged form—hence the need for the mildly alkaline pH value—most probably reacts with ester groups to give an amide-type link, as shown in Fig. 2.

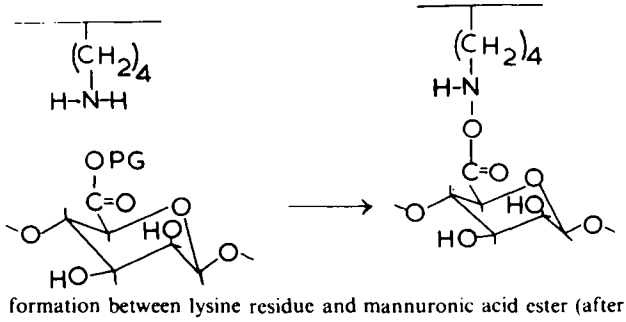

Fig. 2. Amide formation between lysine residue and mannuronic acid ester (after Wilson, 1978).

Although clear evidence, from infrared absorption spectra, can be obtained for this proposal using low molecular weight amines to give highly crosslinked films, there is no direct evidence for the chemical nature of the crossbond in protein gels. Dye binding experiments using Acid Orange 12 (using the conditions suggested by Hurrell & Carpenter, 1975), the binding of trinitrosulphonic acid (using the method of Fields, 1971) and studies of the formol titration show that protein lysine groups are involved and that only about one in six is utilised. Some further work is needed before reliably quantitative figures can be given. If the lysine side chains are blocked (for example, by acetylation or carbamylation), then no gel forming occurs with alginate esters. The lysine residues may not be the only sites for reaction. McDowell (1970) has shown that alginate esters can undergo transesterification with hydroxyl groups of starch, to produce a gel in similar mildly alkaline conditions to those required for reaction with proteins. Transesterification may therefore take place with hydroxyl groups on amino acid residues, such as serine in proteins.

The involvement of the few lysine residues in gel formation poses no nutritional or digestive problem. The lysine availability in such a gel is still high, and the nutritional loss is small compared with the loss that occurs when proteins are heated with simple reducing sugars and browning reactions occur. Moreover, the digestive enzymes, pepsin and trypsin, readily attack the protein in the gel, under the appropriate conditions for pH and temperature, since almost all the sites for attack by these enzymes are still unaffected by gelation. The crossbonds are very stable in acid, but their stability during the later stages of digestion does not seem to have been reported. It is most unlikely that toxic effects or metabolic inhibition are caused by the bonds.

Through the appropriate choice of pH and temperature the gelling reaction can be controlled either to give a very rapid set or to give a delayed set. The reason lies in the alternative, hydrolytic reaction which the ester undergoes once its natural pH (usually about pH 3·5) has been raised beyond neutrality. This extensive reaction thus competes with gelation, by reducing the availability of ester groups. Since alginic acid contains two types of monomer—guluronic acid and mannuronic acid —which do not occur in random sequence to form the polymer, and which have different pK values (pK for guluronic acid is 3·65, whereas it is 3·38 for mannuronic acid)—it is a complex problem to decide which ester is the more stable and which is the more reactive. By using esters of a similar overall degree of glycolation, but different proportions of guluronic to mannuronic acid, it has been shown (Wilson, 1978) that it is the mannuronic ester groups which take part in gelation with proteins. Measurement of gel rigidity as a function of time after mixing, using Saunders & Ward's U-tube method (1954), shows that at room temperature and at a pH of 9·6 (in bicarbonate/carbonate buffer), setting begins a few minutes after mixing and the crosslinking reaction is complete in approximately 90 min. The gels are adhesive and, because no syneresis occurs, they stick to the glass. This is essential. The gels are purely elastic and show no viscous flow up to the maximum

strain, of 0·1, that has been used. Similar times are found for reaction with gelatin (in the presence of molar KSCN to prevent gelation due to gelatin–gelatin interactions), sodium caseinate, egg white, whey and soya isolate, although the final strengths of the gels are very different (see Table 1). In a pH-stat, however, de-esterification continues for at least 24 h, confirming statements in patents that alginates which are highly esterified initially should be used for form gels. (The buffer is, of course, omitted when the pH-stat is used, so that the consumption of standardised sodium hydroxide solution exactly meters the acidity produced by de-esterification.)

TABLE 1
THE DEVELOPMENT OF STRENGTH IN ALGINATE ESTER/PROTEIN GELS

Time (h)	*Shear modulus* ($N m^{-2}$)		
	Soya	*Casein*	*Gelatin*
0·5	0·44	0·96	1·85
1·0	0·44	1·05	2·37
1·5	0·42	1·07	2·57
2·0	0·45	1·06	2·63
4·0	0·43	0·98	2·55
48	0·42	0·84	1·82

Final composition: 3% air dry protein plus 2% ester. pH 9·6 (bicarbonate/carbonate buffer). Temperature, 22°C.

Gels formed under such mildly alkaline conditions are only of use as food gels if the pH is subsequently lowered, often to the range pH 5–7. A patent (No. 1,443,513) published in July, 1976 utilises such gels for making shaped freeze–thaw stable and thermostable foodstuffs. The use of these gels is particularly beneficial when the protein is of little value (i.e. when it would otherwise be usable only in animal feeds or in agricultural fertilisers) and is reconstituted into a foodstuff for human consumption. No refining or purification is required, provided the 'waste' protein is safe for human consumption. An acceptable final pH value is attained by washing the gel with a dilute aqueous solution of an acid and then removing superficial liquid by, say, centrifugation. Many of the examples utilise the gel as an extender, rather than as a product in its own right, so that it is prepared in an appropriate form either by extrusion or by shredding a block prior to the adjustment of pH with acid. It is stated that 15% or more of cod muscle in fish fingers, for example, can be replaced by the gel (as flakes or fibres) without detracting substantially from the appearance, flavour or texture. However, the likelihood of syneresis, even after freezing, is reduced when the gelled extender is present.

In the laboratory it is convenient to change the pH after gel formation simply by leaving the gel, even in large pieces, in a suitable buffer. In this way it can be shown that the intrinsically useful properties of the gel (its elasticity, good water-holding

and stability to processing temperatures) are all maintained. The texture of the gel is maintained, too, and this is not altogether a very good feature, unless the gel is to be used merely as an extender, since in the absence of non-covalent crossbonding it is rather chewy and short, i.e. it has no plastic flow, and it splits open after only modest deformation. Perhaps the nearest equivalent is an agar jelly as used in microbiological assays. Whilst these characteristics are not particularly in demand in the Western world they are nevertheless highly regarded in some Eastern cultures. Protein/alginate ester gels do not melt in the warmth of the mouth, as do gelatin gels, nor do they dissolve through dilution of an ingredient, as when sugar is leached from acid/sugar/pectin gels. When additional non-covalent bonding is also present, as with gelatin, for instance, a more plastic and less chewy product is formed. Here two kinds of gel network co-exist until the temperature is raised above the melting point of the gelatin–gelatin hydrogen bonded network. Such a compound gel bears a resemblance to the mixed gelatin/agar system which, in the past, has been used (e.g. in pie fillings) to overcome problems associated with the rather low melting point of pure gelatin gels. Unlike these mixed gels, the single (protein/alginate ester) gel can withstand freezing and heating without losing its strength or shape.

Two further points require comment. First, the prolonged de-esterification reaction continues to produce free acidity, and carbon dioxide bubbles if a bicarbonate buffer is used. It is possible, by careful choice of buffering ability, to enable the pH to be kept high enough to ensure sufficient gelation and then to fall slowly to give a product having a neutral, or even mildly acidic, pH. Secondly, the mildly alkaline conditions favouring gel formation also favour degradation of the alginate backbone, through the β-elimination reaction which cleaves glycosidic bonds adjacent to uronide ester groups. This reaction is very temperature dependent and sets an upper limit of 40–50 °C for gel formation. Even at these temperatures the gel will be quite quickly broken down unless the pH is reduced to pH 6 or lower.

The proteins used so far in model systems have been disordered rather than globular and it remains to be shown whether stable and highly organised proteins, such as those of blood plasma, can form effective gels by bonding covalently to polysaccharides. Powdered gelatin, or collagen, in suspension, is reactive with alginate esters (Ranganayaki & Stainsby, 1978), but only on the outside of each particle. Further work in this direction may enable a very wide range of proteins to become utilised in new ways for foods.

5. WATER-BINDING

Food gels are required to retain a considerable quantity of water and show no syneresis or weeping throughout shelf-life. Traditional gels vary widely in this capacity, ranging from the very good (e.g. gelatin; agar, above the freezing point) to the rather poor (e.g. unmodified starch; some of the gel networks held together by ions at the junctions). More complex gels, containing several macromolecular

components, can show advantages which accrue from the mutual interactions and entanglements of the polymer. This was clearly shown by Fessler (1957), using a model system: thus, the presence of a polysaccharide (hyaluronate) in a collagen gel conferred an enhanced retention of water when the gel was compressed in an ultracentrifuge. A particularly nice demonstration of this interplay between polysaccharide and protein is mentioned in the review by Comper & Laurent (1978). A natural collagen gel, the vitreous body, contains polysaccharide. If the latter is degraded enzymatically the gel shrinks whereas, if the collagen network is removed, only a viscous solution remains. The inability of the network to hold the extra water after enzymic degradation clearly relates to the macromolecular properties of the polysaccharide solution in the gel.

A useful way of considering this behaviour is to regard the gel as generating a swelling pressure, which is the balance between osmotic forces which encourage water retention, and elastic forces which press the liquid out of the system. Such a swelling can also be countered by mechanical force applied to the gel (e.g. in the ultracentrifuge) or by the chemical potential (water activity) of the water in a surrounding medium (e.g. a solution, in the laboratory, or cells in tissues). The osmotic swelling for the gel is composed of contributions from all the molecules in the system, including all the macromolecules and any counter ions. Experiments with model systems, using uncharged macromolecules initially, have shown that the contribution from the chains forming the network (the worms in Hill's analogue) is equivalent to that for a comparable solution.

The mutual exclusions of the macromolecules, since two or more monomeric residues cannot occupy the same space at the same time, is an entropic interaction and can form an important contribution to the total osmotic swelling— and hence to water retention by the gel. It is particularly marked when one macromolecular component is rather inflexible and requires a large domain. Many polysaccharides fall into this category. At the other extreme, compact globular proteins provide a very small excluded volume effect.

To date, research in this area seems to have been confined to model systems which relate to the extracellular gels in tissues from plants and, more particularly, from animals. It is desirable that the use of such polymeric 'fillers' in food gels should be evaluated as a practical means of ensuring water retention and as an alternative to low molecular weight 'humectants', which need to be at high concentrations. Moreover, the inevitable and large increase in the internal viscosity of the system due to macromolecular exclusion effects must cause changes in rheological behaviour and may lead to useful developments in texture.

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PHYSICAL PROBES OF POLYSACCHARIDE CONFORMATION AND INTERACTIONS

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ABSTRACT

In the solid state most polysaccharides adopt regular chain geometry corresponding to fixed values of the angles between adjacent residues. Such ordered conformations may persist in solution or as the fundamental structural unit in polysaccharide gels or biological tissue. Physical techniques may be used (1) to detect conformational order in solution, (2) to characterise chain geometry and packing, often by reference to known solid-state conformations, and (3) to monitor order–disorder transitions. Characterisation of solution conformation is often facilitated by the use of structurally regular short chain segments, which show the same conformational behaviour as the intact polymer, but are capable of participating in only one ordered intermolecular junction zone.

Nmr relaxation behaviour, measured either directly from the exponential decay of magnetisation, or indirectly from high resolution linewidth, provides a convenient index of conformational rigidity. Polysaccharides show two conformation-sensitive circular dichroism (cd) bands in the vacuum uv at about 150 and 170 nm. These are inaccessible to commercial cd instruments, but may be monitored indirectly by optical rotation, which shows a simple quantitative correlation with the linkage geometry between adjacent sugars in the polymer chain. Accessible pendant chromophores, in particular carboxy groups, also show conformation-sensitive cd behaviour and provide a direct probe of specific cation chelation. The masking effect of such chromophores on the optical activity of the polymer backbone may be eliminated by Kronig–Kramers transform of their observed cd spectra.

Order disorder transitions are conveniently monitored by discontinuities in optical rotation behaviour, intensity of discernible high resolution nmr resonances, differential scanning calorimetry, solution viscosity and, for segments, by light scattering. Transition kinetics show the number of chains involved in the fundamental

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ordered structure, a source of frequent difficulty in solid-state x-ray analysis. Further association of ordered structures may be probed by competitive inhibition studies with short chain segments or, where appropriate, by the stoichiometry of cation chelation.

POLYSACCHARIDE STRUCTURE, SHAPE AND INTERACTIONS

The role of polysaccharides in the development of texture in both natural and manufactured foods may be traced to various levels of structural organisation (Morris, 1979*a*). Most polysaccharides of commercial importance are based on a simple repeating sequence, although this may be interrupted by occasional structural irregularities, or masked by substitution or covalent modification of some of the residues (Rees & Welsh, 1977). Figure 1 shows idealised primary structures for the polysaccharides used as specific examples in this review.

The geometry of the individual sugar rings in a polysaccharide chain is essentially rigid. Overall conformation is therefore determined by the relative orientations of component sugars (Rees, 1977), as defined by the rotational angles Φ and Ψ between adjacent residues (see Fig. 2). In the solid state most polysaccharides adopt sterically regular crystal-like conformations which can be characterised by x-ray fibre diffraction. These structures correspond to fixed values of Φ and Ψ, rather than a statistical distribution of orientations as for random coils in solution, and are stabilised by non-covalent interchain interactions. Polysaccharides in which the bonds to and from each component residue are essentially co-linear (such as alginate) tend to pack together in extended ribbon-like solid-state conformations, while non-colinear bonding 'bends' the chain and favours the formation of hollow helices which often occur as compact multi-stranded structures such as the double helices of amylose, agarose or carrageenan (Rees, 1973). In favourable cases such ordered tertiary structures may persist under conditions of extensive hydration, either in solution or as the primary mode of interchain association in polysaccharide gels (Morris *et al.*, 1977*a*).

In general, ordered conformations are promoted by favourable non-covalent interactions, chain stiffness and efficiency of packing and inhibited by loss of conformational entropy, energy of hydration, intermolecular electrostatic repulsion, structural irregularities and branching. The balance of these opposing drives is often delicate, and may be tipped by relatively small perturbations. For example, thermally induced order–disorder transitions, which may or may not be accompanied by a gel–sol transition, have been observed for a number of polysaccharide systems and show the sharp temperature profile characteristic of a co-operative process (Rees & Welsh, 1977).

In addition to such thermal transitions, the adoption of ordered conformations may be promoted by changes in ionic environment. At the simplest level of

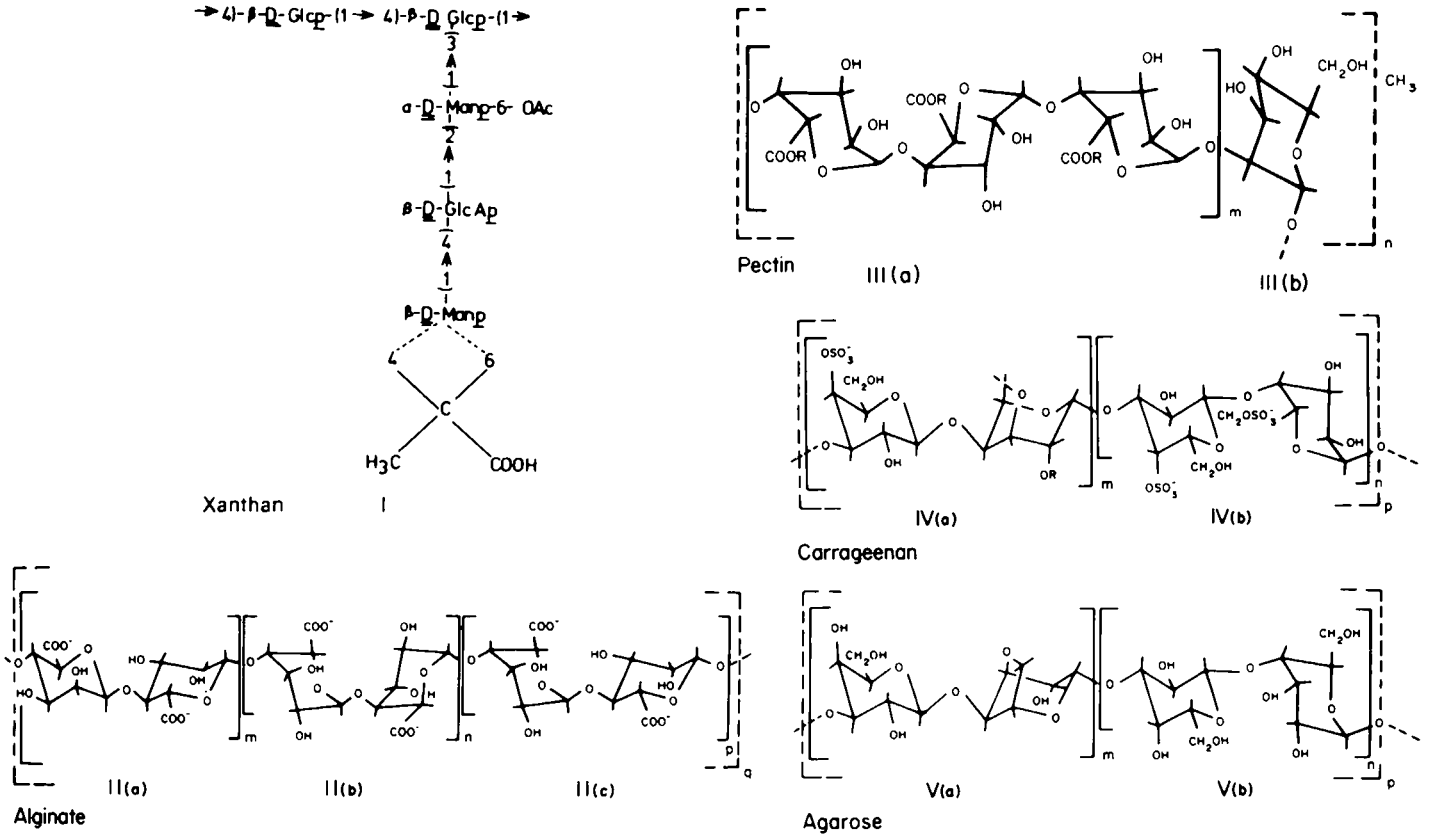

Fig. 1. Polysaccharide primary structures. I. The pentasaccharide repeating unit of xanthan. The level of pyruvate ketal and *O*-acetyl substitution shows appreciable sample to sample variation. II. Alginate block sequences: (a) poly-β-D-mannuronate, (b) poly-α-L-guluronate and (c) mixed sequences in which both component residues are present. III. Pectin primary structure. Poly-α-D-galacturonate sequences (a) which may be substituted to varying degrees by methyl esterification ($R = CH_3$) are interrupted periodically by the presence in the polymer backbone of α-1,2-linked L-rhamnose (b). IV. Carrageenan (kappa: $R = H$; iota: $R = SO_3$). Helix forming structural sequences (a) are interrupted by the occurrence of residues (b) in which the anhydride bridge is absent. V. Agarose primary structure. Helix compatible disaccharide sequences (a) are again interrupted by sequences (b) in the unbridged ring form.

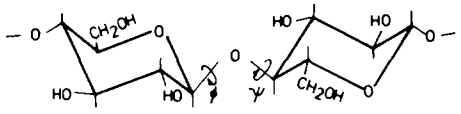

Fig. 2. Polysaccharide chain conformation. The relative orientations of adjacent residues, and hence overall chain geometry, are defined by the rotational angles Φ and Ψ, as illustrated here for cellulose.

understanding, compact ordered conformations of polyelectrolytes may be stabilised under conditions of high ionic strength due to reduction of mutual electrostatic repulsions by charge screening (Morris *et al.*, 1977*b*). More directly, ordered conformations of charged polysaccharides may be stabilised by the incorporation of counterions within the tertiary structure. For example, the primary event in the gelation of alginate is dimerisation of poly-L-guluronate sequences, with specific interchain chelation of calcium, or other divalent cations of appropriate size (Morris *et al.*, 1978). Under hydrated conditions, intermolecular associations of polysaccharides are stable only above a minimum critical chain length necessary for co-operativity, typically in the range 15–20 residues (Kohn, 1975). Thus, as shown schematically in Fig. 3, the traditional concept of a

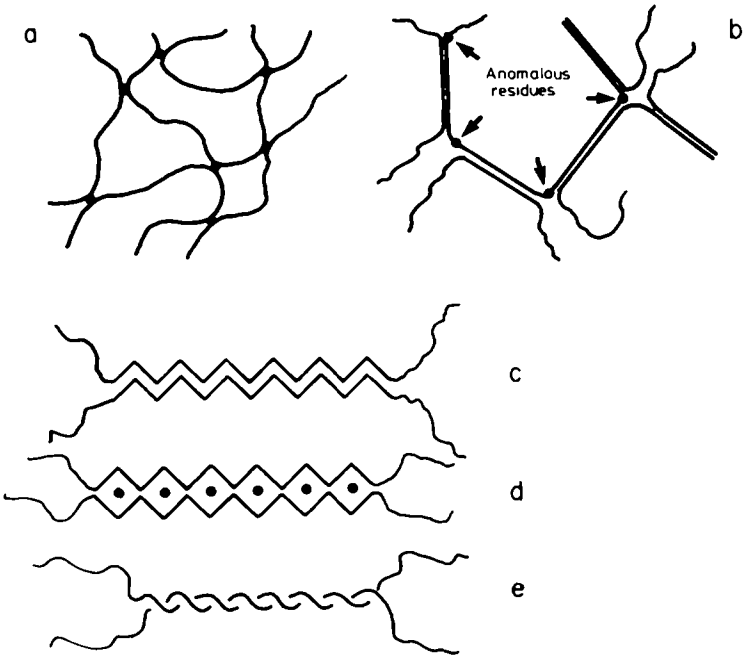

Fig. 3. Schematic representation of polysaccharide gel network models. (a) 'Point-crosslinking' model, (b) 'junction-zone' model. Junction-zone types: (c) nested ribbons, e.g. cellulose, galactomannans, (d) cation 'egg boxes', e.g. alginate, pectin, (e) double helices, e.g. amylose, carrageenan, agarose.

polysaccharide gel network as involving 'point cross-linking' of disordered chains is superseded by a 'junction-zone' model (Rees, 1972*a*), with extensive regions of ordered tertiary structure. Junctions are terminated by the occurrence in the primary structure of residues incompatible with the ordered conformation (Rees & Welsh, 1977). Thus, for carrageenan, double helix formation is interrupted by the presence of 4-linked α-D-galactose residues in the unbridged chair conformation, rather than in the helix-compatible 3,6-anhydro ring form. Similarly, poly-L-guluronate dimerisation in alginate is terminated by chain sequences involving D-mannuronate. Such junction delimiting structural features are important for the formation of gel networks rather than insoluble precipitates, by allowing each chain to participate in ordered associations with several different partners.

While the adoption of ordered tertiary structures by flexible polymer chains involves considerable loss of conformational entropy, subsequent aggregation of such rigid molecular assemblies is entropically far less unfavourable and indeed is to be expected in the absence of over-riding repulsions. Thus, for carrageenan and agar double helices, aggregation increases with decreasing sulphate content, being most evident for uncharged agarose helices (Rees, 1972*b*). Association of charged tertiary structures may be promoted by specific counterions whose radius, charge and chelation geometry are suitable for incorporation within the aggregate. Thus, further association of poly-L-guluronate dimers is promoted by calcium ions (Morris *et al.*, 1978), while aggregation of carrageenan helices is specifically favoured by the presence of potassium ions (Morris *et al.*, 1980*a*). In some cases, such a quaternary structure may be necessary for gelation. Thus, the primary event in the gelation of iota carrageenan is formation of clusters or 'domains' of approximately ten chains held together by double helix junctions. Development of a continuous gel network, however, requires association of these domains by helix helix aggregation, promoted specifically by potassium ions.

THE ROLE OF PHYSICAL TECHNIQUES

Much of the current fundamental understanding of the molecular origin of polysaccharide gelation behaviour has come from the development and application of physical techniques to characterise molecular shapes (conformation) and interactions. The aim of this review is to give a brief outline of established methods of proven effectiveness, to illustrate the way in which they have been used to develop present molecular understanding of polysaccharide behaviour and to discuss in more detail promising lines of future advance. Coverage is by no means exhaustive, or indeed balanced, but rather reflects the personal preferences, experience and prejudices of the author.

As outlined, the physical properties of polysaccharides can be radically altered by the adoption of a fixed conformation, rather than the fluctuating random-coil chain

geometry typical of synthetic polymer solutions. One of the primary objectives in the application of physical techniques to polysaccharide systems is therefore to identify the existence of such ordered structures. Nmr relaxation behaviour offers a convenient general method for detecting conformational rigidity. Thermally induced order–disorder transitions also provide direct evidence of conformational order and may be monitored by discontinuities in the temperature course of optical activity or nmr relaxation and by differential scanning calorimetry. The kinetics of order–disorder behaviour may also assist in the characterisation of the ordered state, by indicating the number of chains involved.

Ordered molecular assemblies stabilised by specific cations may be characterised by stoichiometry of cation chelation and by circular dichroism changes which accompany the site binding of cations to uronic acid residues. Comparison of cd behaviour in solid films and in hydrated situations may also indicate whether or not ordered conformations in solutions and gels are identical to those characterised by x-ray fibre diffraction. For many polysaccharides, structurally regular chain segments may be prepared by selective chain cleavage, and show the same conformational behaviour as the intact polymer, but without network formation. Light scattering studies of such segments in their random coil and ordered conformational states can give direct evidence of the number of chains involved in the ordered assembly, and of its dimensions. Addition of segments to gelling concentrations of the parent molecule can also be used to characterise the nature of gel junctions, by competitive inhibition of intermolecular association of intact chains. These approaches will now be discussed in greater detail, with illustrations of their application to specific polysaccharide systems.

NMR RELAXATION

Nmr relaxation behaviour provides a convenient index of polysaccharide conformational mobility (Bryce *et al.*, 1974). The decay of magnetisation may occur by loss of phase of individual precessing nuclei, and is characterised by the spin–spin relaxation time, T_2. As thermal motions interfere with this process, the relaxation rate is inversely related to the degree of molecular mobility. Relaxation behaviour can be measured directly as the time constant for exponential decay of magnetisation, or indirectly from high resolution linewidth, $\Delta\nu_{1/2}$, from the relationship $T_2 = 1/\pi\Delta\nu_{1/2}$. Thus, small molecules moving freely in solution show sharp, narrow, spectral lines, whereas linewidths for solids are so great that no high resolution spectrum can be detected. The proton T_2 for water is of the order of 1 s, whereas those for mono- and oligo-saccharides in solution are of the order of hundreds of milliseconds. Typical random coil polysaccharides in solution show T_2 values around 50 ms, corresponding to high resolution peaks which, although broader than for monosaccharides, are still clearly discernible. By contrast, such

rigid conformations as the carrageenan double helix show T_2 values around 50 μs even in solution, and the corresponding high resolution linewidth is so great that all peaks are flattened into the baseline. Thus, loss of discernible high resolution signal can be used to monitor conformational ordering, as illustrated in Fig. 4 for the thermal order–disorder transition of xanthan (Morris *et al.*, 1977*b*). For systems

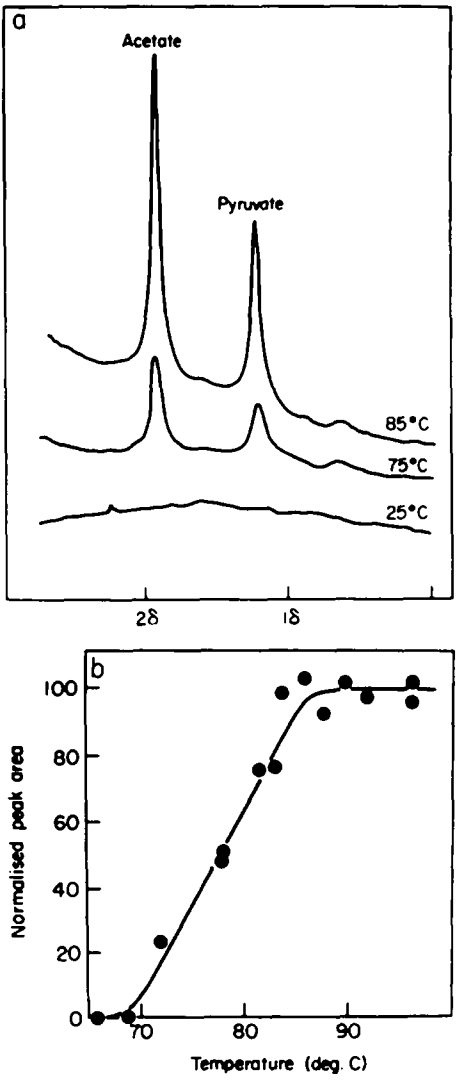

Fig. 4. Detectable high resolution nmr signal as an index of conformational ordering. The collapse of the well resolved upfield resonances (a) from the acetate and pyruvate ketal groups of xanthan may be used to follow the temperature course of the disorder–order transition (b).

which do not show accessible order–disorder behaviour, T_2 measurements, or 'missing' high resolution intensity, may be used directly as an index of molecular rigidity in solutions or gels (Darke *et al.*, 1978).

OPTICAL ROTATION AND CIRCULAR DICHROISM

Single-wavelength optical rotation is now well established as a sensitive and direct probe of polysaccharide conformation and has been used extensively to monitor order–disorder behaviour (see, for example, Fig. 5). Indeed, a simple semi-empirical quantitative relationship has been demonstrated (Rees, 1970) between changes in the glycosidic angles between adjacent residues (Φ and Ψ in Fig. 2) and consequent changes in D-line optical activity. Recent vacuum ultraviolet circular dichroism studies (Liang *et al.*, 1979) suggest that these chiroptical effects have their origin in

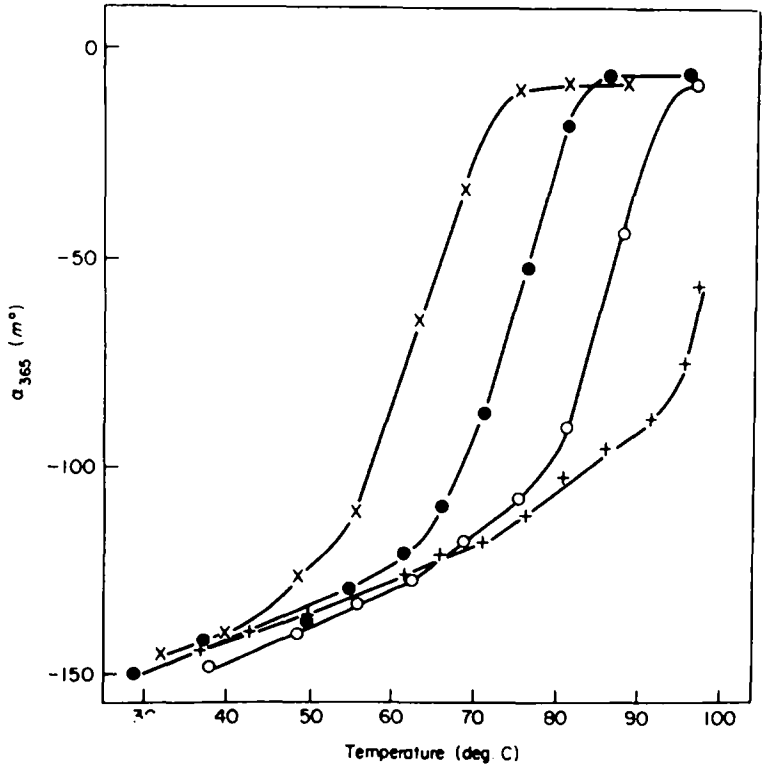

Fig. 5. Optical rotation as an index of polysaccharide comformation. The use of this technique is illustrated in monitoring the increased thermal stability of the ordered conformation of xanthan with increasing ionic strength (from left to right: 0·005, 0·01, 0·025 and 0·5).

two conformation-sensitive electronic transitions centred at about 175 and 155 nm. Figure 6 shows the changes in intensity of the higher wavelength band which accompany the thermally induced gel–sol transition of agarose, and follow the same temperature course as the associated changes in optical rotation at higher wavelengths. The pronounced hysteresis is ascribed to quaternary association (aggregation) stabilising the agarose double helix at temperatures substantially above those at which it would form spontaneously.

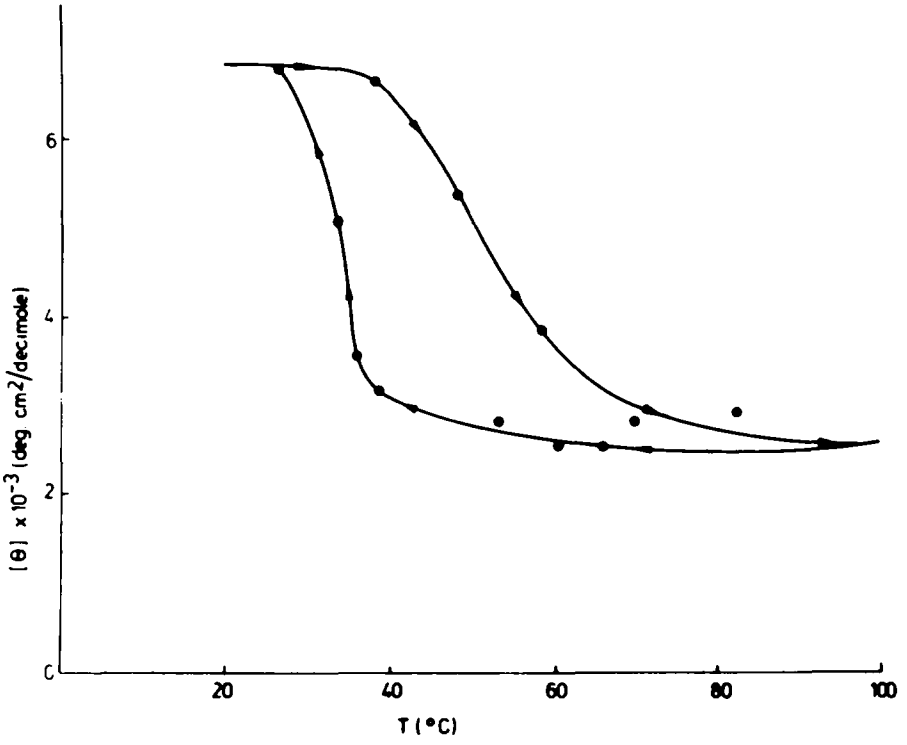

Fig. 6. Thermally induced order–disorder transition of agarose, as monitored by the conformation-sensitive vacuum ultraviolet circular dichroism band at 180 nm.

Polysaccharides with carboxy or acyl substituents show circular dichroism (cd) bands which are accessible to normal commercial equipment, and may be diagnostic of chain composition, conformation and interactions (Morris *et al.*, 1975). Figure 7 shows the very different cd behaviour of carboxylate chromophores in the three polyuronate structural sequences present in alginate (Morris *et al.*, 1980*b*). To a good first approximation the relative amounts of D-mannuronate and L-guluronate present in a particular alginate sample may be estimated from the ratio of peak height to trough depth (Fig. 8). Comparison of the cd spectrum of alginate mixed

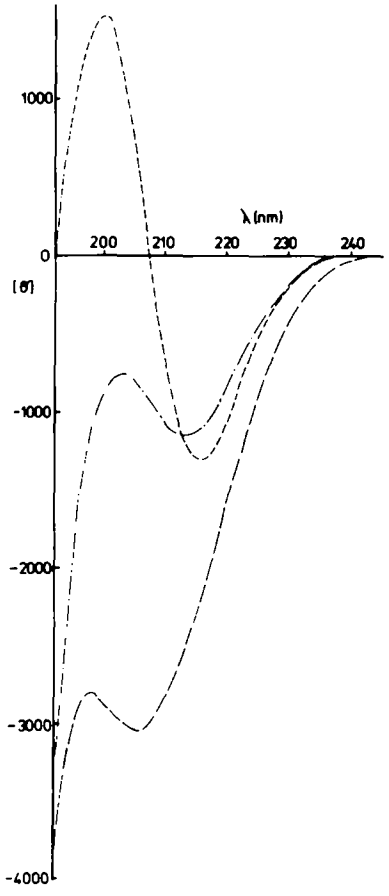

Fig. 7. Cd of alginate blocks approximating in structure to poly-L-guluronate (— · ·), poly-D-mannuronate (– – –) and mixed (– · – · –) chain sequences.

sequences with a linear combination of half the observed spectra of each of the two homopolymeric block types (Fig. 9), however, shows the sensitivity of cd behaviour to the nature of adjacent residues in the polymer chain. Matching of observed spectra by linear combination of the known spectra for the three component structural sequences, using an iterative computer curve-fitting process, thus provides a direct index of alginate block composition.

Interchain chelation of cations on calcium gelation of alginate is accompanied by large cd changes (Fig. 10) which characterise and quantify chain–chain association. Comparison of alginate gel spectra with those of solid films suggests closely similar chain geometry and packing in both cases (Morris *et al.*, 1973; Grant *et al.*, 1973; Bryce *et al.*, 1974). By contrast, calcium pectate gels show (Gidley *et al.*, 1979) large

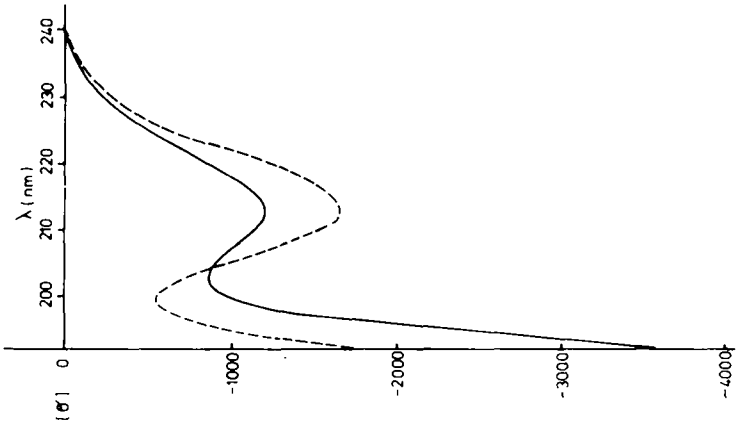

Fig. 9. Comparison of the cd behaviour of alginate mixed sequences (——) and a spectrum (– – –) synthesised by linear combination of 50 % of the spectra for each of the homopolymeric block types.

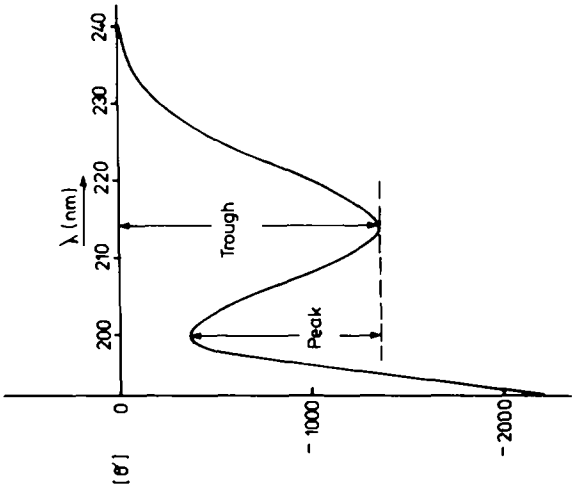

Fig. 8. Characterisation of alginate cd behaviour by the ratio of peak height to trough depth.

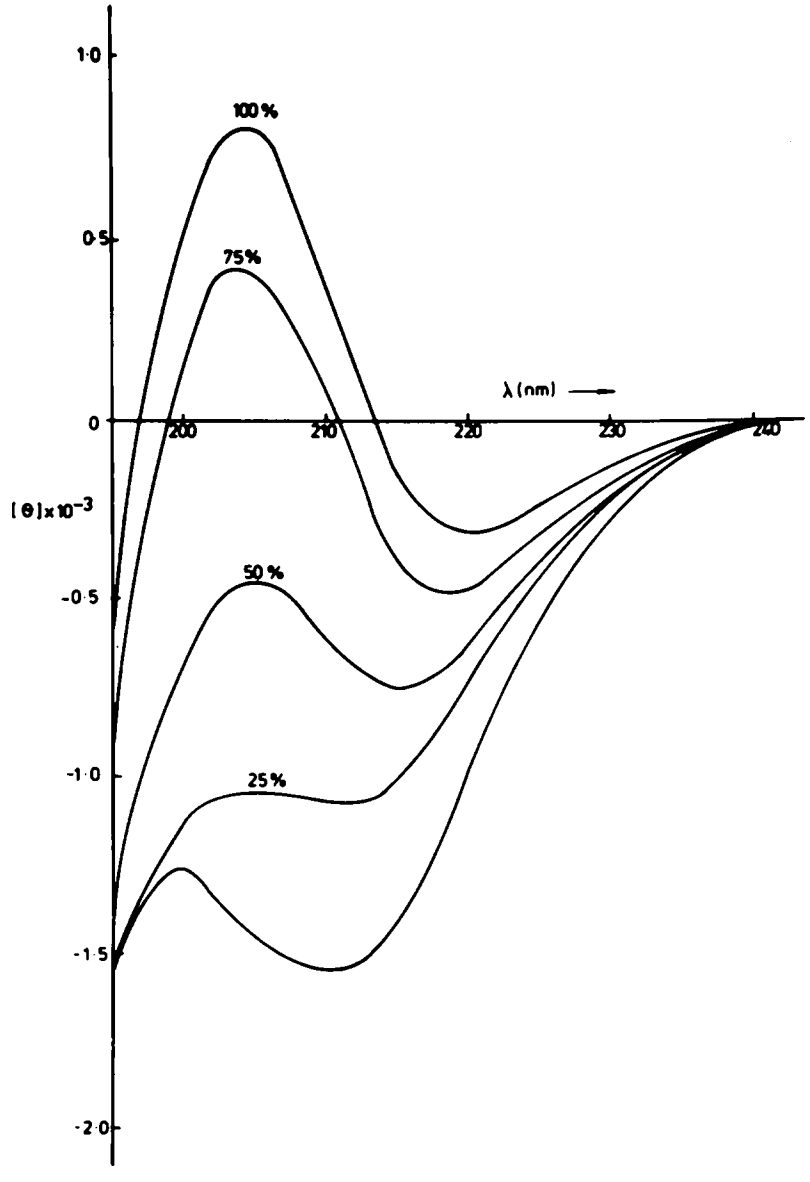

Fig. 10. Alginate cd behaviour in the presence of Ca^{++} concentrations equivalent to various percentages (as shown) of the total stoichiometric counterion requirement of the alginate.

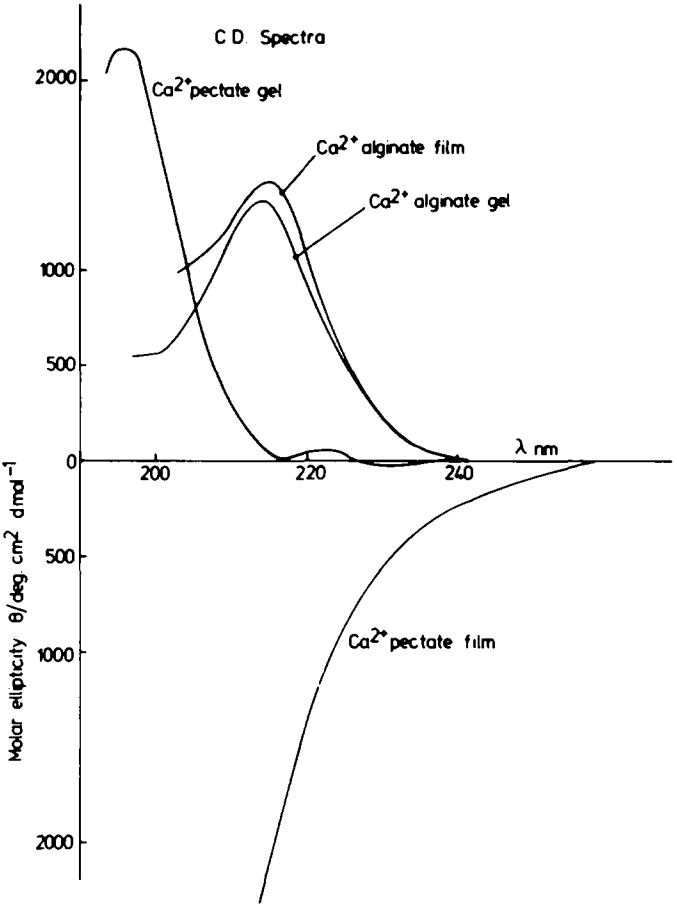

Fig. 11. Comparison of the cd behaviour of calcium alginate and pectate in the gel state, and as solid films.

changes in cd behaviour on drying down to the solid state (see Fig. 11). This is consistent with x-ray diffraction evidence of threefold chain symmetry in the solid state, and evidence from cation binding studies (discussed later) of twofold chain geometry in gel junction zones.

While carboxy groups and other accessible chromophores may, as shown, be of considerable value in probing local geometry, they also obscure the conformation-sensitive optical activity of the polymer backbone transitions in the vacuum ultraviolet. These transitions may be 'unmasked', however, by the combined use of cd and optical rotation. Both forms of optical activity have their origin in the same electronic transitions and are related in a well-defined and predictable way, as shown in Fig. 12. The contribution to overall optical rotation behaviour from accessible

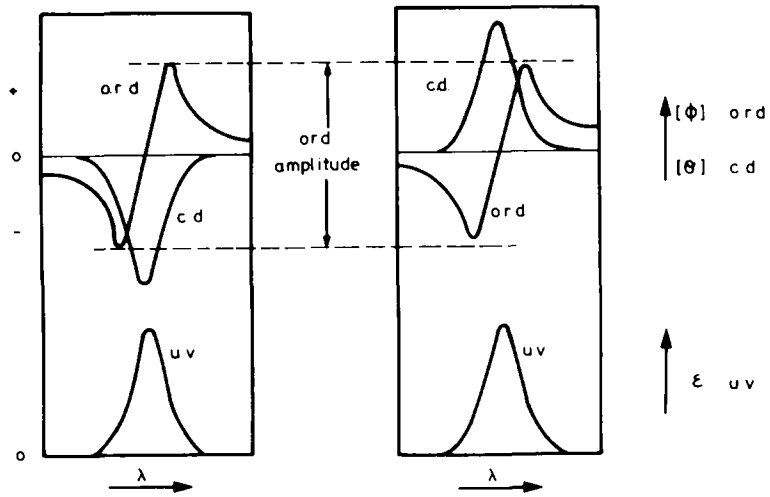

Fig. 12. Uv, cd and optical rotation (ord) spectra for a single electronic transition. Left: laevorotatory molecule—negative cd, Right: dextrorotatory molecule—positive cd. Ord amplitude = $1{\cdot}22[\theta]_{max}$

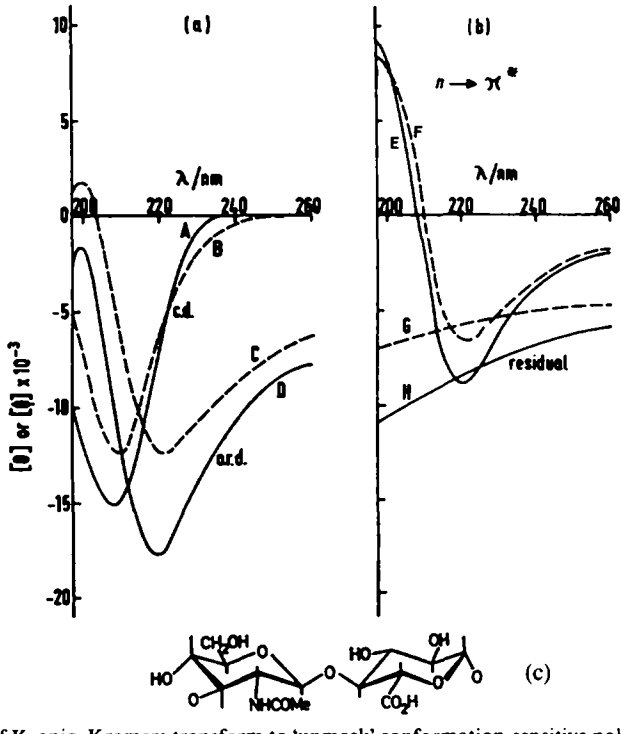

Fig. 13. Use of Kronig–Kramers transform to 'unmask' conformation-sensitive polysaccharide backbone transitions in the presence of accessible chromophores. Use of ord behaviour (curves C and D) to monitor for possible changes in conformation of hyaluronate between neutral (——) and acid (- - -) pH is complicated by contributions to overall optical activity from transitions of the carboxy and *N*-acetyl chromophores. Kronig–Kramers transform of the observed cd spectra (A and B) of these chromophores gives their ord spectra (E and F) which may be subtracted from net optical rotation values to show the optical activity (G and H) of deeper lying transitions.

chromophores may be derived quantitatively by the Kronig–Kramers transform of their observed cd spectrum, thus allowing the optical activity of deeper lying transitions to be determined by subtraction, as illustrated in Fig. 13 (Balazs *et al.*, 1977).

DISORDER–ORDER TRANSITION KINETICS

The dynamics of conformational transitions may be studied by applying a sudden perturbation which shifts the equilibrium state of the system from one conformation to another, and following the rate of conformational change (Morris *et al.*, 1980*b*). The nature of the perturbation used is dependent on the particular system under investigation, but rapid changes in temperature ('*T*-jump') or ionic environment ('salt-jump') are most commonly used. Figure 14 illustrates the principle of the salt-jump approach taking the disorder–order transition of xanthan as an example. Since, as shown in Fig. 5, the ordered conformation is stabilised to progressively higher temperatures on increasing ionic strength (presumably by screening of segment–segment electrostatic repulsions), conformational ordering may be induced isothermally by rapid mixing of salt solution with a solution of the polysaccharide in the low-salt disordered state, and the transition kinetics monitored.

In principle any conformation-sensitive physical technique may be used to follow transition dynamics, but optical activity has proved particularly suitable for

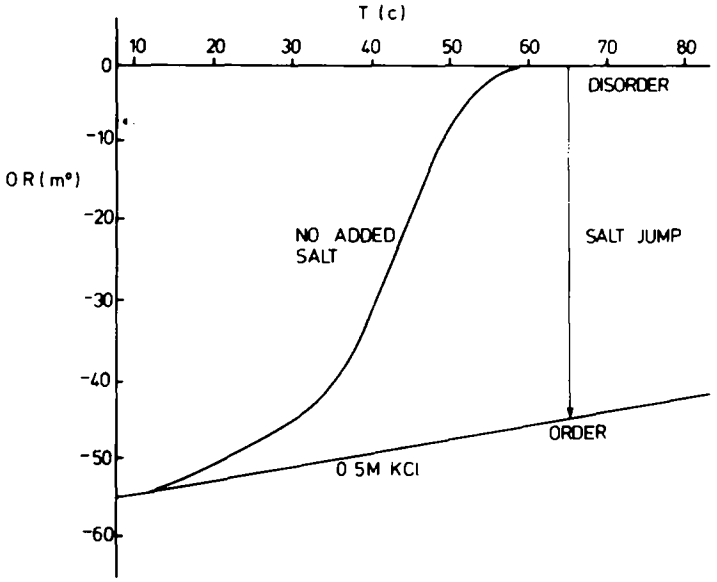

Fig. 14. Salt-induced disorder–order behaviour of xanthan.

following the very rapid kinetics (typically < 1 s) of conformational change in polysaccharide systems (Norton *et al.*, 1978). Experimentally this may be done using a polarimetric stopped-flow apparatus, in which polysaccharide and salt solutions are held in separate syringes, which are discharged simultaneously through a high-speed mixer into a flow-through polarimeter cell. The mechanism of conformational ordering may then be explored by analysis of the kinetics of optical rotation change after cessation of flow.

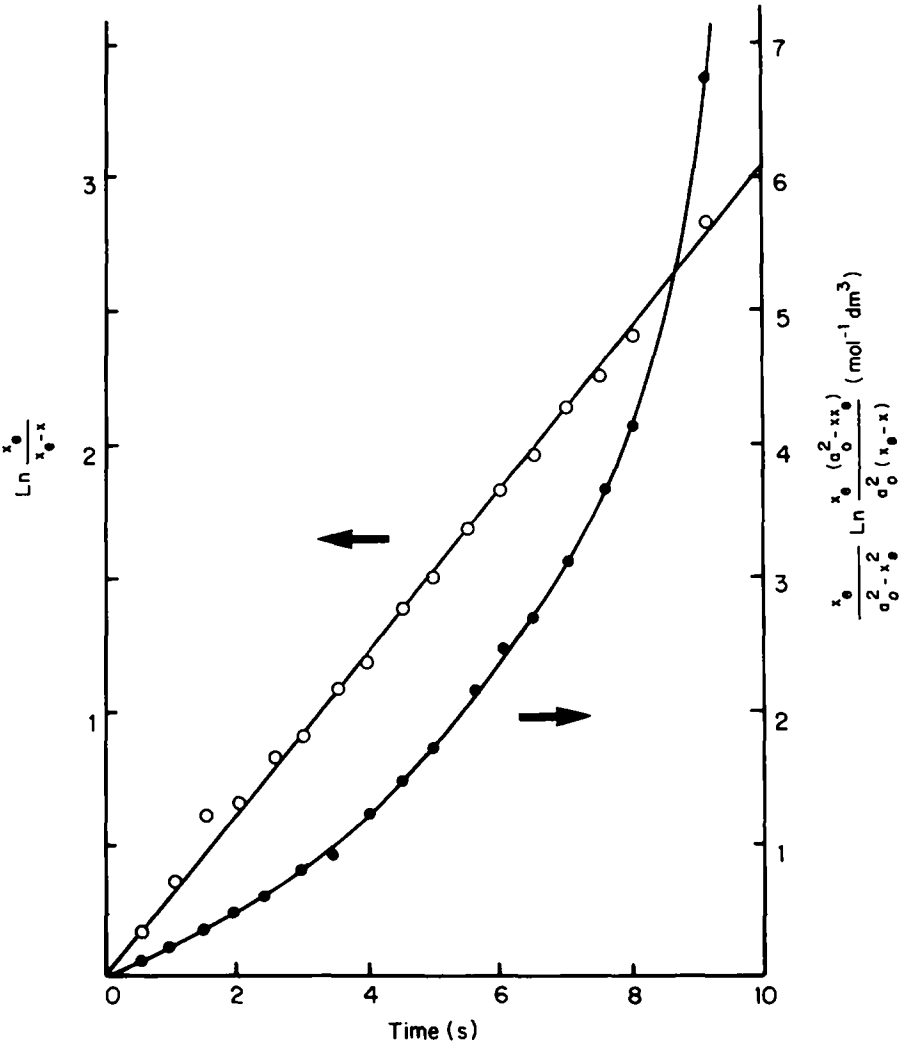

Fig. 15. Dynamics of the xanthan disorder–order transition analysed in terms of first-order (○) and second-order (●) kinetics.

Transition kinetics have proved especially useful in the elucidation of the number of chains involved in polysaccharide ordered structures. In the particular case of xanthan, both double and single helical models have been proposed and x-ray diffraction evidence, while favouring the single-stranded model, is not conclusive. Figure 15 shows analysis of the kinetics of the salt-induced xanthan disorder–order transition in terms of the two reaction schemes:

$$\text{coil} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{single helix} \tag{1}$$

and

$$\text{two coil} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{double helix} \tag{2}$$

using respectively the following rate equations.

$$\ln \frac{x_e}{x_e - x} = (k_1 + k_{-1})t \tag{1}$$

$$\frac{x_e}{a_0^2 - x_0^2} \ln \frac{x_e(a_0^2 - xx_e)}{a_0^2(x_e - x)} = k_1 t \tag{2}$$

where a_0 is the total residue-repeat concentration and x and x_e are the residue-repeat concentrations in the helix form at time t and at equilibrium, respectively.

Data plotted according to the second-order treatment (eqn. (2)) show marked departure from linearity, but the experimental results are in good agreement with the first-order kinetic scheme (eqn. (1)), consistent with single-stranded order, stabilised by side chain–main chain interactions as characterised in the solid state. The dynamics of conformational ordering in iota and kappa carrageenan have also recently been studied by polarimetric stopped-flow, and both show second-order kinetics, consistent with previous evidence from other techniques of double-helical order (Norton *et al.*, 1978, 1979).

LIGHT SCATTERING

In addition to its familiar role as a primary method for molecular weight determination, light scattering has also proved useful in investigation of polysaccharide ordered structures, in particular by comparison of the light scattering behaviour of structurally regular chain segments in their disordered and ordered conformational states. The polysaccharide system which has been most extensively studied in this way is iota carrageenan. Optical rotation and nmr relaxation evidence show that the extent of conformational ordering is largely independent of the counterion, although bulk properties show wide variations, from

mobile solutions in the presence of large organic cations such as $(CH_3)_4N^+$, through viscoelastic pastes in the presence of Li^+ or Na^+, to rigid gels with K^+ or Rb^+, and somewhat weaker gels with Cs^+ and NH_4^+. Light scattering shows exact doubling of molecular weight for iota carrageenan segments in the $(CH_3)_4N^+$ salt form on cooling through the disorder–order transition. In the presence of Na^+ ions, the measured molecular weight in the low-temperature ordered state is somewhat more ($\sim$2·2) than twice that in the high-temperature disordered conformation, while in the presence of K^+ five- or sixfold molecular weight increase is observed (Jones *et al.*, 1973; Morris *et al.*, 1980*a*).

For unsegmented iota carrageenan, where each chain contains several structurally regular sequences capable of participation in intermolecular double helix formation, the disorder–order transition in the $(CH_3)_4N^+$ salt form is accompanied by an approximately tenfold molecular weight increase. These results have contributed to the development of a 'domain' model for carrageenan gelation. In terms of this model, the primary gelation event involves association of chains through double helical junction zones, into small soluble clusters (domains) of limited extent, while development of a continuous gel network requires further association of these domains by helix–helix aggregation, which is promoted by K^+ and the larger Group I metal ions, and completely abolished in the presence of $(CH_3)_4N^+$ as sole counterion, with Li^+ and Na^+ showing only limited effectiveness in promoting aggregation. The domain model removes the serious topological problems associated with the formation of a cohesive network solely through double helical crosslinks (Morris *et al.*, 1980*a*).

DIFFERENTIAL SCANNING CALORIMETRY

Order–disorder processes in polysaccharide systems are frequently accompanied by appreciable enthalpy changes (Reid *et al.*, 1974). In the case of thermally induced transitions, such as those of xanthan, carrageenan and agarose, differential scanning calorimetry (DSC) may be used to characterise the temperature course of the transition. This technique has provided valuable additional insight (Morris *et al.*, 1980*c*) into the mechanism of carrageenan gelation. Figure 16 shows the temperature course of the order–disorder transition of iota carrageenan segments in the non-aggregating $(CH_3)_4N^+$ salt form, as monitored by both optical rotation and DSC. For both techniques heating and cooling scans are closely superimposable, consistent with a simple two state all or none transition mechanism. In the presence of K^+ ions, however, DSC results (Fig. 17) show clear evidence of two distinct molecular processes separated in their transition midpoints by approximately 15 °C. The lower temperature event shows no hysteresis and follows the same temperature course as was observed for the $(CH_3)_4N^+$ salt form, while the higher temperature

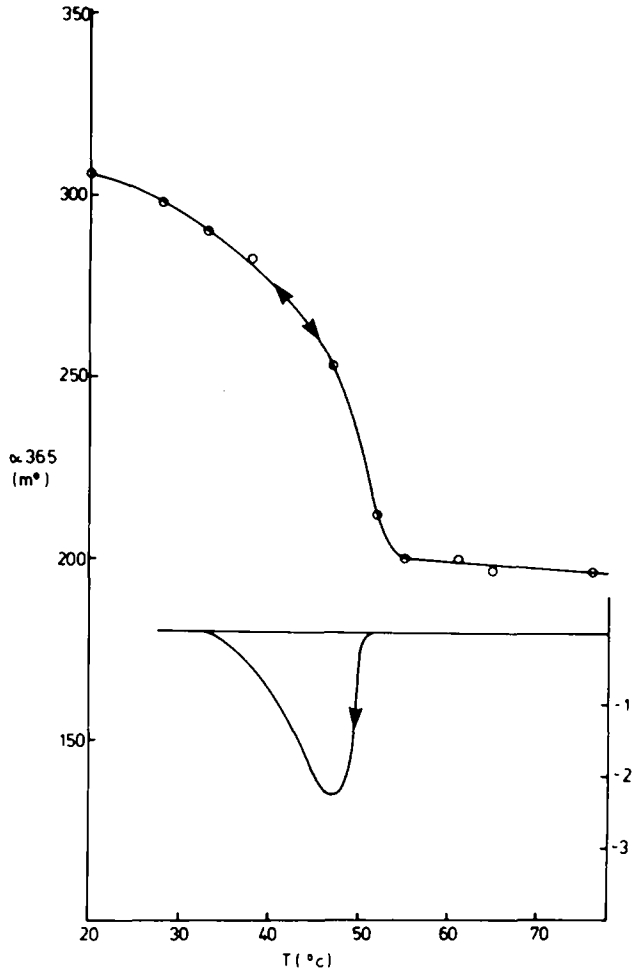

Fig. 16. Order–disorder behaviour of iota carrageenan in the $(CH_3)_4N^+$ salt form, as monitored by optical rotation (upper curve) and DSC (lower curve).

process exhibits detectable thermal hysteresis, indicative of stabilisation of the ordered conformation by aggregation. The temperature course of this second process is close to that observed for conformational ordering in kappa carrageenan, which occurs only in the presence of cations (such as K^+) which have been shown by light scattering to promote helix–helix aggregation in iota. It therefore appears that the two thermal events observed in the characterisation of the order–disorder behaviour of K^+ iota carrageenan by DSC correspond to coil-isolated double helix and coil-aggregated helix, respectively.

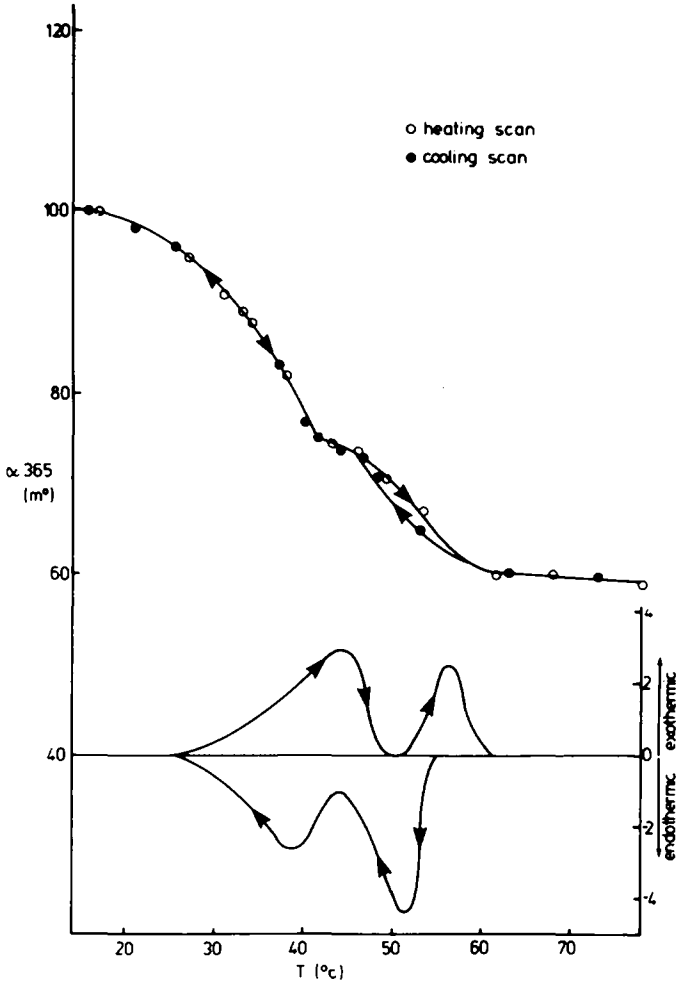

Fig. 17. Order–disorder behaviour of iota carrageenan in the K^+ salt form. Indications from optical rotation (upper curve) of two separate processes are confirmed by DSC (lower curves).

STOICHIOMETRY OF CATION BINDING

The principle of this approach to the characterisation of ordered polysaccharide structures is illustrated schematically in Fig. 18 for the particular case of alginate. It is clear from other evidence that the principal mode of intermolecular association in calcium alginate gels involves cation chelation by poly-L-guluronate chain sequences. (Smidsrød, 1974). X-ray fibre diffraction studies (Atkins *et al.*, 1973; Mackie, 1971) show a buckled twofold conformation for poly-L-guluronic acid,

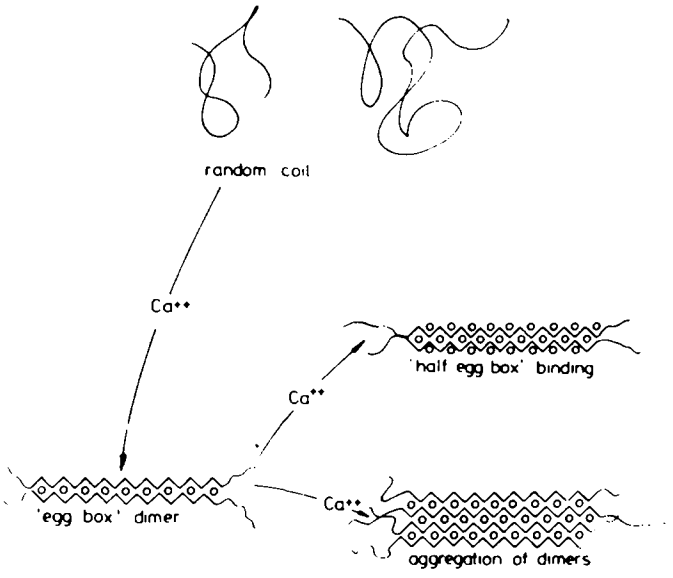

Fig. 18. Schematic representation of calcium ion chelation to alginate poly-L-guluronate sequences. It is evident from inspection of this scheme that dimerisation involves co-operative 'egg-box' binding of calcium ions to only the interior faces of the participating chains (50 % stoichiometric requirement) while, on more extensive aggregation, this ratio will tend towards 100 %. The level of bound calcium might also be raised by non-co-operative 'half egg-box' binding to exterior faces of dimers.

which appears to persist in all of the salt forms so far studied. In this favoured twofold conformation, polyguluronate chains display (Grant *et al.*, 1973) a regular array of electronegative cavities, whose size and geometry appear to be compatible with chelation of calcium. Hence, calcium binding has been interpreted in terms of an 'egg-box' model, with specific site binding of cations between long, structurally and sterically regular polyguluronate chain sequences.

In terms of this model we can envisage 'egg-box' junctions that involve only two chains at one extreme and, at the other extreme, tend to infinite sheets of calcium polyguluronate. As shown in Fig. 18, the ratio of uronate residues to co-operatively bound calcium for 'infinite sheet' junctions is 2:1 (stoichiometric equivalence), whereas for chain–chain dimers the corresponding ratio would be 4:1 (half the stoichiometric calcium level). Thus, in principle, stoichiometry of cation binding offers a route to the determination of the average number of chains in each polyguluronate junction zone. To measure co-operative interchain chelation in isolation from less specific electrostatic effects, calcium binding was studied (Morris *et al.*, 1978) in the presence of various concentrations of sodium ions, using an equilibrium technique in which solutions of sodium alginate were dialysed against mixed solutions of sodium and calcium chloride. As shown in Fig. 19, the level of bound calcium (i.e. calcium ions not available for equilibration across the

membrane) decreased rapidly with increasing Na^+ concentration up to Na^+:Ca^{++} ratios of about 30:1, above which no further decrease was observed up to sodium chloride saturation.

The concentration of bound calcium resistant to displacement by swamping concentrations of sodium corresponded closely to half the stoichiometric cation requirement of the poly-L-guluronate sequences of the alginate samples studied.

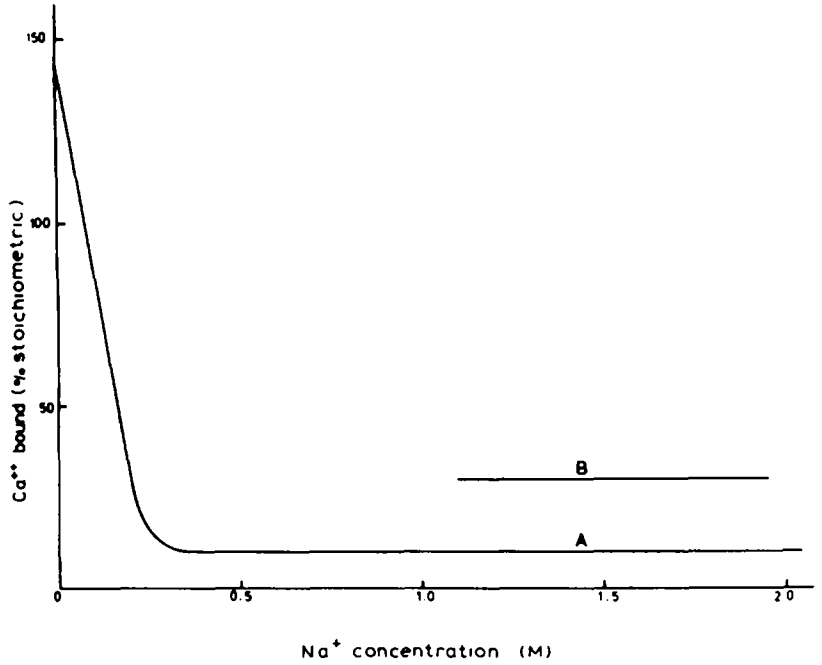

Fig. 19. Equilibrium dialysis investigation of Ca^{++} chelation by alginate chains. The level of bound calcium (expressed as a fraction of the total stoichiometric requirement of the alginate) decreases with increasing concentration of sodium ions, as shown. For both alginate A (20·7% polyguluronate) and alginate B (58·6% polyguluronate), the concentration of bound calcium resistant to displacement at higher levels of sodium is equivalent to half the stoichiometric requirement of the polyguluronate chain sequences alone, consistent with preferential dimerisation of these sequences (see Fig. 18).

These results therefore indicate very strong preferential chelation of calcium ions between pairs of polyguluronate chain segments to form dimeric junction zones rather than larger aggregates. Closely similar results have recently been obtained for poly-D-galacturonate segments from pectin, indicating that calcium pectate gelation also involves dimerisation of chain sequences in a twofold conformation, in contrast to the threefold conformation characterised in the solid state. This interpretation is consistent with circular dichroism evidence (see above) of a large change in chain geometry on drying down calcium pectate gels to solid films (Gidley *et al.*, 1979).

COMPETITIVE INHIBITION

Competitive inhibition is widely used to explore and characterise specific binding events in biochemical systems. Familiar examples are in studies of antibody–antigen, lectin–hapten and enzyme–substrate recognition. The technique has recently been applied to polysaccharide gels and appears to offer a powerful general method for the characterisation of chain–chain interactions (Morris *et al.*, 1980*d*). As illustrated in Fig. 20, the formation of a cohesive gel structure may be inhibited by the presence of molecular species, such as short chain segments, which are capable of participation in only one stable intermolecular junction zone, and thus occupy binding sites on other chains without contributing to the network.

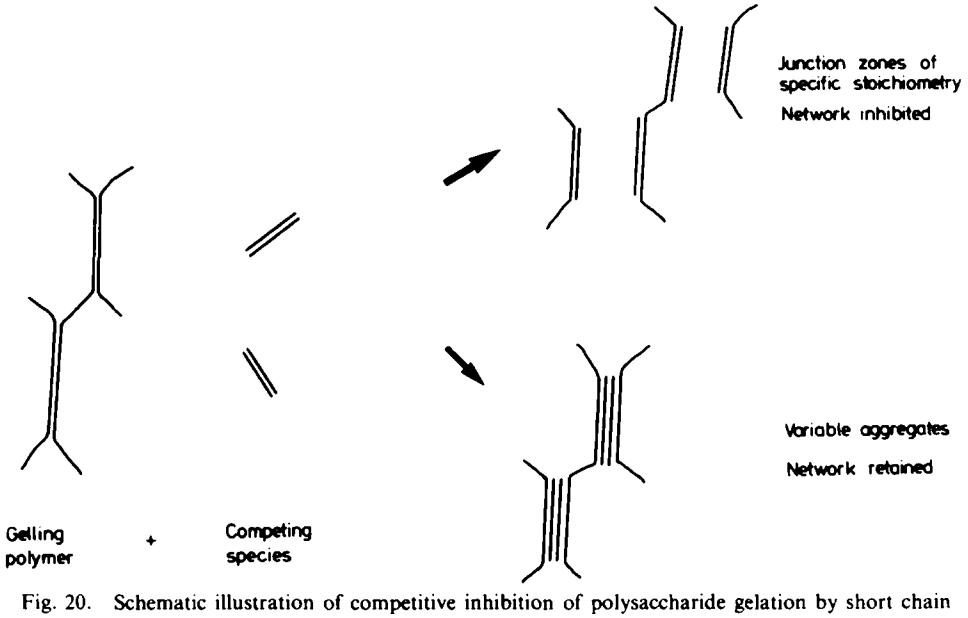

Fig. 20. Schematic illustration of competitive inhibition of polysaccharide gelation by short chain segments.

Thus, calcium alginate gels are weakened drastically by the presence of poly-L-guluronate chain segments. Addition of poly-D-mannuronate or mixed blocks, however, has relatively little effect on gel strength, consistent with other evidence that association of these sequences makes little contribution to the gel network.

Addition of iota carrageenan segments to gelling concentrations of the intact polymer also inhibits network formation, but the effect is progressively decreased by the addition of cations (such as K^+) which promote helix–helix aggregation until, at very high K^+ concentrations, inhibition is abolished completely. Similarly, kappa carrageenan gels, where aggregation is even more pronounced, show no inhibition

with homologous chain segments. A likely interpretation of this behaviour is that, as indicated in Fig. 20, short segments may be incorporated within large aggregates of variable stoichiometry without displacing other chains. It therefore appears that the competitive inhibition approach represents a powerful general method for the characterisation of intermolecular junction zones, in terms of both the nature and number of participating chain segments.

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THE FORMATION AND STRUCTURE OF MILK PROTEIN GELS

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ABSTRACT

The formation of gels from milk proteins is irreversible. Most are not suitable for optical or rheological approaches.

Various gels from milk products are discussed: those made by the action of rennet on whole milk, yoghurt, those from heated milk and by the storage of heated milk and gels made from whey protein of very high nutritional quality.

INTRODUCTION

Gels formed from milk proteins are involved in the manufacture of certain dairy and non-dairy foods. Their formation is irreversible, in contrast to most other food gels. Those formed from casein, such as junket and yoghurt, are soft and brittle, with a tendency to synerise. They are also opaque. This means that most milk gels are not amenable to study by the optical and rheological methods used for many gels. However, the size and composition of the natural particles make them ideal for electron microscopy and this technique, together with empirical rheological measurements, has proved useful.

The requirements for these gels to form and the chemical mechanisms by which the characteristic structures are produced will be reviewed. One justification for these studies is the assumption that the structure must determine the gross textural properties. The nature of this connection is rather unclear in most instances, but will be discussed as far as information is available.

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FORMATION OF GELS FROM WHOLE MILK BY RENNET ACTION

The first stage of cheesemaking is the gelation of whole milk by treatment with an enzymic coagulant. This is normally rennet, an extract from calf stomachs containing chymosin (EC 3.4.23.4) and bovine pepsin (Garnot *et al.*, 1972). Subsequently, the water and water-soluble components (whey) are largely removed, but the fat is retained in the curd. The final curd structure consists of areas of fat in a continuous protein matrix (Hall & Creamer, 1972) and is probably crucial to the development of the traditional flavour and texture during maturation of the cheese.

Enzymic action of rennet

The coagulation of milk by rennet comprises an enzymic phase, followed by aggregation. First, κ-casein, which constitutes about 12·5% of the total casein in milk (Davies & Law, 1977), is hydrolysed at a single peptide bond (Mackinlay & Wake, 1971). A highly acidic macropeptide, containing phosphorus and a variable amount of carbohydrate and constituting about 30% of the κ-casein monomer, is released, leaving a basic, hydrophobic moiety, *para*-κ-casein (Mercier *et al.*, 1973).

Almost all the casein in milk is present in micelles. These are roughly spherical, highly hydrated particles (Bloomfield & Morr, 1973) of about 120 nm average diameter (Schmidt *et al.*, 1973), and containing about 2% calcium phosphate-citrate in addition to casein (Boulet *et al.*, 1970). Rennet did not appear to affect the structure of these, as viewed in electron micrographs, before aggregation occurred (Green *et al.*, 1978*a*). However, it did reduce the electrophoretic mobility of the micelles, indicating that the surface negative charge was approximately halved (Green & Crutchfield, 1971; Pearce, 1976). This can be explained by the conversion of κ-casein to *para*-κ-casein.

Aggregation phase

The aggregation of micelles could be followed by viscometry up to the visually observed clotting time and also by electron microscopy. The latter was a particularly useful technique for this study. The milk sample was treated with rennet for the required time and then fixed with glutaraldehyde, which blocked further aggregation. Then it was solidified in agar, sectioned and examined (Green *et al.*, 1978*a*). Pairs of micrographs taken at 90° to each other showed that the structure of the sample was the same in all directions (Green *et al.*, 1978*a*). Thus, the gel was of the non-oriented, network type.

The increase in viscosity of rennet-treated milks can be considered as a measure of the aggregation of the casein micelles. This process did not start until the enzymic reaction was almost complete (Fig. 1), at close to 60% of the visually observed clotting time in normal milk at 30°C (Green *et al.*, 1978*b*). Observations of the electron micrographs showed that the gel structure was built up steadily after 60% of the visual clotting time. Small chains or groups formed first and began to be

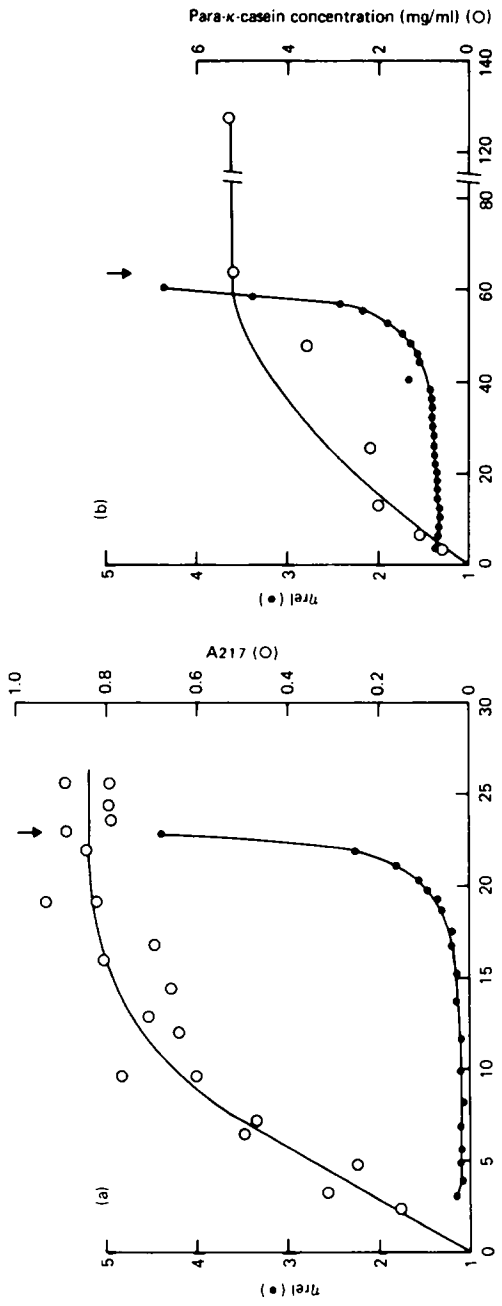

Fig. 1. Time courses of viscosity of rennet-treated skim-milk (●) and the enzymic action of rennet (○) at 30°C. Viscosity determinations and measurements of the enzymic phase by the release of 12% (w/v) trichloroacetic acid-soluble peptides (A_{217}) were made as described previously (Green *et al.*, 1978*b*). *para*-κ-casein concentrations were determined by polyacrylamide gel electrophoresis using a purified sample of *para*-κ-casein as standard.

assembled into a network at the visual clotting time. Eventually, the network consisted of loose strands about five micelles thick separated by wide spaces. The loose packing of the micelles enabled much serum to be trapped and some small groups were not bound to the network.

Quantitative information can also be obtained from the electron micrographs. The size distribution of the aggregates at each time gave the average degree of aggregation. This was proportional to time between 60 and 100% of the visual clotting time, but increased more slowly at later times (Fig. 2). This was consistent with a Smoluchowski mechanism (Overbeek, 1952), in which aggregation depended on the collision of randomly diffusing aggregates, rather than on their chemical reactivities. The aggregation rate constant was apparently much less than expected for the number of micelles present in milk, as also found from measurements of turbidity (Payens, 1978). However, the rate of micellar aggregation is proportional to rennet concentration, even long after κ-casein hydrolysis is complete (Hansson *et al.*, 1949; Claesson & Claesson, 1970; Kirchmeier, 1972; Olson & Bottazzi, 1977; Payens, 1977; Green *et al.*, 1978*b*). This implies that the rate at which the micelles aggregate is somehow predestined early in the coagulation process.

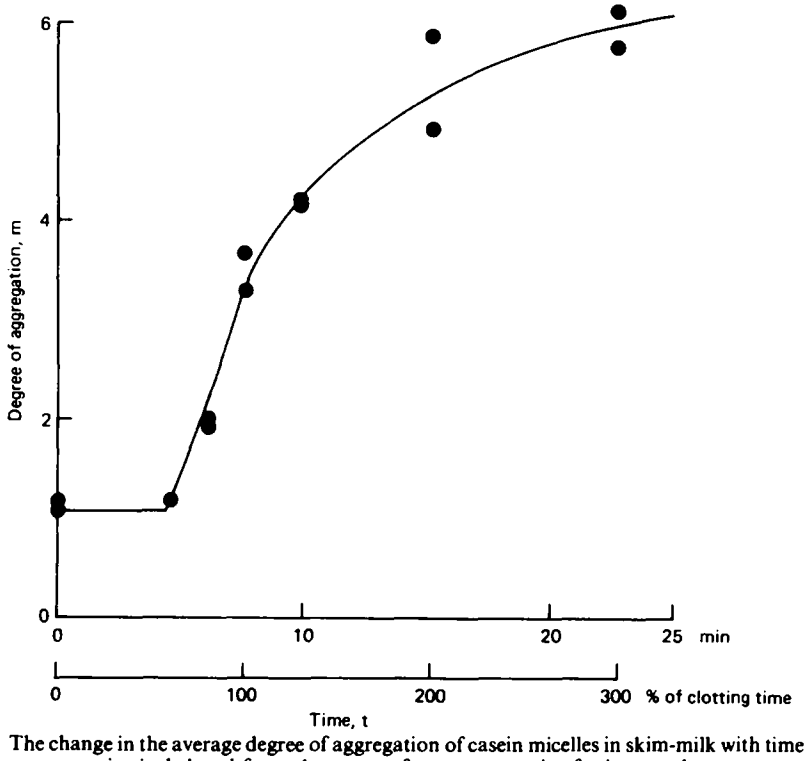

Fig. 2. The change in the average degree of aggregation of casein micelles in skim-milk with time. Each point is deduced from the count of one stereo pair of micrographs.

As aggregation does not begin until most of the κ-casein is hydrolysed (Green *et al.*, 1978*b*), it has been suggested (Payens, 1977; Green & Marshall, 1977) that rennet-treated micelles interact only at defined sites on the surface. Quite specific reactions may be involved. For example, modification of about 30% of the lysyl or arginyl residues of κ-casein prevents its self-aggregation after hydrolysis to *para*-κ-casein (Hill & Craker, 1968; Hill, 1970).

Curd firming

The rate of aggregation of the micelles probably also affects the structure of the gel. The technologically important properties, the fat- and water-holding capacities, depend on the conditions under which the gel is formed. This probably reflects variations in structure. However, these relationships are only beginning to be investigated.

The structure of the gel can be described by the average size of the pores and strands in it, which can be deduced from the micrographs by a standard stereological method (Underwood, 1969). However, this only describes the gel at a particular point in time. This is because the gel formed by adding rennet to milk does not reach a final rigidity, but continues getting firmer over several hours, by which time considerable syneresis has also occurred. Firming appears to involve the formation of more linkages between micelles, so as to form a stronger network, and also fusion between micelles (Kimber *et al.*, 1974). It is easy to see how this leads to shrinkage of the curd. In turn, this causes squeezing out of the whey and distortion of the fat globules, which contain semi-liquid fat under most cheesemaking conditions, and rupture of their membranes. Cheesemaking technology is probably concerned simply with controlling the extent and direction of these processes and gearing them to fermentation, so that the final green cheese has the correct composition and structure.

Recently, D. S. Hatfield of NIRD has developed an instrument based on one produced at CSIRO (Vanderheiden, 1976). A diaphragm, placed in the milk, oscillates at low frequency and the displacement induced in a receiver is monitored. This should measure the rigidity of the gel. We are also testing a commercial probe viscometer, which measures the damping by the sample of a high frequency vibration, induced by slight changes in the length of the probe. The outputs of both instruments start to rise before the visually observed clotting time and continue to rise at rates which follow first order kinetics. It remains to compare them quantitatively.

YOGHURT GELS

Yoghurt is formed by the acid fermentation of milk. Its important textural characteristics are its firmness and ability to retain water, which are determined by

the structure of the gel (Knoop & Peters, 1975; Kalab *et al.*, 1976; Davies *et al.*, 1978).

The addition of acid to casein micelles causes progressive solubilisation of the calcium phosphate–citrate (Pyne, 1962) and aggregation of the casein at the approach to the isoelectric point. In milk this is associated with some disruption of the micelles, which is greater if the pH falls rapidly and if there is agitation. In yoghurt, lactose is fermented to lactic acid, causing the pH to drop to about 4 in 4–5 h. During this process, the micelles group together and partly coalesce (Kalab *et al.*, 1976).

The final product was firmer, with less tendency to syneresis, if the milk was pre-heated to about 90 °C for 10 min. This caused the appearance of appendages on the casein micelles (Davies *et al.*, 1978). Their formation was dependent on the presence of β-lactoglobulin, but was inhibited by sulphydryl blocking agents. Thus, they consisted of denatured β-lactoglobulin, linked by disulphide bridges (Davies *et al.*, 1978), presumably to the κ-casein of the micelles.

These partially protected the micelles against disruption on acidification, so that there was less coalescence (Davies *et al.*, 1978). Thus, the gel network consisted of thinner strands, making up a smaller mesh, than those obtained from unmodified milk. This finer network presumably gives a firmer gel with greater water-holding capacity.

GELS FROM HEATED MILK

Heated milk gels, prepared by heating concentrated suspensions of dried non-fat milk in aqueous salt solutions, could form a base for new products. The firmness of the gels, measured by means of a penetrometer, correlated closely with the ultrastructure, observed in electron micrographs (Kalab & Harwalkar, 1974). A gel containing 17 % protein was little firmer than one containing 14 % protein, and both contained casein micelles as individual entities linked by bridging material, possibly denatured β-lactoglobulin. On the other hand, a gel containing 20 % protein was 4·2 times firmer than one containing 17 % protein and the structure was very different. In the 20 % protein gel, most of the micelles were fused together to form chains or clusters. Further, the presence of $CaCl_2$ in a 17 % protein gel resulted in some fusion of the micelles and a doubling of the firmness compared with the control without added salt. Conversely, the presence of sodium hexametaphosphate in a 17 % protein gel caused some disruption of the micelles and a reduction in firmness to 40 % of the control value.

GELATION ON STORAGE OF HEATED MILKS

Gel formation in milk products can also be disadvantageous. It tends to occur on prolonged storage of sterilised milk concentrates (evaporated milks) and ultra-high-temperature (UHT)-treated milks and is a major factor limiting the shelf-life of these

products. It is characterised by coalescence of the casein micelles into larger particles (Andrews *et al.*, 1977; Harwalkar & Vreeman, 1978*a*), which become linked by bridges. There tended to be some proteolysis (Harwalkar & Vreeman, 1978*b*) and also protein polymerisation, probably resulting from Maillard-type reactions and the formation of disulphide bonds (Andrews, 1975). However, it is not clear exactly what chemical changes are responsible for destabilisation of the casein micelles and gelation.

WHEY PROTEIN GELS

Proteins can be isolated from whey, a by-product of cheese manufacture, in a nearly pure form. They are of very high nutritional quality and are most effectively used as an ingredient of human food. The ability of the concentrates to form stable gels on heating may allow their use in non-milk foods, such as meat products.

R. M. Hillier, while working at NIRD, studied a number of whey protein concentrates isolated by adsorption to carboxymethylcellulose at pH 4 and elution at pH 9. Different isolates had different gelling capacities, but all produced gels at concentrations of 8–12% protein in water at 60–90 °C. The gel structure broke down above 95°, but reversibly, so that a gel reformed on cooling.

In water, the gelation only occurred at above pH 7. At pH 4–7 there was very

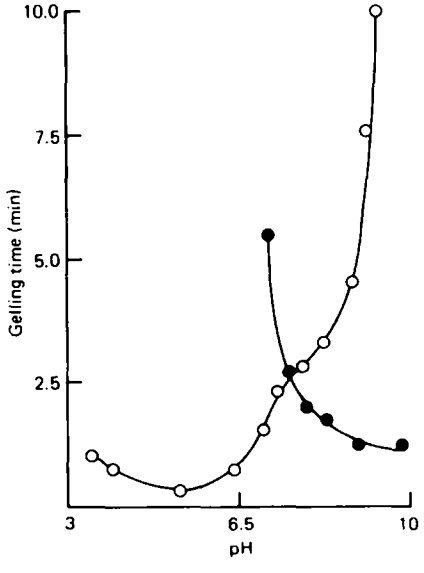

Fig. 3. The effect of pH on the gelation of whey protein powders in water (○) and 0·1 M phosphate buffer (●). Gelling time is that required to coagulate or gel 1 ml of 10% (w/v) protein solution at 80 °C. Different powders were used for the two curves.

rapid coagulation, probably because of the proximity to the isoelectric point of β-lactoglobulin at pH 5·2 (Fig. 3). When the electrostatic effects were masked by the presence of 0·1M phosphate buffers, the rate of gelation increased with pH in the range pH 7–9 (Fig. 3). This suggests that gelation may be accelerated by the presence of ionised thiolate in the protein. In accord with this, the gelling capacity of different powders was approximately proportional to the sulphydryl content of the powder (Table 1). If the sulphydryl groups were partially reacted by titration with

TABLE 1

THE RELATIONSHIP BETWEEN THE GELLING BEHAVIOUR OF WHEY PROTEIN POWDERS, THE PROPORTION OF PROTEIN IN THE NATIVE STATE AND THE TOTAL CONTENT OF SULPHYDRYL GROUPS[a]

Nature of gel	*Gel time (min)*	*Native protein (% of total)*	*Total—SH (mM)*
Very opaque	2·5	29	1·33
	3·0	81	1·59
Opaque	2·5	48	0·72
	3·8	23	0·42
Clear	4·0	32	0·27
	14·0	15	0·24
	21·0	13	0·28
Very clear	5·8	7	0·13
	9·0	24	0·10

[a] R. M. Hiller (unpublished results).

Hg^{2+}, the gelling capacity was reduced proportionally. Further, the gel structure was disrupted by treatment with the sulphydryl compounds, dithiothreitol and 2-mercapto-ethanol, which reduces disulphide linkages, but not by urea. These results indicate that gel formation was dependent on $RS^{-}RSSR$ interchange and the gel was stabilised by disulphide linkages.

Whey powders formed two types of gel. Clear gels were elastic and held water effectively and opaque gels were brittle and leaked water rapidly. Clear gels tended to be those that were formed more slowly (Table 1). Most probably, they were those with a smaller mesh size, having a more even spacing of intermolecular linkages. They tended to be associated with a high degree of protein denaturation in the powder (Table 1). The gel structure was also affected by the salt concentration. The whey protein gel formed in 0·2M NaCl was much coarser and composed of larger aggregates than that formed in the absence of salt (Hermansson, 1977). These results raise the interesting possibility of influencing the structure—and thus the properties—of these gels by small changes in the composition of the mix.

CONCLUSION

Studies of milk protein gels seem to me to be approaching an interesting stage. We now have a reasonable idea of the mechanism of formation of most of them, and are

beginning to interrelate the conditions during gel formation, the structure and the textural properties. This should lead to the means to control more closely those properties that are important in the acceptability of foods.

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Food Chemistry 6 (1980) 51-61

THE INFLUENCE OF THE STRUCTURE OF STARCH ON ITS RHEOLOGICAL PROPERTIES

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ABSTRACT

Some of the differences between various starches are considered and the mechanisms of gel formation are described.

The requirements of many food situations are to thicken, clarify and change texture rather than to form gels—these require amylopectin starches.

The importance of stabilisation of rheological properties is emphasised. These properties can be modified by other materials such as salt, fat, sugars, proteins and gums.

INTRODUCTION

Starch is a naturally occurring polysaccharide consisting entirely of anhydroglucopyranose residues linked together in one of two ways. The most common one is the 1-4 glycosidic link which, when occurring exclusively, produces a linear chain-like molecule called amylose. The second type of linkage is the 1-6 glycosidic link which, in conjunction with 1-4 linkages, yields a branched structure called amylopectin.

These two fractions occur in differing amounts in starches from different botanical sources, as can be seen from Table 1. The molecular weights of the two fractions also vary, depending upon the source. The cereal starches generally show the lowest molecular weights and the root and tuber starches show the highest (Whistler & Paschall, 1956, p. 358).

The difference between the molecular weight distribution, abundance and molecular weight of these fractions leads to different properties. This can be seen in the shape of the granule (Knight, 1969) which is the partially-crystalline unit in which starch occurs in its native form.

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TABLE 1
AMYLOSE AND AMYLOPECTIN CONTENTS OF SOME COMMON STARCHES

Starch	*Amylose content*	*Amylopectin*
Maize	26	74
Wheat	25	75
Rice	17	83
Sorghum	26	74
Potato	24	76
Sago	27	73
Waxy maize	1	99
Waxy sorghum	1	99
Tapioca	17	83
Amylomaize	50	50
(high amylose)	75	25

In most of its applications, however, starch is used in an aqueous environment as a hydrophilic colloid.

PHYSICAL PROPERTIES

In order to render starch in the form of a sol, the granular dispersion is heated in water to a point where the birefringence of the granule is lost and water begins to enter the granule, breaking down the intermolecular hydrogen bonds. At this point the viscosity rises, as shown by the familiar Brabender profile in Fig. 1.

There are two theories as to what precisely is happening at the molecular level. Traditionally, it was believed that the water was entering the granule and causing it to swell. An alternative theory advanced by Miller *et al.* (1973) is that the granules only swell slightly but exude a material which forms an inter-granular matrix which is the cause of the viscosity increase. This theory demands that intact granules still exist at 95 °C and these workers have obtained evidence of this in wheat, waxy maize and potato starches by the use of freeze-drying techniques followed by electron microscopy.

Cereal starches are reported to undergo a two stage gelatinisation process (Whistler & Paschall, 1956, p. 297) and in our laboratories we have observed a double viscosity peak by extending the conventional Brabender hold at 90 °C for longer periods of time. This behaviour is indicative of different forces existing in the highly associated and the amorphous regions of the granule. Root and tuber starches do not show multi-stage swelling and hence are more uniformly associated.

On the traditional theory the swollen granules break down under the action of shear forces with a reduction in viscosity, leaving a colloidally dispersed phase which, on cooling, may increase in viscosity again. This increase in viscosity on

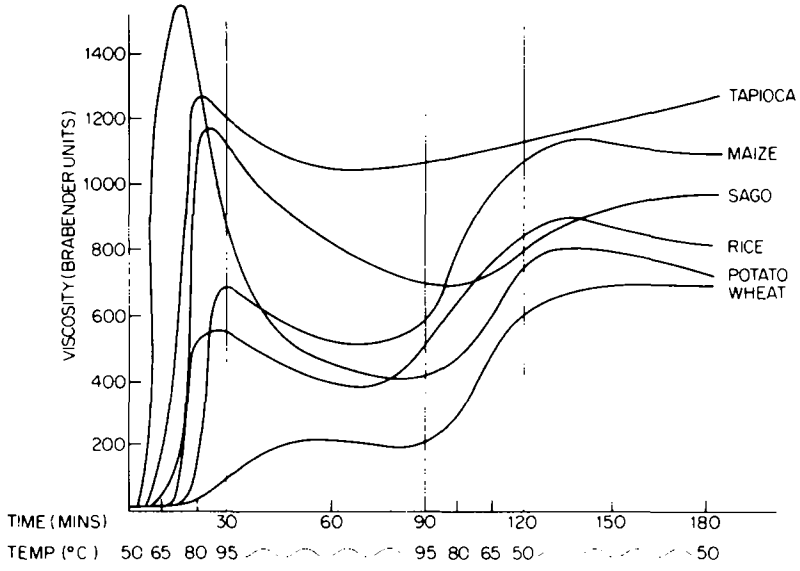

Fig. 1. Brabender viscosity profiles of natural starches.

cooling may result in a phenomenon called retrogradation. This is associated with the amylose fraction which, being linear in nature, has the ability to form junction zones and reassociate, re-establishing intermolecular hydrogen bonds. This process results in a loss of clarity and, depending on concentration, can form a gel in the accepted sense of the term. These gels are elastic but their yield values are low and they do tend to exhibit syneresis and are not stable to freeze–thaw cycling.

Amylopectin, being a branched molecule, does not have the same ability to form junction zones and therefore causes viscosity increases without leading to retrogradation. In these properties it is similar to gum arabic.

If the starch molecule has been treated with acid the branching is reduced and there is an increase in the linear nature of the starch. The number of 'molecules' available to form junction zones therefore increases and the gel strength also increases. Commercially modified starches which have been acid treated are used in the production of starch jellies in the sugar confectionery industry. In these products gels are quite rigid.

The effect of this retrogradation and its increased gel strength can be seen by studying the Brabender traces of an acid thinned starch (Fig. 2). High amylose-containing starches also exhibit these tendencies very markedly although the high gelatinisation temperatures which these starches possess make their commercial use limited.

The converse of this argument holds true for starches which have functional groups introduced into the molecule. Here the ability to form junction zones is

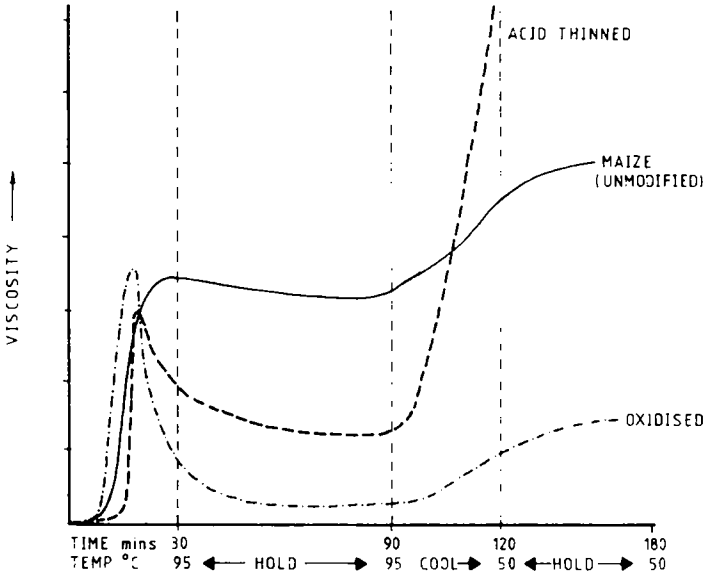

Fig. 2. Brabender profiles—Modified maize starches.

reduced. If esters and ethers are produced the effect may be totally steric in nature. On the other hand, if oxidation of the primary alcohol group at carbon atom 6 is affected the nett negative charges on the molecules resulting from the carboxylate anions may give an electrostatic repulsion which inhibits gel formation. If the degree of modification is low, however, enough uninterrupted areas will remain in the chain to allow gel formation to take place. As a result of this the gel strength is reduced and the texture of the gel is much softer. Typical of gels formed with oxidised starches are the lemon curd type preparations.

TABLE 2
PROPERTIES OF THICK BOILING STARCHES

	Maize	*Wheat*	*Waxy sorghum*	*Potato*	*Tapioca*	*Sago*
Hot paste viscosity	Medium	Medium/low	Medium/high	Very high	High	Medium/high
Type of gel						
(a) clarity	Opaque	Opaque	Clear	Clear	Clear	Moderately clear
(b) texture	Short	Short	Soft cohesive	Very cohesive	Soft	Soft
Stability to retrogradation	Poor	Poor	Good	Fair	Fair	Fair
Freeze–thaw stability	Poor	Poor	Fair	Poor	Poor	Poor
Resistance to shear	Fair	Fair	Poor	Poor	Poor	Poor

Since amylose favours gel formation, whereas amylopectin does not, the relative abundance of these materials and their molecular weight affect the gel properties of a starch. Since these parameters vary according to the botanical source of the starch it is not surprising that the paste properties of starches of different botanical sources differ from each other. These differences can be seen in Table 2.

Since the requirement in many food applications is for thickening, clarity and texture, rather than gel formation, the amylopectin starches such as waxy maize are much used (Howling, 1974). Unmodified waxy maize, however has an unsuitable texture for most applications and is not resistant in its thickening properties to the action of shear forces, low pH conditions and high temperature.

CHEMICAL MODIFICATION

In the modern food industry these conditions abound and hence there is a need to stabilise the rheological properties of these starches. This can be done by the chemical modification of starch with a bifunctional reagent, e.g. adipic acid, epichlorohydrin or phosphate groupings.

The effect of this chemical reaction is to strengthen the starch granule by augmenting the intermolecular hydrogen bonds with covalent bonds. The result of this process on the rheological properties can be seen in Fig. 3 and the increased

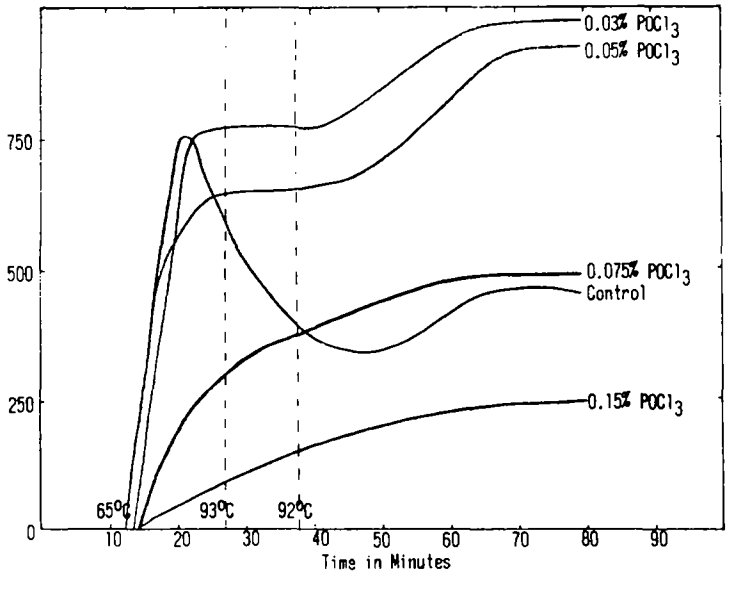

Fig. 3. Effect of cross-linking of Brabender based viscosity of waxy maize starch.

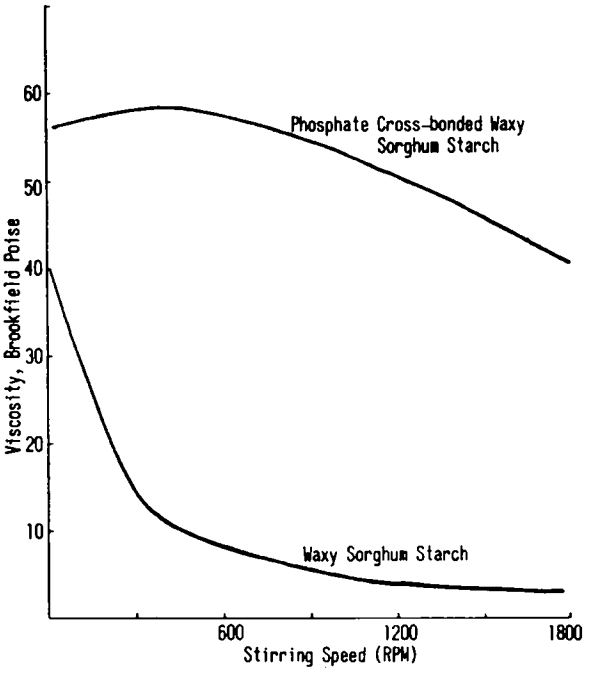

Fig. 4. Viscosity of 5% aqueous pastes versus stirring rate.

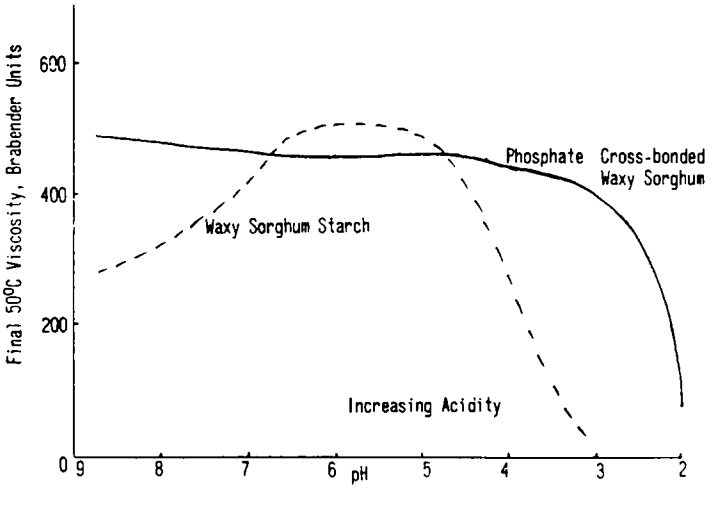

Fig. 5. Effect of acidity on final 50°C Brabender viscosity.

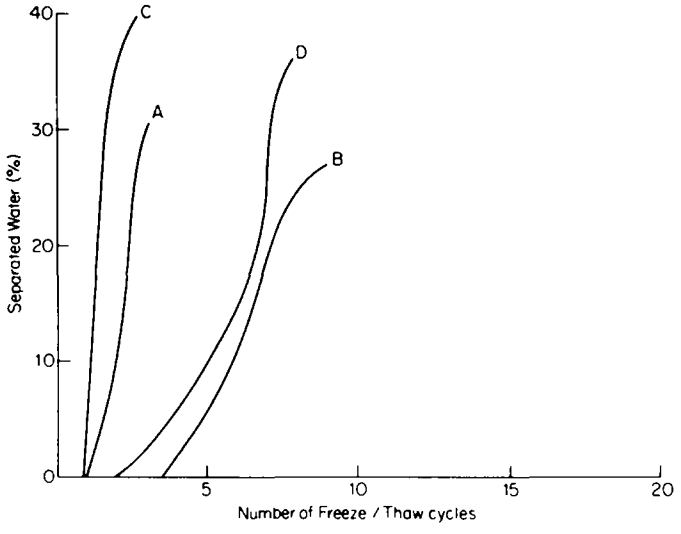

Fig. 6. Modified waxy starches—comparison of freeze–thaw stability. 5% D.C. paste in 37° Brix solution at pH 3·5. Curve A: 0·045% crossbonded waxy maize. Curve B: 0·045% crossbonded waxy maize + 5% acetylation. Curve C: 0·08% crossbonded waxy maize. Curve D: 0·085% crossbonded waxy maize + 5% acetylation.

stability of the viscosity to shear forces and low pH conditions is illustrated in Figs. 4 and 5.

The effect of cross-linking starches reduces the gel strength of the system and this can be seen by the freeze–thaw stability of the products.

This difficulty can be overcome by introducing a mono functional group to the C6 position. The benefit of this can be seen in Fig. 6.

PHYSICAL MODIFICATION

Physical modifications can be made to the starch granule which will alter its properties. Physical disruption of the granule is the basis of all these methods which include milling, spray drying, jet cooking and roll drying. The formation of pre-gelatinised starches which will re-disperse in cold water depends on the disruption of the granule to form a sol which is dried before retrogradation can set in and junction zones form between the linear fragments. These products form a clear sol again on the addition of water but the viscosity of the starch paste may have been reduced by the shear forces employed in the cooking and drying. The use of thermal conversion in jet cookers to reduce the viscosity of starch is also commercially exploited.

OTHER MOLECULES

Up to this point the properties discussed have been confined to starch and water pastes but the rheological properties of starch may be modified by other materials present in the system. These materials may be impurities in the commercially produced starch or other components of the system. A review of these properties with special reference to wheat starch has been published by Olkka & Rha (1978).

(*a*) *Salt*

The effect of salt concentration on the swelling power—and hence the properties —of starch has been known for many years and is exploited in the manufacturing process for starch modification. The presence of sodium chloride has the effect of inhibiting the swelling and subsequent breakdown of the starch granule and hence affects the gelatinisation temperature of the starch, its hot paste viscosity and resistance to shear. In these properties the function of sodium chloride has the opposite effect to that of sodium hydroxide, and in the modification of starch the balance of these two reagents is critical. The mechanism of the salt effect is not clear and calcium chloride and potassium chloride tend to have an opposite effect to that of sodium chloride (Metcalf & Gillies, 1966). The position of the electrolyte in the Hofmeister series has been correlated with their behaviour.

(*b*) *Fat*

The presence of trace amounts of fat in maize starch has a profound effect on the gel properties of this starch. If maize starch having a fat content of 0·6 % is submitted to solvent extraction with methanol to remove the last traces of fat, then the tendency to retrogradation is very much increased and the gel properties enhanced. The product also swells more freely and uniformly with little evidence of the two stage pattern of the original starch. The natural fatty acids in corn starch have the effect of inhibiting granule swelling by forming an insoluble complex with the linear fraction.

Non-molecular bound fat outside the granule tends to form a barrier to water absorption and reduces the tendency of the granule to swell. The presence of surfactants or emulsifying agents in the system can alleviate this condition (Collinson & Elton, 1961), although this is not always the case.

(*c*) *Sugars*

Sugars are common constituents of food products and their effect on the rheology of the systems derives from the ability which the sugars have to bind water. They compete with the starch for the available moisture and hence reduce the amount of water available to enter the granule and therefore limit the swelling. This, in turn, reduces the strength of the gels. At first sight this might be seen as an adverse property but in fact a reduction in gel strength can lead to a reduction in syneresis

and retrogradation and the presence of sugars in starch systems generally has a beneficial effect. Another benefit of sugars is that an increase in soluble solids increases the boiling point of a system and this can give benefits in terms of the efficiency of cooking. This is particularly important when high gelatinisation temperatures are encountered—for example, with amylomaize.

(d) Other colloids

Other colloids have a significant effect on starch rheology and interact directly with it. Non-ionic starches tend to have a nett negative charge and hence the interaction with proteins is pH dependent. Above the isoelectric point of the proteins, where they also have a negative charge, there is little interaction but, at pH values below the isoelectric point of the protein, coacervation can take place.

In food systems containing undenatured proteins—e.g. milk starch–protein interactions have been noted (Grant, 1968). These have been attributed to the fact that the starch–protein complex inhibits swelling and the mechanism of gelatinisation. The practical effect of this can be seen in a failure to develop the full viscosity and a granularity or sandiness in the texture of the milk product. Denatured protein does not interact in the same way with starch.

Starch can interact with other polysaccharide molecules present in a food system. In work in our laboratory on a confectionery gum preparation with a modified starch/gum arabic/sugar system a turbidity was observed which increased with the starch concentration but which was not present when the starch alone or the gum arabic alone was used. This interaction, which could be reduced significantly by alternative modifications to the starch, is believed to be due to an interaction of the two colloids. In this case they both have a similar charge, resulting in the precipitation of the colloid. This is an example of simple coacervation at high concentrations where the availability of water is a limiting factor and proceeds via a mutual salting out effect of the two colloids.

Both the previous examples of starch colloid interactions were deleterious to the systems in which they had been used and are to be avoided by the food manufacturer. Starch colloid interactions may be used beneficially, however; for example to produce synergism in viscosity.

A UK patent (Anon, 1978) has been published recently which depends on the discovery that blends of xanthan gum, guar gum and starch produce aqueous sols with synergistic behaviour in that their final viscosity is higher than would be predicted from the individual components used. In this publication a blend of 55% xanthan gum, 22·5% guar gum and 22·5% modified starch is used at 0·25% in a french dressing preparation. In our own laboratory K. Marshall (Private communication) showed similar synergism with starch and xanthan gum, as can be seen in the Brabender trace (Fig. 7) and in the synergism coefficient data in Fig. 8.

The final interaction which I should mention concerns starch itself and is an extension of the well known phenomenon of retrogradation. This concerns the

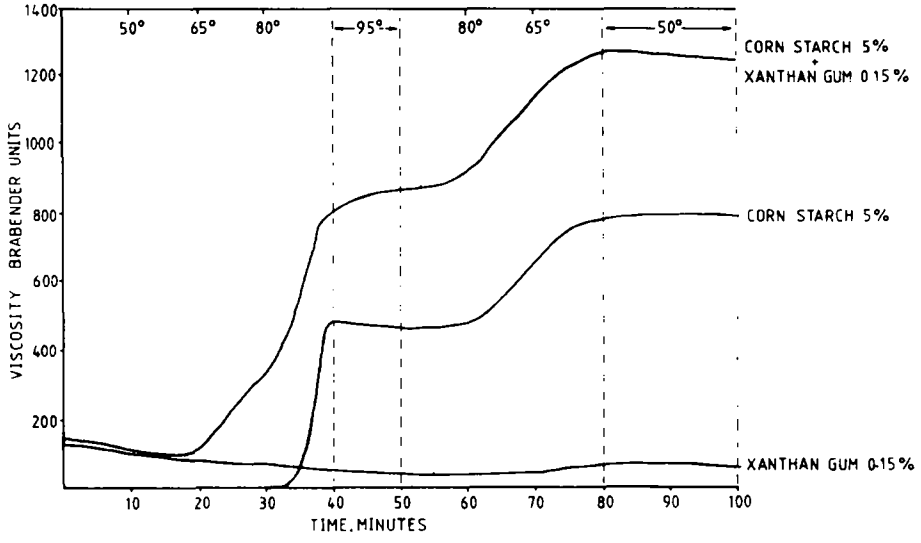

Fig. 7. Brabender viscographs at pH 5·5.

formation in solution of reformed amylose particles (RAP's) which have been identified by T. Davies in our laboratories (Private communication). These particles, which are 20–50 μ in diameter and can be seen by light or electron microscopy, are believed to be examples of the interaction between long chain fatty acids and amylose. They are formed under specific storage conditions; namely, when a maize starch pasted sol is held above 75 °C after a high temperature jet cooking. Non-lipid containing starches, e.g. potato starch, do not exhibit this phenomenon.

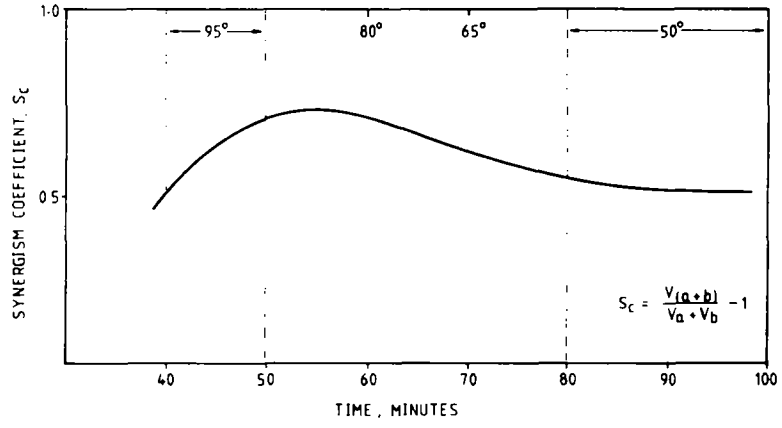

Fig. 8. Viscosity synergism of corn starch (5%) + xanthan gum (0·15%).

CONCLUSION

That the structure of a molecule should affect its rheological properties is obvious to all. Precisely how is what makes life interesting.

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Food Chemistry 6 (1980) 63–76

THE RELATIONSHIP BETWEEN THE COVALENT STRUCTURE OF THE *XANTHOMONAS* POLYSACCHARIDE (XANTHAN) AND ITS FUNCTION AS A THICKENING, SUSPENDING AND GELLING AGENT

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ABSTRACT

The ability of xanthan gum to form highly viscous and pseudoplastic solutions which are generally stable to variations in pH, temperature and salt concentrations is well documented. It has only recently been possible, however, to attempt an explanation for this behaviour on a molecular basis following publication of the revised chemical structure of the polysaccharide. The realisation that the polymer is, in essence, a derivative of cellulose has led to a number of propositions for the conformations adopted by the chains in solution. Temperature-induced changes of viscosity and optical rotation have been related to both inter- *and* intra-*molecular conformational transitions, whilst viscosifying power has been found to depend on the degree of pyruvylation of the polysaccharide. From our work in this area we propose a rationalisation of these conflicting views and an explanation for the anomolous solution viscosity variations of the polyelectrolyte with ionic strength. The effect of molecular interactions on the suspending power of the solutions and the synergistic interactions with the galactomannans guar and locust bean gum is discussed.*

INTRODUCTION

In the extracellular polysaccharide from *Xanthomonas campestris*, nature has fashioned a polymer with such remarkable properties that the large scale cultivation of the micro-organism and industrial application of the alcohol precipitated product, xanthan gum, have become practical realities (Jeanes, 1968).

The natural habitat of the gram-negative bacterium is in the vascular system of plants of the brassica family, and it was originally isolated from the rutabaga plant. Infected areas become slippery following bacterial growth and polysaccharide

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biosynthesis. Various functions of bacterial slimes (extracellular or capsular polysaccharides) have been proposed but usually no single purpose for their secretion can be identified. It is likely that the polysaccharides have multi-functional roles, acting as food reserves, offering protection from dehydration, ultra-violet radiation and, in the case of capsular polysaccharides at least, attack by bacteriophage. To most bacteria in the environment these advantages are essential to ensure survival, but the reasons behind the multiplicity of structural types which are elaborated by the different species remains something of a mystery. Evolutionary pressures have designed and shaped the *Xanthomonas* polysaccharide (xanthan) to suit a purpose and, in the process, have endowed it with properties that withstand extremes of pH and temperature far in excess of those likely to be encountered in the life cycle of the bacterium.

One unusual property of xanthan is the synergy it exhibits with the galactomannans, especially guar and locust bean gum. This interaction has been attributed (Morris, 1977) to a specific association of molecules of xanthan with those of the galactomannan gum and this affinity for the cellulose-like helix of the mannan chain has been interpreted (Morris *et al.*, 1977) in functional terms as being the 'specific glue' which holds the bacterium to the tissues of the host plant.

DEVELOPMENT AS A FOOD ADDITIVE

Recognition of the unique properties and the commercial potential of xanthan gum dates back to the late 1950s with the pioneering efforts of Jeanes and her co-workers at the Northern Regional Research Centre of the US Department of Agriculture. Their original studies have now been summarised (Jeanes *et al.*, 1976) but refinements continue to be made either on microbiological aspects of cultivation (Kidby *et al.*, 1977) or on characteristics of the polysaccharide produced (Sandford *et al.*, 1977). Following commercial production, xanthan gum found a diverse industrial market (McNeely & Kang, 1973) and was accepted by the US Federal Food and Drug Administration (Anon, 1969) as a permitted food additive in 1969. It has since been widely used as a food stabiliser, emulsifier, thickener, suspending agent and foam enhancer, and it is now accepted for food use in many countries, including the UK.

The principal properties exhibited by solutions of xanthan gum which are of importance to the food industry (Glicksman, 1969) are:

(1) A high viscosifying power.
(2) A high degree of pseudoplasticity.
(3) A high tolerance to salts.
(4) Very high stabilities towards extremes of temperature, pH, ionic strength and shearing forces.

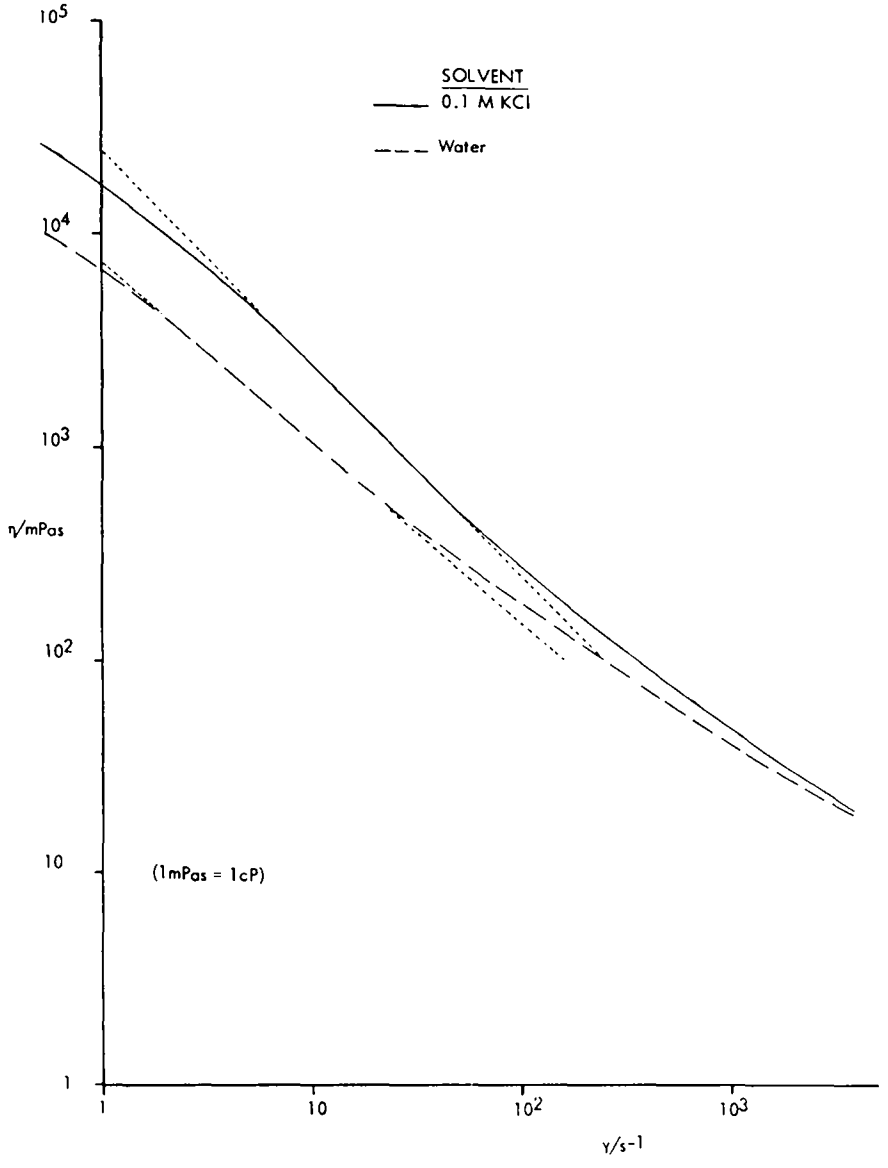

Fig. 1. Log (viscosity) versus log (shear rate): 1%, 25°C.

(5) An ability to suspend particulate matter.
(6) The synergy with galactomannan gums.
(7) A resistance to enzymic degradation.

Depending upon the actual application, several of the above properties may be sought. In salad dressings, for example, which are oil-in-water emulsions, the xanthan gum serves as an emulsion stabiliser, a suspending agent (if particulate matter is present) and to give desirable flow properties. The latter includes a pleasant mouthfeel, which is associated (Szczesniak & Farkas, 1962) with the high degree of pseudoplasticity of the emulsion. Additionally, the gum is required to withstand high salt concentrations and low pH conditions for prolonged periods. Sauces and gravies are other products which are improved by the incorporation of xanthan gum, and it is also used in baked, canned and frozen foods and in dairy produce.

Xanthan solutions are non-Newtonian (i.e. the viscosity is dependent upon the shear rate) and exhibit typical power-law fluid characteristics. It is convenient to express the flow properties of a solution by the consistency index (K) which is the intercept of the linear region of the curve with the y-axis, and the flow behaviour index (n) calculated by subtracting the slope of the linear region from unity. These two parameters are obtained by plotting log (apparent viscosity) against log (shear rate) as in Fig. 1 and extrapolating the linear region through the ordinate.

STRUCTURAL AND CONFORMATIONAL THEORIES

Progress continues to be made on the elucidation of the molecular origins of the chemical and physical properties of the polysaccharide, but there are issues which still remain unresolved. In the wake of the discovery that the covalent structure of xanthan (Jansson *et al.*, 1975), is as shown in Fig. 2, physical and chiroptical studies

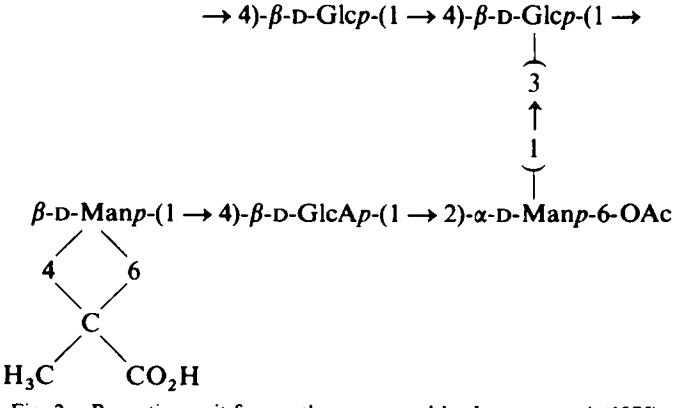

Fig. 2. Repeating-unit for xanthan proposed by Jansson *et al.* (1975).

have been used in attempts to define the rheological behaviour of solutions of the polysaccharide in terms of the conformational characteristics of the polymer.

The sharp decrease in optical rotation observed by Rees (1972) upon heating 1 % solution of xanthan in the absence of salt to about 55 °C was found to coincide with a viscosity transition reported by Jeanes *et al.* (1961). From this behaviour it was suggested that the polymer possesses an ordered secondary structure, a theme pursued by Holzwarth (1976) who showed that the transition midpoint increases as log (Na^+). In later experiments, Holzwarth & Prestridge (1977) presented electron microscopic evidence that the native structure is multi-stranded with two, or perhaps three, strands arranged in a right-handed twist. This proposal contrasts with the conclusion suggested from x-ray diffraction of xanthan fibres that the structure is a fivefold single helix (Moorhouse *et al.*, 1977).

The thermally induced transition between the rigid native conformation and a more flexible form has also been demonstrated by high-resolution nuclear magnetic resonance spectroscopy of xanthan in solution (Morris, 1977). Freely moving molecules show sharp nmr spectra while, for rigid polymers, the line width is so great that the spectrum is so flattened that no peaks are visible. Monitoring the area under the acetate and pyruvate peaks, Morris (1977) found increases which coincided with the transitions in optical activity and solution viscosity. Under normal conditions of use, the ordered form is stabilised to temperatures in excess of 100 °C by the higher ionic strengths encountered and therefore the viscosity of xanthan gum solutions can be regarded as temperature-insensitive for most practical purposes. A diagrammatic representation of the *intramolecular* change is shown in Fig. 3.

An alternative explanation for the temperature-induced phenomena described above has been given by Holzwarth & Prestridge (1977) who visualised the effects of heating xanthan in solutions as being direct consequences of *intermolecular* dissociation of a multistranded assembly (Fig. 4). Upon cooling it was proposed that many short strands combine to make up the native structure, although the nature of the interchain attraction was not apparent.

In a joint study (I. H. Smith, K. C. Symes, C. J. Lawson and E. R. Morris, unpublished results) between researchers at Tate and Lyle Ltd, Group R & D, and Unilever Research (Colworth House), further data on the changes of viscosity of xanthan solutions as a function of polysaccharide concentration, ionic strength, pH and the effects of variations in some molecular parameters on rheology have been obtained. These results are interpreted assuming the existence of equilibria between individual molecules and aggregates, the latter being the favoured form under certain defined conditions.

It is proposed from this work that at elevated temperatures disaggregation occurs, leading to the predominance of the monomeric form of the polysaccharide which then, at T_m, undergoes the intramolecular conformational change between rigid rods and random coils as previously described. The temperature-related changes in optical activity, viscosity and nmr line-width are thought to be associated with this

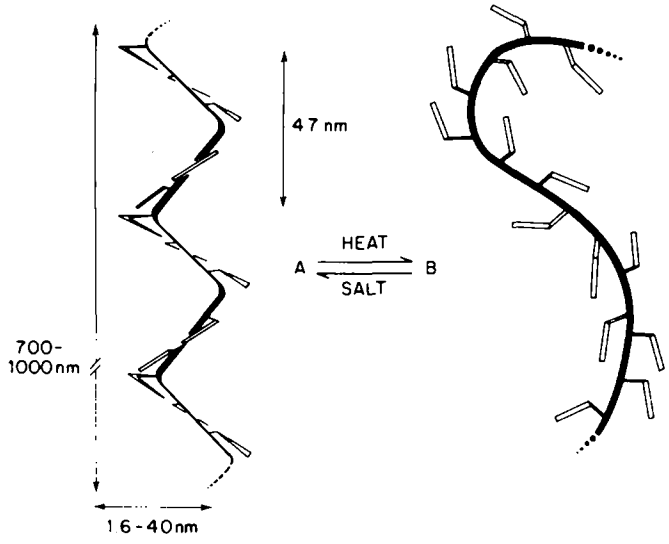

Fig. 3. Intramolecular conformational change which occurs when aqueous solutions of xanthan are heated above T_m.

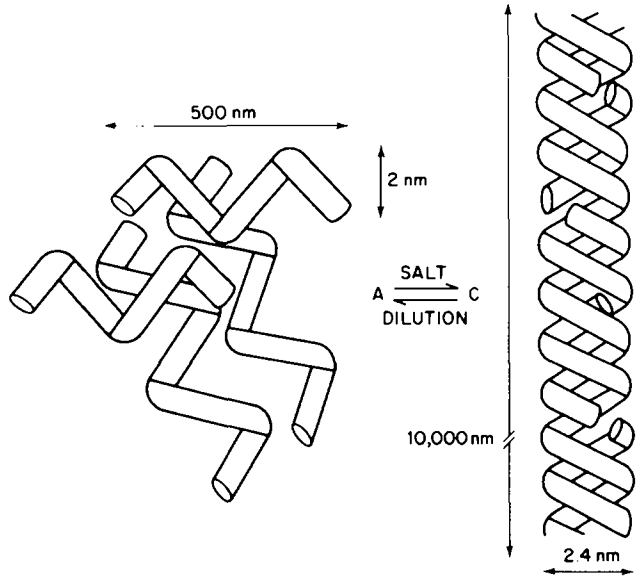

Fig. 4. A possible monomer (A)–aggregate (C) equilibrium. The configuration of the aggregate is uncertain, although evidence for (C) has been provided by Holzwarth & Prestridge (1977).

transition. At ambient temperatures and relatively high concentrations of the polysaccharide, however, the individual molecules in the form of rigid rods (or sub-units as referred to by Holzwarth & Prestridge (1977)) are presumed to associate into aggregates. The degree of aggregation has also been found to depend upon the ionic strength of the solution, the pyruvic acid content of the polysaccharide and the shear rate exerted upon the solution (I. H. Smith, K. C. Symes, C. J. Lawson and E. R. Mossis, unpublished results). An enhancement of solution viscosity is presumed to follow molecular aggregation and at low shear rates this effect is maximised giving rise to the suspending ability of the solutions. This property has already been ascribed (Morris, 1977) to the existence of a tenuous intermolecular polysaccharide network.

IONIC STRENGTH EFFECTS

In their comprehensive studies of xanthan solution rheology, Jeanes *et al.* (1961) discovered the anomalous response of viscosity to increases in salt concentration. Defining polysaccharide concentrations above 0·5 % w/v as high, those between 0·1 0·5 % w/v as medium and those below 0·1 % w/v as low, it was demonstrated that the viscosities of solutions at high concentrations increased upon the addition of salt whereas, at low xanthan concentrations, a decrease was observed. Typically, solutions of polyelectrolytes show sharp decreases of viscosity with increasing ionic strength, with this effect becoming minimal only at very high polymer concentrations (2–3 %).

On re-examination (I. H. Smith, K. C. Symes, C. J. Lawson and E. R. Morris, unpublished results) the unusual increase in solution viscosity upon the addition of salt to purified (dialysed) xanthan at high concentration can be seen to depend upon the degree of ionisation of the polyanion (Fig. 5). When the carboxylate groups are fully protonated the response to added salt is the reverse of that found for solutions of the neutral salt, i.e. an initial high viscosity becomes lower upon increasing the ionic strength. Protonation of the carboxylic acid groups should minimise the repulsion between chains and, by virtue of hydrogen bonding, may even accentuate the attractive forces. The decrease then observed with increasing ionic strength may be caused by the disruption of these hydrogen bonds. Merely adding acid to neutral xanthan solutions does not expose this pattern of behaviour because the ionic strength in the acidified solution under these circumstances is too high for its observation. Indeed, in most practical applications sufficient salt is present for the viscosity to be essentially independent of pH and ionic strength.

EFFECT OF POLYSACCHARIDE CONCENTRATION

Viscosity measurements of xanthan solutions with and without added salt (Fig. 6) confirm that at the higher concentrations it is the salt-containing solution which is

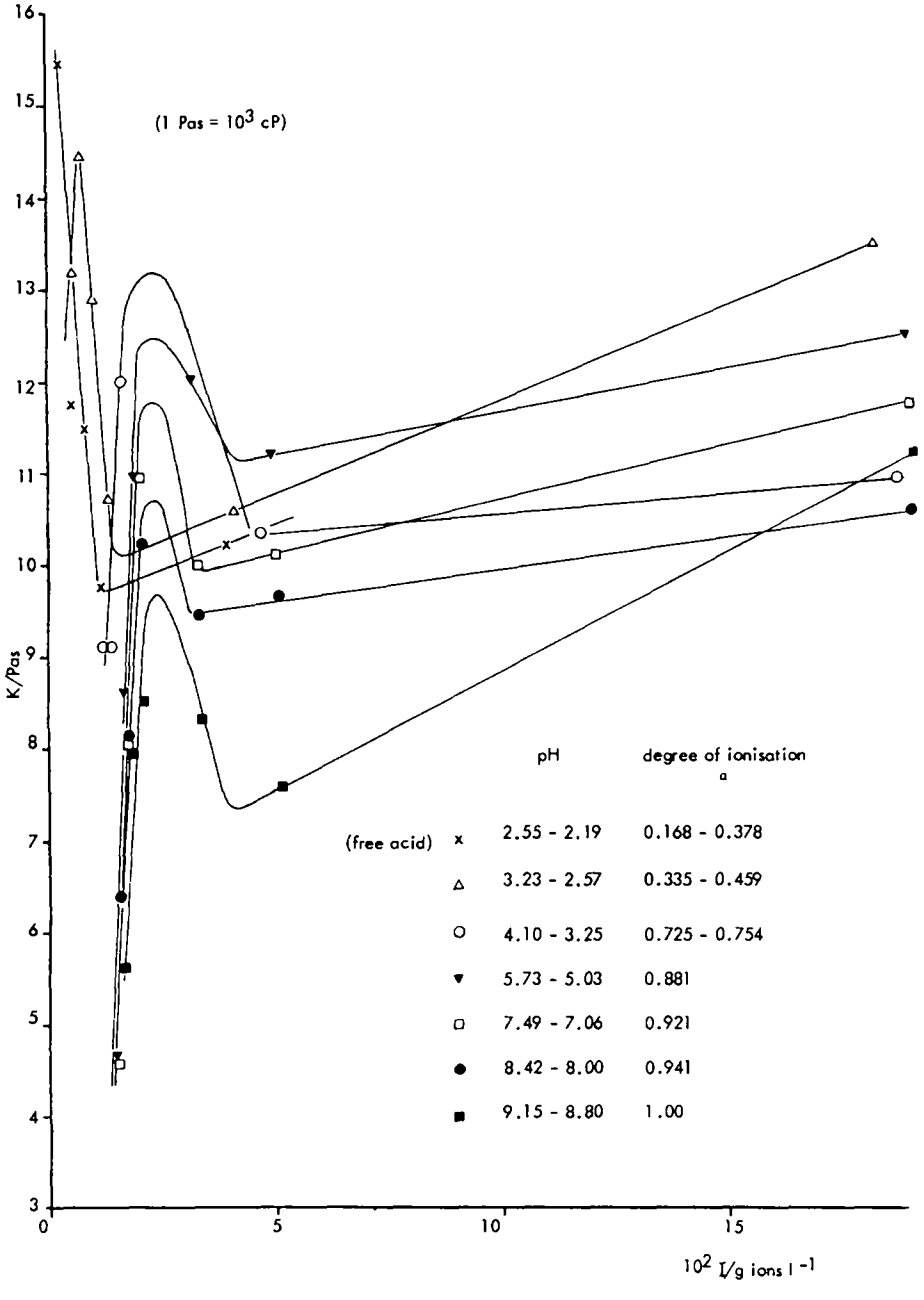

Fig. 5. Consistency index as a function of pH and ionic strength: 1% xanthan, 25°C.

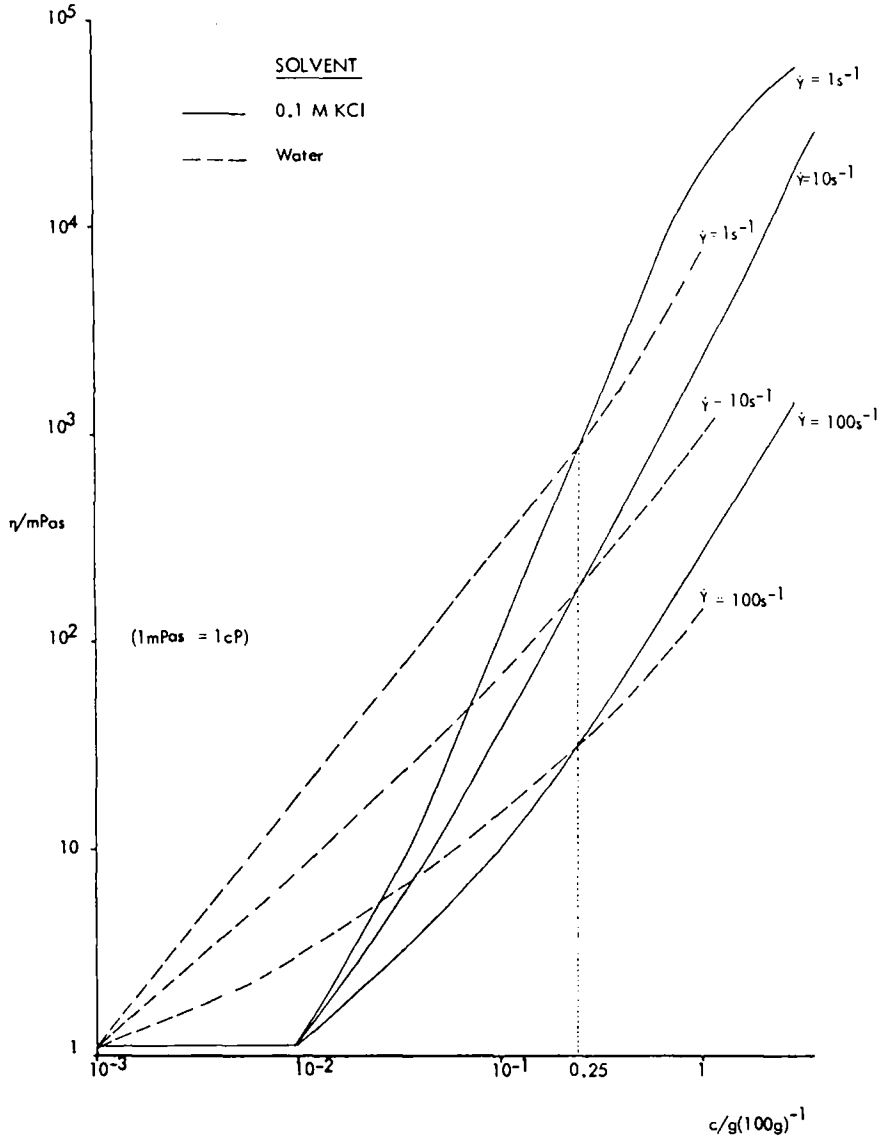

Fig. 6. Log (viscosity) versus log (concentration) for xanthan at 25°C.

the more viscous whilst the reverse is true at the lower concentrations. The more rapid rise in viscosity versus concentration for the solutions containing salt can be attributed to the progressive increase in the extent of molecular interaction which can occur when the ionic strength of the solution is sufficient to reduce ionic repulsions. At the crossover point of the lines in Fig. 6 presumably the tendency for

high ionic strengths to lower viscosity by lowering electroviscous contributions (Pasika, 1977) is counterbalanced by the extent of aggregation of the xanthan molecules.

EFFECT OF MOLECULAR PARAMETERS

The viscosity of polymer solutions is known to depend critically upon molecular weight (M) (Holzwarth, 1978) but estimates of M and its distribution for xanthan have varied widely. Holzwarth (1978) has calculated a typical native-xanthan sample to have $M_w = 15 \times 10^6$ with a polydispersity index $M_w/M_n = 2{\cdot}8$ from sedimentation coefficients and intrinsic viscosity measurements. (M_w = weight average molecular weight; M_n = number average molecular weight). However, it was recognised that at high shear stresses and at concentrations of 0·01 % w/v or greater the differences in viscosity measurements resulting from differences in M are obscured. It is probable that at concentrations where molecular associations are appreciable the solution viscosity will be related in part to the extent of molecular interaction.

Studies by Sandford *et al.* (1977) led them to conclude that xanthans with a high degree of pyruvylation give more viscous solutions than those less heavily substituted and were, therefore, of higher quality (Cadmus *et al.*, 1978). In their experiments Smith *et al.* (unpublished results) describe results which support this conclusion, but only under conditions of high ionic strength. By compensating for differences in M by selection of pairs of xanthans with similar intrinsic viscosities but varying in their degree of pyruvylation (x = proportion of side-chains bearing this substituent), it was found that as x increases so also does the viscosity of the salt-containing solution. However, in the absence of salt, the more highly substituted polymer solutions were the least viscous. In Fig. 7 the relationship is shown between the salt sensitivity of viscosity to the degree of pyruvylation for a number of xanthans originally freed of extraneous salts by dialysis and it can be seen that the polysaccharides with greater than about 30 % of the side-chains substituted show large positive changes of viscosity on going to the higher ionic strengths.

The rationale proposed (Smith *et al.*, unpublished results) to explain this behaviour is based upon a theory of monomer aggregate equilibria. Under conditions of low ionic strength (as in solutions of the polysaccharide containing no added salt) ionic repulsions between carboxylate anions will act to keep the individual polymer molecules separated. However, if there is sufficient salt in the solution to raise the ionic strength to the point at which interchain repulsions are minimised, the latent tendency for the molecules to aggregate is expressed and a dramatic rise in the consistency index of the solution follows. Since the final viscosity achieved can also be related to the degree of substitution by pyruvic acid by taking into account molecular weight effects, the substituent is thus implicated in both repulsive and attractive interactions, depending upon the ionic strength.

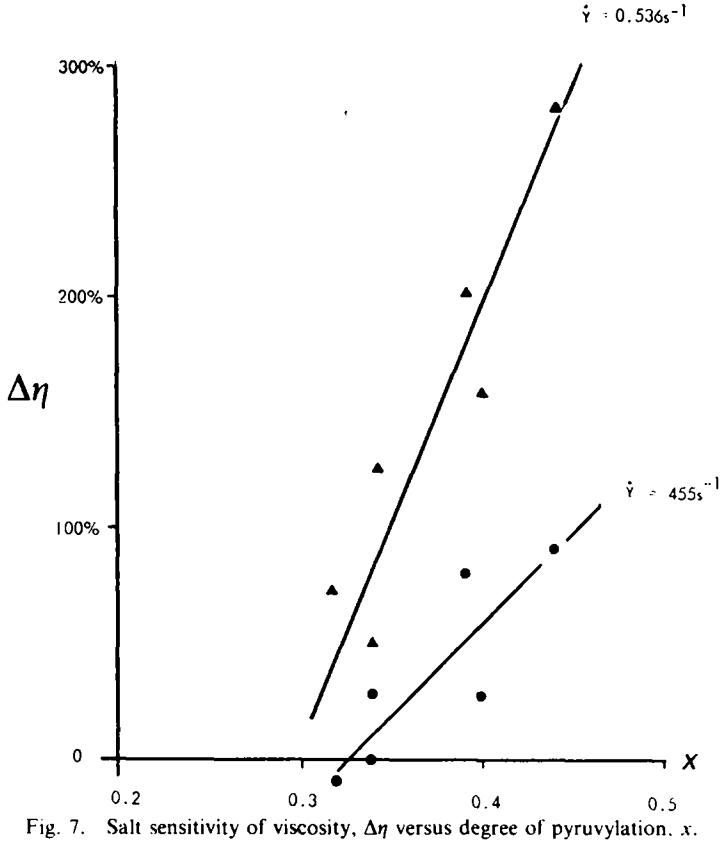

Fig. 7. Salt sensitivity of viscosity, $\Delta\eta$ versus degree of pyruvylation, x.

By shifting the position of the equilibrium, lowering the concentration of the polysaccharide would be expected to favour the monomeric form, but careful measurements (Holzwarth, 1978; Rinaudo & Milas, 1978) have led to diverging opinions as to the exact condition existing at infinite dilution. Whichever form exists at extremely low dilution, individual molecules (Rinaudo & Milas, 1978) or a double or treble-stranded species (Holzwarth, 1978), it is quite conceivable that the polysaccharide molecules associate, to a greater or lesser extent, at higher concentrations as dictated by the ionic strength of the solution and the degree of pyruvylation of the xanthan in question.

SYNERGISTIC INTERACTIONS WITH GALACTOMANNAN GUMS

It is well known that xanthan gum forms extremely cohesive heat reversible gels upon mixing and cooling hot solutions with locust bean gum (Schuppner, 1971), but

little is known about the factors which influence the gel texture characteristics. The molecular origins of the synergistic interaction between xanthan gum and the galactomannans guar, tara and locust bean gum has been interpreted (Dea & Morris, 1977) in terms of junction zone formation between the so-called 'smooth' regions (mannan main-chain with no galactose substituents) of the galactomannan and the xanthan molecules in their native conformation. Regions of the mannan chain highly substituted by galactose residues are thought to provide interruptions in this co-operative association, giving a matrix structure, and hence a gel, rather than a precipitate, results.

Recently, Painter *et al.* (1979) have used periodate oxidation techniques in studies on the structure of locust bean gum and guar and proposed somewhat different galactose substitution patterns than those described previously (Dea & Morris, 1977). The difference, however, would not be expected to alter the basic proposal for junction zones and interruptions.

The breaking strength and, to a lesser extent, the melting point (50 °–60 °C) of the gel is influenced by the presence of inorganic salts added to the original gum mixture. Figure 8 shows the increase in the strength of a xanthan gum–locust bean gum gel which is observed with increasing the salt concentration.

It is claimed (Anon, 1977) that partially de-acetylated xanthan gum gives gels which are stronger than those obtained with the normal acetylated product. It is difficult to rationalise these results from a molecular point of view, since the actual attractive

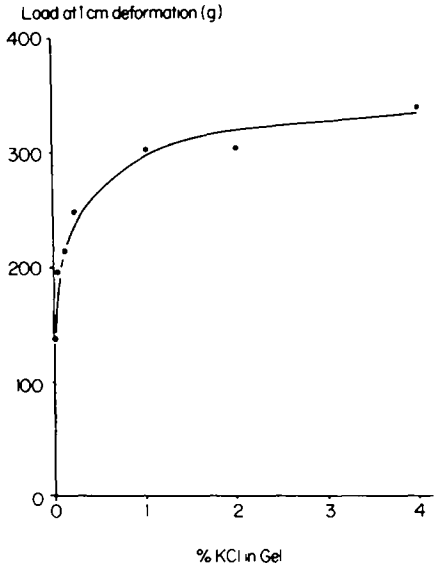

Fig. 8. Strength of xanthan gum–locust bean gum gel (1:1 mixture. 1 % total polysaccharide) as a function of added salt content.

interaction at the junction zones has not been clearly defined. Consequently, the effects of any variations are not easily predicted. Indeed, x-ray studies on oriented films prepared from synergistic xanthan gels have failed to reveal diffractions specifically associated with junction zones (Dr E. D. T. Atkins, personal communication; see also Dea & Morris, 1977).

The absence of syneresis and the smooth texture of the gel, combined with its freeze–thaw stability and compatibility with almost all other food ingredients, have resulted in a wide range of food (and non-food) uses for this gel system being described (Anon, 1976). Amongst them are salad and dessert gels, tomato aspic, cold milk gels, canned and frozen puddings, sour or imitation cream, party dips and whipped topping.

Even so, it is unlikely that the full potential has yet been realised for this versatile gelling system. Possible variations in the structure of each gum employed, their relative proportions and final concentrations all make for a wide choice of final texture which can be achieved and it is therefore a system with considerable potential to the food industry as a whole.

ACKNOWLEDGEMENTS

The valuable contributions of Drs I. H. Smith and E. R. Morris to the experimental results and conclusions reported here are gratefully acknowledged. Mr B. Lockwood is thanked for the data comprising Fig. 8, and for helpful discussions. Messrs J. E. Rudland and J. M. Brown provided excellent technical assistance.

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Food Chemistry 6 (1980) 77–88

CARRAGEENAN, PECTIN AND XANTHAN/LOCUST BEAN GUM GELS. TRENDS IN THEIR FOOD USE

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ABSTRACT

There are unlikely to be any completely new gelling agents, due to the high cost of proving them acceptable; exploitation of such agents as carrageenan and pectin is likely to be intensified.

Both of these gelling materials, together with xanthan/locust bean gum gels are discussed in detail. Their values in food formulations are considered, with some estimate of their future development.

INTRODUCTION

The primary function of a gel in foods is to bind/solidify the free water in the food. The free water may originate from a liquid ingredient, e.g. milk, or may be cell water being exuded from a food ingredient. Water is thus exuded from berries when osmotic equilibrium is being sought in jam manufacture. In ham preservation water is exuded as a consequence of boiling.

FOOD INGREDIENTS AND FOOD ADDITIVES AS GELLING AGENTS

Some food ingredients produce gels, casein and egg albumen being representative of the protein gelling agents and amylose being the typical carbohydrate gelling agent.

Gelatine is normally classified as a food ingredient, which requires that the substance be consumed as such. Other polymers with the ability to produce gels and found in biological materials are classified as food additives when they are not themselves consumed as such.

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Only a very limited number of polymers which have the status of permitted food additives are able to gel. An EEC directive on 'Gelling and thickening agents, emulsifiers and stabilisers' lists only five (Table 1). In view of the facts that (i) it took a minimum of three years' extensive toxicological testing, at an estimated cost of US$100 000, to clear xanthan for food use in the USA; (ii) that it took ten years from the time when xanthan was first accepted as a food additive in the USA until it

TABLE 1
GELLING AGENTS IN EEC STABILISER DIRECTIVE

Hydrocolloid	*E-number*
Alginates	400 to 404
Agar	406
Carrageenan	407
Pectin	440a
Amidated pectin	440b
Xanthan	
suggested transferred from Annex 2 to Annex 1	

appeared on the EEC list of permitted additives (the so-called Annex 1 list); (iii) that the 'climate' for food additives has become even more cold since xanthan was approved, it is unlikely that any completely new gelling agent will appear on the list of permitted additives.

It can therefore be predicted that the manufacturers of gelling agents and the food industry will seek to exploit the full potential of presently permitted gelling agents. Finding new raw material sources for these gelling agents and studying combinations of them will be ways of extending the possibilities.

This paper discusses the two most important—both in volume and value—of these gelling agents, namely carrageenan and pectin.

The justification for the use of gelling agents in food is 'technological need'. Suppliers of gelling agents must therefore base their development activity on uncovering the current and future 'technological needs' of the food industry, expressed or unexpressed. It must be emphasised that a technological need is only met when it is met at an acceptable cost.

CARRAGEENAN

Five families of red seaweeds are listed as carrageenan raw materials.

Carrageenan is not a well-defined substance, but rather a designation (a family name) for a group of salts of sulphated galactans. Various 'types' or 'fractions' of carrageenan are defined according to idealised structures and designated by Greek letters (λ, κ, ι, etc.) for lambda, kappa, iota, etc. (Fig. 1). Nature never comes up with

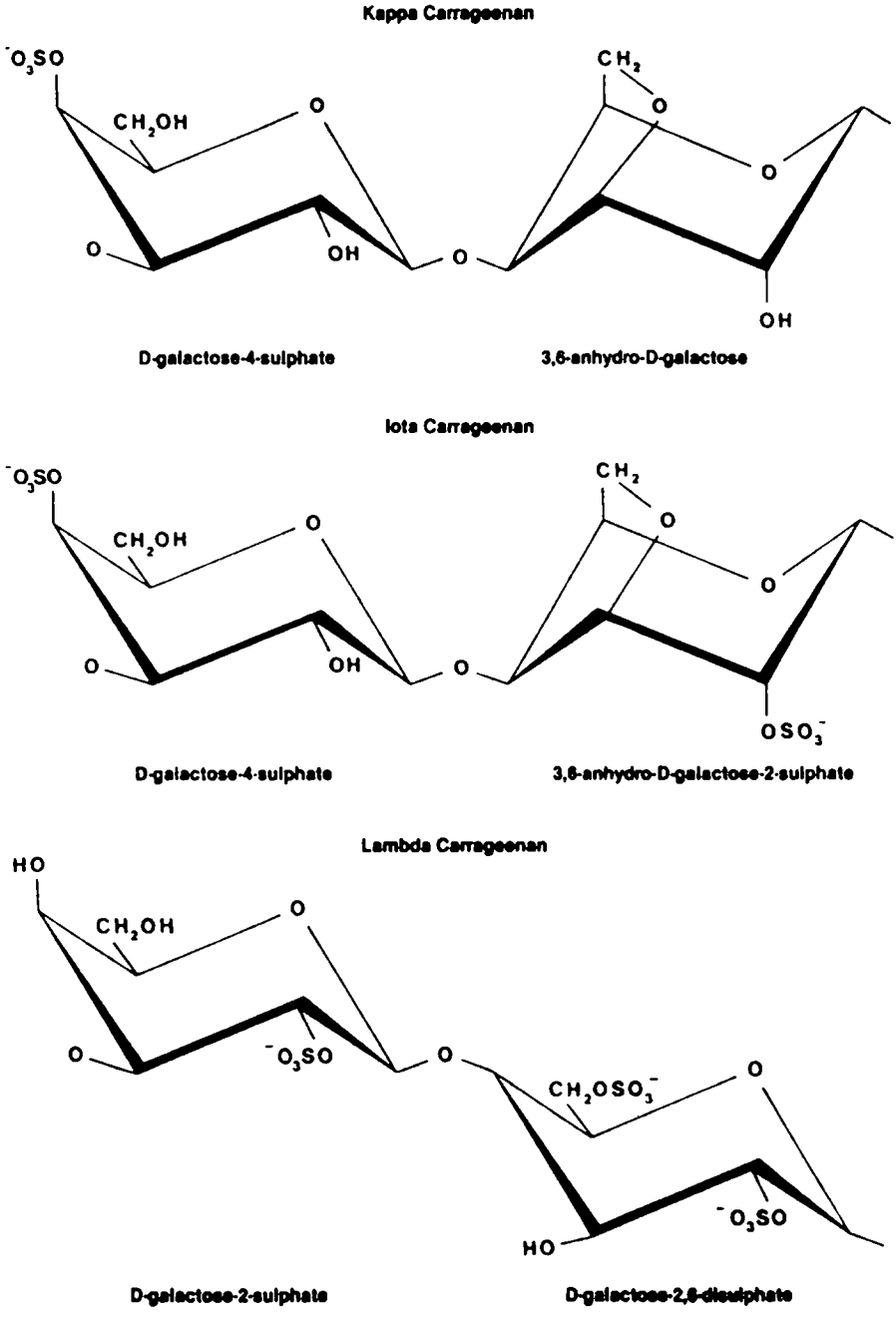

Fig. 1. Carrageenan. Repeating units in 'ideal' fractions.

pure fractions, but 'kappa-like', 'iota-like' and 'lambda-like' carrageenans occur in varying ratios in various red seaweeds. By selection of seaweeds it is therefore possible to obtain carrageenans which are predominantly the kappa type, etc.

Blends with controlled intermediate properties are produced by blending extracts or seaweeds before the extraction step.

With all carrageenan raw materials being harvested or collected solely for the purpose of making carrageenan, and most of these seaweeds further being collected/harvested by hand from natural seaweed beds, future availability and price stability of carrageenan could be questioned.

The carrageenan manufacturers therefore started development programmes to grow their own raw material, following two different paths: (1) cultivation in shallow tanks on shore, fresh seawater being circulated in the growth tanks and (2) farming in the sea, attaching seedlings to nylon strings held by supporting structures.

These programmes have been successful and kappa and iota type raw materials are available from these new sources ensuring not only the supply of carrageenan, but also promising a new means of tailor-making new carrageenans by breeding.

The largest single use of carrageenan in foods is probably as a gelling agent in gelled milk desserts.

The original product, the cooked pudding, used (kappa) carrageenan as a gelling agent, utilising the unique so-called 'milk reactivity' of carrageenan, which allowed kappa carrageenan to be used at a concentration of less than 0·2%. A water dessert

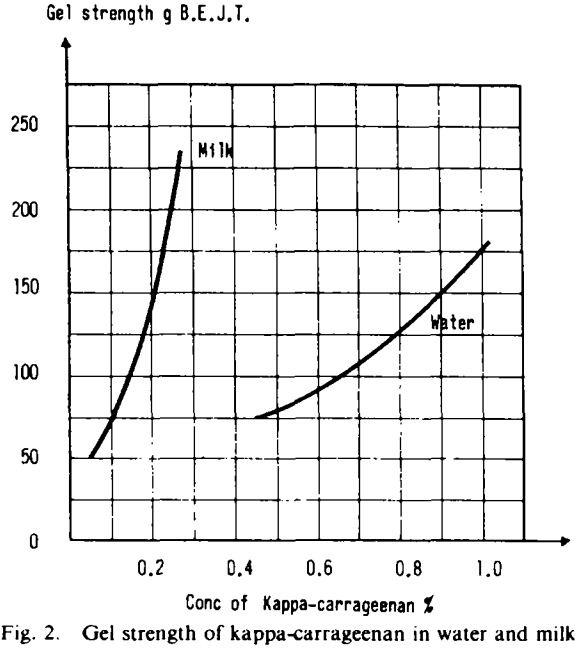

Fig. 2. Gel strength of kappa-carrageenan in water and milk.

gel would have required 1·0 % carrageenan to produce the same firmness (Fig. 2 and Table 2).

It is often claimed that kappa carrageenan reacts with casein or, more specifically, kappa-casein, to form a casein–carrageenan gel.

A better explanation is probably that casein—or, rather, native casein micelles—act as reinforcing elements for the potassium-kappa-carrageenan gel. If potassium is dialysed out of a kappa-carrageenan-milk gel and replaced by sodium, the gel strength follows the potassium level—it drops. This effect can be reversed by bringing back potassium ions—dialysing against a potassium salt.

TABLE 2
THE CARRAGEENAN–MILK GEL STORY
MILK-DESSERT TYPE

Product characteristics	*Cooked pudding (powder)*	*Ready-to-eat pudding pasteurised*	*Ready-to-eat with whipped cream topping*	*Ready-to-eat two-layer dessert pudding/fruit gel*
Consumer price	Low	High	High	High
Convenience	Low	High	High	High
Shelf-life	Long as powder hours as pudding	Two weeks at 5 °C	Four to six weeks at 5 °C	Four to six weeks at 5 °C
Texture	Gelled	Soft gel	Weak thixotropic gel	Soft gel/gel
Gelling agent	Kappa-carrageenan	Kappa/lambda carrageenans	Iota-carrageenan	LM-pectin
Type and conc.	0·20 %	0·32 %	0·5 %	0·6/1·0 %

The milk gel made with a strong kappa-carrageenan tends to exude water when stored for more than a few hours. When a longer shelf-life is required—for example, 7 days at 5 °C—a weaker kappa type carrageenan is used at a higher concentration (~0·4 %), ensuring better water binding at a still acceptable cost.

New and more strict requirements had to be met by the gelling agent when the UHTST sterilised aseptically packed pudding with whipped cream topping was developed.

The gelling agent must tolerate UHTST sterilisation, allow subsequent cooling to 10 °C for filling and form a gel, immediately after filling, strong enough to tolerate deposition of a whipped cream layer on top of the gel. Iota-carrageenan had the properties that were sought. Iota-carrageenan gels at a much higher temperature than 10 °C, but the gel is thixotropic, it recovers after pumping fast enough to support a topping a moment after it is filled.

The next generation in the ready-to-eat dessert sector is a two- or three-layer product composed of a fruit sauce/gel at the bottom, a milk pudding/custard next and a whipped cream topping.

This product presents a new challenge to the gelling agent in the milk pudding. As acid diffuses from the fruit layer into the milk gel, pH is reduced. With carrageenan being used as a gelling agent, pH reduction gives rise to the formation of an insoluble carrageenan/milk protein complex which may appear as a firm cheese-like border layer. This reaction of carrageenan caused by the extreme electronegativity of the molecule, may rule out carrageenan in this specific application and call for the use of the next substance to be discussed—pectin.

PECTINS

This plural term covers a group of substances, all derived from citrus peel or apple pomace, characterised in Fig. 3.

These pectins are further supplemented by so-called amidated pectin produced by treating pectin with ammonia in an alcohol/water slurry, resulting in amide groups partly replacing methyl ester groups. A typical amide pectin composition is: 20% amide substitution, 30% methyl esters, 50% free acid (Fig. 4).

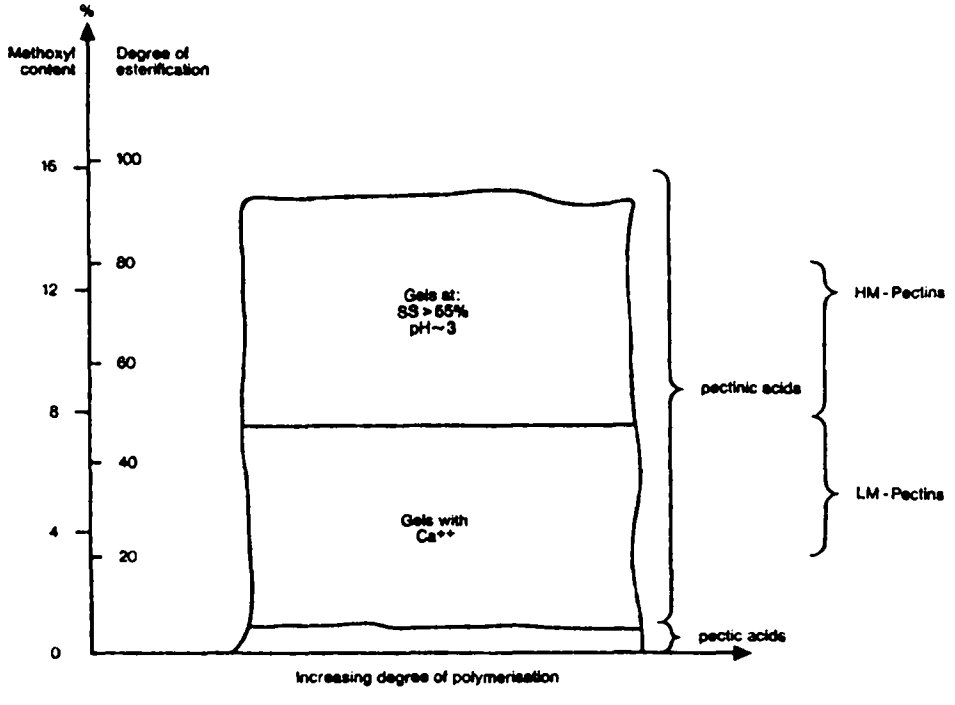

Fig. 3. Pectins.

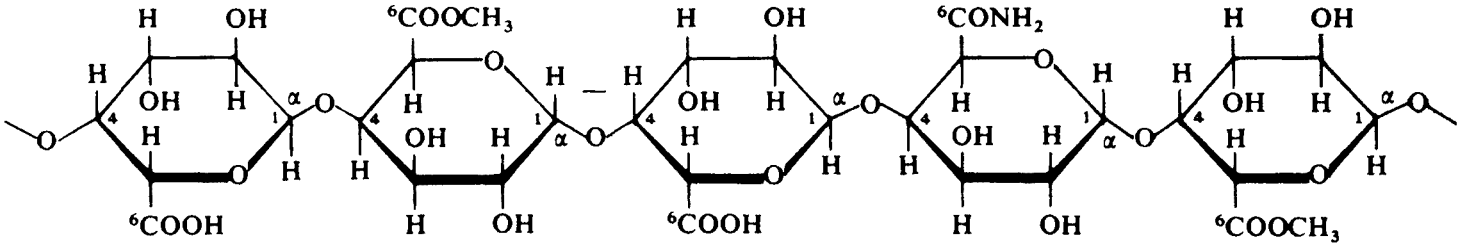

Fig. 4. Amidated low methoxy pectin.

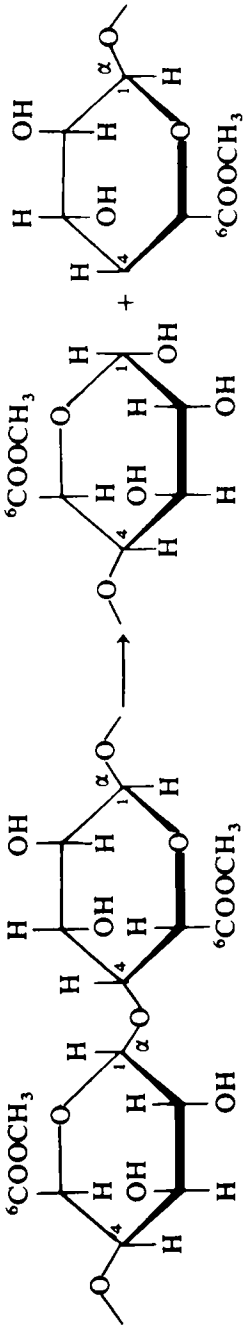

Fig. 5. β-elimination.

HM-pectin

High methoxyl pectin enjoys an exclusive position in conventional jams and has, over the last few years, gained ground in confectionery jellies at the expense of agar.

Gelation of HM-pectin requires the presence of sugar to a minimum of 55% soluble solids and simultaneously a pH below 3·5. This limits the use of HM-pectin to those areas already mentioned and there is no greater growth potential for HM-pectin as a gelling agent.

There are high growth rate areas for HM-pectin where other properties than the gelling ability are utilised, but these areas should not be dealt with here, since we are discussing food gels.

LM-pectin

Gels by a calcium mechanism, independent of sugar solids. An obvious application area for LM-pectin is in jams with reduced ($< 55\%$) sugar content. Such jams are becoming increasingly popular in developed countries with nutrition-conscious consumers.

In jams made for blending into yoghurts and similar flavoured milk products, LM-pectin is preferred as a gelling agent, giving the short, pumpable texture required for proper blending into the milk portion.

LM-pectin produces gelled milk desserts with a soft and smooth texture. In particular, LM-pectin is used in powdered or liquid preparations to be blended with cold milk to produce a truly gelled texture.

LM-pectin is less suited to food processes involving retorting. High temperatures cause the so-called β-elimination (Fig. 5), the effect of which is chain cleavage and loss of gelling ability.

LM-pectin requires calcium to gel and, if the LM-pectin is of the so-called acid demethylated type, it requires more calcium than is present in most fruit. Calcium must therefore be added in the jam-making process. This is unnecessary when the so-called amidated LM-pectin is used. Requiring less calcium for gelation, it is also more tolerant to an overdose of calcium. Further, the gel produced by amidated LM-pectin is more elastic and more transparent than that made from acid demethylated LM-pectin, showing the obvious advantage of amidation.

Amidated pectin is now a separate 'E-number' on the EEC list of approved gelling agents; E 440b. It has a preliminary ADI of 25 mg/kg body weight/day—and a permanent—and probably higher—ADI awaiting the 1980 JECFA (FAO/WHO Joint Expert Committee on Food Additives) meeting (March–April 1980) to make a judgement based on a complete report on toxicology which was forwarded to JECFA at the end of 1979. LM-pectin—and particularly amidated LM-pectin—has good growth potential as a general purpose gelling agent in formulated foods, being more stable under acidic conditions than agar and carrageenan and producing heat reversible gels, whereas alginates and pectates produce irreversible gels.

PECTATES

Complete de-esterification of pectin with acid is not possible as the de-esterification rate falls drastically with DE. Alkaline de-esterification gives β-elimination as a side reaction, resulting in low molecular weight pectates.

A US patent (2.132.065) to Sunkist describes how high molecular weight pectates can be produced. Methylesterase present in citrus peel is activated by pH adjustment and de-esterifies the pectin in the peel completely in 30 min. From the resulting 'crude pectate' a purified pectate may be produced by extraction with water containing a Ca-complexing agent.

High molecular weight pectate was available for several years. It had only limited application, however, and is no longer produced.

Recently, there has been renewed interest in pectate and crude pectate in the UK. Pedigree Petfoods Ltd has patented the use of crude pectate as a binder in pet food formulations. Dr Mitchell, of the University of Nottingham, has published work on the use of pectates as thickeners in sauces for canned foods.

Pectates are in direct competition with alginate, both hydrocolloids requiring calcium for gelation and the production of heat irreversible gels.

The future of pectate therefore depends on the demonstration of superior properties and/or lower prices.

XANTHAN/LOCUST BEAN GUM GELS

Xanthan is not, by itself, a gelling agent. However, combining xanthan and (non-gelling) locust bean gum produces a gel. It is difficult to envisage a xanthan locust bean gum gel consumed as such—it is too cohesive.

The xanthan/locust bean gum combination will find use as a stabiliser for food suspensions, emulsions and foams. The combination produces a yield value in the water phase at very low use concentrations.

There is a potential long-term supply problem for locust bean gum, which may affect xanthan (and—to a lesser extent—kappa-carrageenan) use.

There is interest in methods of producing locust bean gum characteristics, particularly synergism with xanthan and carrageenan, starting from guar gum.

DEMANDS FOR GELLING AGENTS IN FUTURE FOODS AND FOOD PROCESSES

Scarcity of protein, particularly animal protein, will lead to price increases and intensified efforts to develop restructured, simulated and fabricated foods.

Food processing was developed when water was plentiful and energy cheap. There is a need for new and less water- and energy-intensive food processes.

Future 'fabricated' foods and new food processes will probably first be accepted

for non-human use—for pets and food animals. Humans will probably never accept 'eating nutrition', but will rather demand traditional foods or starve.

To make meat trimmings, isolated plant proteins and single cell proteins acceptable as human foods requires that these nutritive substances become structured. To bind them in a matrix requires a gelling agent which should preferably (a) be soluble in cold water, (b) produce a gel without the application of heat and (c) produce a non-heat reversible gel (which remains a gel under all subsequent heat processing conditions).

These demands are only met by the alkali metal alginates and pectates. These uronates gel with calcium ions, administered by diffusion or slowly released from insoluble Ca salts in the food—for example, by decreasing the pH.

Restructured onion rings made from onion cutting waste (from making real onion rings!) and sodium alginate, extruded into a calcium chloride setting bath, are the prototypes of a 'restructured' food.

Artificial fruit is an example of a simulated product made with alginate or pectate.

Intermediate moisture foods are not new; jams and certain sausage products are examples of foods which exclude bacterial growth because their water activity has been reduced below 0·90. New, however, is the use of the IMF principle in pet foods —the water activity in meat-based products being reduced by the infusion of sugars, glycols and salts.

This area might seem a new potential area for HM-pectin, which relies on a certain concentration of solids in the application medium for its gelation. A recent French paper describes how high concentrations of easily soluble proteins (derived from milk) can be incorporated into pectin jellies (confectionery jellies), thereby making this sweet nutritious food! I must express a personal doubt as to whether this idea is viable. High concentrations of solutes are probably acceptable to humans only in small doses, as snacks. Secondly, the health effects of too many easily assimilated carbohydrates are questionable.

Use of the intermediate moisture principle in a food intermediate is a new idea which is more likely to find application. A British patent, No. 1,483,507, describes how a gelled milk dessert *base* is formulated. A calcium-sensitive gelling agent, LM-pectin, -pectate or alginate is contained in a flavoured sugar syrup with an a_w below 0·90. This syrup is blended into three parts of cold milk to make an instant gelled milk dessert with the texture of a cooked pudding. The preserving effect of the sugar is utilised in the base and the sugar concentration is reduced to 25% of the concentration in the base before consumption, thus making use of the IMF principle without having to accept its disadvantages.

CONCLUSION

New carrageenans with useful gelling characteristics are most likely to occur as a result of seaweed breeding and the cultivation of such new red seaweeds in tanks or in

the sea. If no new carrageenans are produced, seaweed cultivation will at least ensure supply of carrageenan raw material at controlled prices.

Among the pectins, LM-pectins, including amidated LM-pectins, promise the highest growth rate. LM-pectins will find use as gelling agents in formulated foods, particularly low-pH formulated foods and where heat reversibility is required.

Xanthan/locust bean gum does not have much score as a gelling agent for gels to be consumed as such; the gel is too cohesive. Xanthan/locust bean gum combinations will find use as stabilisers for multiphase foods.

Xanthan gum is probably the last hydrocolloid which will be permitted for food use in the foreseeable future, the cost of toxicological testing of any new hydrocolloid being too prohibitive. This situation forces the food industry, in co-operation with the manufacturers of gelling agents, to study all combinations of permitted hydrocolloids for possible useful properties and synergistic effects.

The growth potential for gelling agents in general is closely linked to the future of formulated foods.

Food Chemistry 6 (1980–81) 89–95

THE EFFECT OF DIFFERENT VARIETIES OF SOYBEAN AND CALCIUM ION CONCENTRATION ON THE QUALITY OF TOFU

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ABSTRACT

The Japanese food tofu (bean curd) was prepared by the traditional method using 15 varieties of soybeans. The tofu quality was evaluated on the basis of colour, taste and texture. The texture measurements were subjective and instrumental (hardness and cohesiveness).

A slight variation in the texture of tofu was due to the 7S and 11S proteins in the soybean but an important factor affecting the texture of tofu was found to be the amount of calcium ions added during processing. The firmness and coarseness of the product increased with increases in the amount of calcium ions added and a linear relationship was found between the protein content of the beans and the calcium sulphate concentration required for good quality tofu.

INTRODUCTION

The consumption of soybeans for food in Japan has been estimated at one million tonnes per year (Ministry of Agriculture and Forestry, Japan, 1978). The non-fermented bean curd, tofu, makes up 40% of this total. Tofu is the gel-like precipitate obtained by adding calcium sulphate to heated soybean milk (Smith & Circle, 1972). Japan imports 81% of its requirements of soybeans for food use.

Soybeans are grown in the exporting countries for their yield, disease resistance and oil characteristics. However, previous studies have shown that a variety of soybean affects the quality of the tofu. Saio *et al.* (1969) prepared tofu from the separate 11S and 7S fractions of defatted soybean meal. The tofu prepared from 11S protein was significantly harder in texture than tofu prepared from 7S protein and Watanabe (1978) has suggested that the differences in the quality of tofu prepared from varieties of soybean are due to the ratio of 11S proteins which have markedly different sulphydryl group contents.

Food Chemistry 0308-8146/80/0006-0089/$02·50

Saio *et al.* (1969) and Tseng *et al.* (1977) have also shown that the phytic acid (as measured by phosphorous content) and protein content of soybeans influences tofu quality. In the studies described here, the effects of 7S and 11S proteins as well as the protein and phosphorous content and level of calcium ion addition on the quality of tofu were determined.

MATERIALS AND METHODS

Soybean samples

Fifteen varieties of soybeans were all grown under the same agricultural conditions (soil, climate, fertiliser, etc.). The soybeans were stored for 8 weeks at 4 °C and 10 % humidity.

Chemical analyses

Protein content was determined by the Kjeldahl method (AOAC, 1975) Gel electrophoresis was used to determine the 11S and 7S protein content of the soybeans by the method of Larsen (1967). Phosphorous contents were determined by the method of Fiske & Subbarow (1925).

Preparation of tofu

One kilogramme of soybeans was soaked for 15 h in 4 litres of distilled water at 15 °C. After draining off the water, the beans were macerated in a Waring blender at high speed with 4 litres of distilled water. The resultant soymilk was filtered through cheesecloth and the liquid heated to boiling point. The soymilk was allowed to cool to 70 °C and while stirring, a saturated solution of $CaSO_4, \frac{1}{2}H_2O$ was added until the soymilk began to coagulate. At this stage, the suspension was allowed to form a curd without further stirring. After 1 h, the curd was transferred to a wooden box (30 × 30 cm) with a detachable bottom and lid, lined with cheesecloth. The lid was placed on top of the curd and a 5 kg weight was used to apply pressure to the lid. After 2 h, the curd had attained the required firmness and consistency of tofu.

Texture measurement

Sections of tofu were cut to a standard size of 30 × 30 × 30 mm and the texture was determined using an Instron Food Testing Instrument Model 1140. The drive speed of the compression anvil was 100 mm/min. A multiple bite was used to compress the sample to a 3 mm thickness with a force range of 20 kg.

The hardness was measured by the area of the peak obtained by compressing the sample from 30 mm to 3 mm. The sample was then compressed again and the cohesiveness was determined by the ratio of the areas of the two peaks obtained by compression of each sample twice. The successive compression is an objective measurement of texture since it simulates chewing.

Subjective assessment

Tofu quality as determined by taste, colour and texture was carried out by Dr S. Yen, experienced in tofu quality grading. Poor quality tofu samples were those judged to have a coarse texture.

RESULTS AND DISCUSSION

Table 1 lists the protein and phosphorus contents of some soybean varieties. Samples of tofu containing 2·7% calcium sulphate (Table 2) were found to be very coarse in texture and the taste and colour of the samples were poor. This level of calcium sulphate was used by previous workers (Smith & Circle, 1972; Saio *et al.*, 1969). Lower concentrations of calcium sulphate were used in a further experiment (Table 3) and it was found that the calcium sulphate concentration had a pronounced effect on the subjective quality of the tofu. The quality of the tofu was markedly affected by the amount of calcium sulphate used in its manufacture and Fig. 1 shows

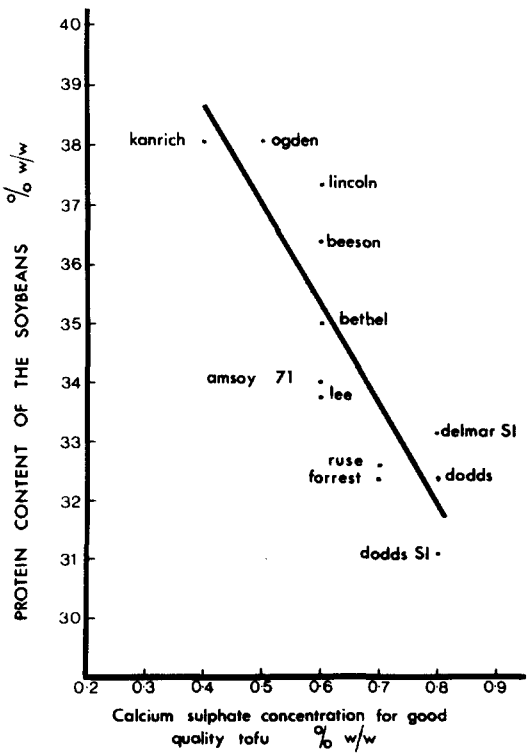

Fig. 1. Effect of calcium sulphate concentration on tofu quality.

TABLE 1
PROTEIN AND PHOSPHORUS CONTENT OF SOYBEAN VARIETIES

Variety	*Crude protein content (g/100 g)*	*7S/11S protein ratio*	*Phosphorus content (g/100 g)*
Dodds Row	34·5	0·98	0·72
Bethel SI	35·4	0·79	0·80
Dodds	32·4	0·98	0·76
Amsoy 71	34·0	0·66	0·68
Ruse	37·1	0·92	0·82
Lincoln	37·3	0·85	0·61
Forrest	32·4	0·90	0·88
Ogden 41	38·1	0·85	0·64
Kanrich	38·2	0·88	0·59
Beeson	36·4	0·92	0·63
Ruse	32·6	0·92	0·78
Bethel 242	35·0	0·80	0·80
Forrest SI	35·1	0·90	0·77
Amsoy 339	34·4	0·66	0·86
Lee	33·8		0·54

TABLE 2
QUALITY ASSESSMENT OF TOFU PREPARED FROM DIFFERENT VARIETIES OF SOY BEANS AND CONTAINING 2·7% W/W CALCIUM SULPHATE

Variety	*Objective assessment*			*Subjective assessment*		
	Hardness (kg)		*Cohesiveness*	*Taste*	*Colour*	*Texture*
	1st chew	*2nd chew*				
Bethel SI	24·1	20·6	0·23	poor	light yellow	coarse
Forrest 122	19·1	15·8	0·19	poor	cream	coarse
Ruse 144	15·0	12·2	0·22	poor	light yellow	coarse
Dodds 101	12·7	11·3	0·28	poor	cream	coarse
Ogden 41	15·2	13·2	0·16	poor	cream	coarse
Ruse	17·7	15·0	0·21	poor	light yellow	coarse
Lincoln	19·6	16·8	0·17	poor	cream	coarse
Amsoy 71	19·7	17·6	0·18	poor	cream	coarse
Amsoy 339	19·1	17·2	0·27	poor	cream	coarse
Lee	20·9	17·8	0·19	poor	cream	coarse
Dodds SI	12·1	10·4	0·18	poor	cream	coarse
Forrest SI	15·7	14·1	0·23	poor	cream	coarse
Dodds B.R.	14·3	12·8	0·22	poor	cream	coarse
Forrest 246	17·1	13·4	0·17	poor	cream	coarse
Bethel 242	19·1	16·6	0·23	poor	cream	coarse
Beeson	22·6	19·4	0·24	poor	cream	coarse

TABLE 3

THE EFFECT OF DIFFERENT CONCENTRATIONS OF CALCIUM SULPHATE, $\frac{1}{2}H_2O$ ON TOFU QUALITY

Variety	*Protein* % *w/w*	*Level of* $CaSO_4\frac{1}{2}H_2O$ *addition* (%)	*Tofu quality*					
			Subjective			*Objective*		
			Taste	*Colour*	*Texture*	*Hardness (kg)* *1st chew*	*2nd chew*	*Cohesiveness*
Kanrich	38·1	0·4	fair	cream	excellent	0·83	0·38	0·16
Kanrich	38·1	0·6	fair	cream	coarse	1·6	1·05	0·32
Kanrich	38·1	0·8	fair	cream	coarse	2·13	1·45	0·25
Kanrich	38·1	1·0	fair	cream	very coarse	5·0	3·63	0·33
Ogden 41	38·1	0·4	fair	cream	good	—	—	—
Ogden 41	38·1	0·6	fair	cream	coarse	0·63	0·35	0·17
Ogden 41	38·1	0·8	fair	cream	coarse	1·95	1·33	0·30
Ogden 41	38·1	1·0	fair	cream	very coarse	3·57	2·31	0·22
Lincoln	37·3	0·4	fair	cream	good	—	—	—
Lincoln	37·3	0·6	fair	cream	optimum	0·04	0·03	0·27
Lincoln	37·3	0·8	fair	cream	coarse	2·0	1·5	0·27
Lincoln	37·3	1·0	fair	cream	very coarse	2·19	1·56	0·23
Beeson	36·4	0·4	fair	cream	curd did not set	—	—	—
Beeson	36·4	0·6	fair	cream	optimum	2·06	1·44	0·27
Beeson	36·4	0·8	fair	cream	moderately coarse	5·63	4·38	0·34
Beeson	36·4	1·0	fair	cream	very coarse	5·63	3·75	0·25
Bethel	35·0	0·4	fair	cream	curd did not set	—	—	—
Bethel	35·0	0·6	fair	cream	optimum	1·58	1·08	0·35
Bethel	35·0	0·8	fair	cream	moderately coarse	2·31	1·5	0·20
Bethel	35·0	1·0	fair	cream	very coarse	5·00	3·56	0·27
Amsoy 71	34·0	0·4	fair	cream	curd did not set	—	—	—
Amsoy 71	34·0	0·6	fair	cream	optimum	1·06	0·63	0·28
Amsoy 71	34·0	0·8	fair	cream	moderately coarse	2·38	1·62	0·24
Amsoy 71	34·0	1·0	fair	cream	very coarse	5·44	5·63	0·21

TABLE 3—*contd.*

Variety	*Protein* %w/w	*Level of* $CaSO_4\frac{1}{2}H_2O$ *addition* (%)	*Tofu quality*					
			Subjective			*Objective*		
			Taste	*Colour*	*Texture*	*Hardness (kg)* 1st chew	2nd chew	*Cohesiveness*
Lee	33·8	0·4	fair	cream	curd did not set	—	—	—
Lee	33·8	0·6	fair	cream	optimum	0·47	0·38	0·24
Lee	33·8	0·8	fair	cream	moderately coarse	0·85	0·69	0·25
Lee	33·8	1·0	fair	cream	very coarse	1·4	1·0	0·24
Delmar SI	33·2	0·4	fair	cream	curd did not set	—	—	—
Delmar SI	33·2	0·6	fair	cream	slightly below optimum	—	—	—
Delmar SI	33·2	0·8	fair	cream	optimum	0·39	0·19	0·22
Delmar SI	33·2	1·0	fair	cream	moderately coarse	1·19	0·88	0·29
Ruse	32·6	0·4	fair	cream	curd did not set	—	—	—
Ruse	32·6	0·6	fair	cream	good	0·37	0·06	0·15
Ruse	32·6	0·8	fair	cream	coarse	1·31	0·74	0·17
Ruse	32·6	1·0	fair	cream	very coarse	3·16	2·09	0·19
Forrest	32·4	0·4	fair	cream	curd did not set	—	—	—
Forrest	32·4	0·6	fair	cream	curd did not set	—	—	—
Forrest	32·4	0·8	fair	cream	good	1·70	1·08	0·28
Forrest	32·4	1·0	fair	cream	moderately coarse	2·13	1·5	0·38
Dodds	32·4	0·4	fair	cream	curd did not set	—	—	—
Dodds	32·4	0·6	fair	cream	curd did not set	—	—	—
Dodds	32·4	0·8	fair	cream	excellent	0·72	0·39	0·28
Dodds	32·4	1·0	fair	cream	coarse	1·38	0·81	0·17
Dodds SI	31·1	0·4	fair	cream	curd did not set	—	—	—
Dodds SI	31·1	0·6	fair	cream	curd did not set	—	—	—
Dodds SI	31·1	0·8	fair	cream	excellent	0·52	0·26	0·23
Dodds SI	31·1	1·0	fair	cream	coarse	1·24	0·75	0·26

that there was a significant relationship between the calcium sulphate added to the curd to obtain good quality tofu and the protein content of the original soybean; protein = 45·4 − 16·9 (calcium sulphate added %w/w), $r = -0{\cdot}87$ ($p < 0{\cdot}01$), for optimum tofu quality.

There was no significant correlation between the ratio of 7S to 11S proteins or phosphorous content and tofu quality. This was not unexpected since previous workers (Saio *et al.*, 1969) measured differences in cohesiveness between tofu samples of the order of 0·03 compared with the differences of 0·15 (for good and poor, i.e. coarse quality tofu due to differences in calcium sulphate concentrations) found in the present studies.

The results therefore indicate that variations in the protein content of soybean varieties may lead to differences in tofu quality, but these differences may be overcome by varying the amount of calcium sulphate used.

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TOXICITY OF CYANOGENIC GLYCOSIDES

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ABSTRACT

The toxicity of cyanogenic glycosides is reviewed in the context of their metabolic involvement with goitre and tropical ataxis neuropathy (TAN). The significance of toxicity data is logically related to the nutritional adequacy of diet.

INTRODUCTION

Cyanide toxicity in plants first came to light when, over a century ago, the transport animals of General Kitchener's army in the Sudan suffered heavily from poisoning after eating the herbage of the Upper Nile. This prompted the Imperial Institute to undertake research; soon it published a series of papers in which it isolated cyanogen compounds in *Lotus arabicus* and *Sorghum vulgare* which finally led to a systematic study of cyanogenesis in plants. The earliest description of cyanide toxicity in man—from ingestion of *Pois d'Achery* (*Phaseolus lunatas*) was given by Davidson & Stevenson (1884) in Mauritius—who established a method for determining the cyanide content in those plants. This method was subsequently extended to cassava.

Clark (1935) was among the first to investigate cases of poisoning by cassava and concluded that a diet high in the root could give rise to pellagra-like symptoms with lesions of the mouth and skin, dimness of vision, blindness, complications of the nervous system, albuminuria, fatty degeneration of the liver and, ultimately, death. He attempted to reproduce these symptoms in rats by feeding them with cassava

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leaves or gari (processed cassava flour) and found that those on cassava leaves soon died while those on gari lost weight and developed photophobia, albuminuria and dysophoea. Potassium cyanide produced symptoms similar to those of gari, followed by death. Autopsy showed fatty degeneration of the liver. Clark concluded that many deaths in Trinidad, Uganda and northern Nigeria were caused by the cyanide in cassava root.

Moore (1934) was of the opinion that the high incidence of frank optic atrophy associated with ataxia and mental disorder in West Africa was due to the consumption of cassava, while Carmody (1900) had reported many cases of cassava toxicity among Trinidadians. Oyenuga & Opeke (1957) found that pigs fed boiled cassava or guinea corn performed better than those fed raw cassava and that, at the bacon stage, some of the latter in fact became lame and lost weight.

Oshuntokun (1968) reported that patients with ataxic neuropathy had a reduced concentration of serum sulphur amino acids and that their plasma thiocyanate concentration (11·4 mM/100 ml) was nearly six times that of the control (2·0). The plasma cyanide concentration was similarly raised from 0·027 to 2·42 mM/kg body weight/24 h (Oshuntokun & Aladetoyinbo, 1970). The patients improved when admitted to hospital and given food free from cassava derivatives. When they were discharged and resumed a cassava-rich diet they suffered relapses. This seemed to implicate cassava consumption in the aetiology of tropical ataxic neuropathy. This hypothesis gained further support from a survey carried out by Oshuntokun *et al.* (1968) which indicated that in Ososa, a village with a high consumption of cassava, the prevalence of tropical ataxic neuropathy (TAN) was 17 per 1000 while in Akinmorin, a village with a low consumption of cassava, it was less than 1 in 1000.

As far back as nearly forty-five years ago, in Nigeria, Oluwole (1935) suspected that it was the high consumption of cassava that led to symptoms of defective vision, stomatitis and puritus in schoolchildren. He succeeded in curing the children by reducing by half the amount of cassava food in their school lunches.

Ekpechi (1964) found a high incidence of goitre amongst the inhabitants of the Enugu areas where cassava is eaten in a dry state. He attributed this to the cyanide content of the cassava which is converted into thiocyanate which then results in antithyroid activity. He confirmed this by feeding rats a 100% cassava diet for 7 days and found a significant decrease in glandular stores of stable iodine, significant higher thyroid weight and higher thyroidal ^{131}I uptake, all due to a synthetic block in the conversion of monoiodothyroxine to diiodothyroxine. De Lange (1973) also observed a decreased ^{131}I uptake by the thyroids of persons who ingested cassava compared with those who ate rice only, confirming the goitrogenous nature of cassava. He used this to explain the higher incidence of goitre in the northern area of Idjwi Island in the Republic of Zaire compared with the south.

Cassava therefore seems to be the cause of both TAN and goitre. Before going into the details of the recent work on cassava and cyanide toxicity, it may be worth while to review the two diseases and their causes.

TROPICAL ATAXIC NEUROPATHY

One of the most perplexing problems faced by neurologists is the cause of a group of paraplagias, known to be common in the tropics, which affect the central nervous system and which are common only in the lower socio-economic group of the population. The general symptoms have been described by several authors and although they vary from region to region, they tend to have certain features in common—i.e. a varying combination of lesions of the skin, mucous membranes and the nervous system. In Nigerians the essential neurological components of the disease are myelopathy, bilateral optic atrophy, bilateral perceptive deafness and polyneuropathy (Oshuntoku, 1973). In about 39 % of the patients stomatoglositis is present. Other syndromes, such as motor neurone disease, Parkinson's disease, cerebellar degeneration, psychosis and dementis, have also been found associated with TAN (Asuni, 1967; Oshuntokun, 1968, 1969). The disease is peculiar to the lower income group who consume 'gari' three times daily.

These symptoms are similar to those which have occurred in prisoner-of-war camps and which are also known to be due to malnutrition and/or dietary toxins (Spillane, 1947; Money, 1958), i.e. angular stomatitis, glossitis, pellicular hyperkeratosis, etc. The fact that there is a diminished excretion of vitamin B_2 and also that the serum vitamin B_2 is usually significantly reduced (Oshuntokun, 1969) suggests that avitaminosis B_2 might be a possible cause. In addition, high blood pyruvate levels were recorded in patients who had been on hospital diets for 2–3 weeks and these were subsequently reversed by parenteral vitamin B therapy, again pointing to possible thiamine deficiency in view of the angular stomatitia, dermatitis and other typical symptoms. Moore (1930, 1932, 1934, 1937) suspected riboflavin deficiency. He found that these symptoms disappeared and that visual acuity was improved after treatment with Marmite, which is rich in vitamin B_2. Treatment with yeast, used by Clark (1935), which appeared to be very effective, especially when applied at an early stage, seems to confirm avitaminosis. However, this does not rule out the possibility of toxins acting as antivitamins or in other ways. Monekosso (1962) suggested that the clinical and epidemiogical studies strongly pointed to a metabolic dysfunction involving the vitamin B complex. Similar studies in Liberia (Kanettgen, 1955) and Malaya (Landor & Pallister, 1935) have led to the same conclusion.

The similarity of a number of other syndromes to TAN can be understood from the fact that cyanide in high doses is known to inhibit cytochrome oxidase which results in biochemical changes similar to those which would be obtained by other agencies capable of impairing the transfer of electrons released during oxidative phosphorylation to molecular oxygen. Deficiency of vitamin B_1, for example, causes impaired oxidative decarboxylation of keto acids; deficiency of nicotinic acid and vitamin B_2 will result in the impairment of oxidative nucleotide systems such as nicotinamide-adenine dinucleotide or nicotinamide-adenine dinucleotide

phosphate, dependent dehydrogenasesand flavin mononucleotide or flavin adenine dinucleotide dependent oxidases, while iron deficiency can impair the function of the cytochrome system. It is therefore not surprising that most of the neurological symptoms are associated with deficiency.

Abnormalities of pyruvate tolerance have been reported in cases of TAN (Monekosso & Annan, 1964), but this may not necessarily be due to the cyanide because the same abnormalities have been reported in vitamin B_{12} deficiency states (Buckle, 1966). Hill (1977) reported that blood lactic acid was elevated in rats dosed orally with 50 mg of linamarin (the cyanogenic glucoside in cassava which releases cyanide on hydrolysis) per 100 g body weight or 0·6 mg KCN per 100 g body weight (64 mg and 43 mg per 100 ml, respectively) compared with controls (23 mg), while the pyruvic acid was essentially unchanged. The resulting increased ratio of lactate/pyruvate was taken as indicative of severe cardiorespiratory failure. As discussed by Oke (1977), ingested cyanide is detoxified in the body by means of the enzyme, rhodanase, which converts the cyanide to thiocyanate with the help of sulphur obtained from methionine derived from food.

Since there is no difference in the rhodanase activity between patients suffering from TAN and the control, it would appear that the main limiting factor is the methionine for detoxification. Tewe (1975) found that when rats and pigs were given balanced diets containing 750 and 500 ppm of cyanide as KCN, the cyanide had no effect on the animals except possibly for a non-significant decrease in weight which might be due to a reduced intake because of the bitterness of the KCN. If, however, the diet was deficient in protein, the performance of the pigs was considerably reduced, with pathological lesions in the bones and thyroid gland. The cyanide alone did not cause any marked pathological lesions except for a non-significant increase in the thyroid gland. Thus, it can be deduced that protein deficiency—rather than the presence of cyanide in the diet—is the aetiology of the neurological maladies in the cassava-eating people of the tropics. One can therefore not justifiably say that TAN is an inevitable consequence of cassava consumption, even if the cassava is not prepared so as to reduce the cyanide or linamarin content.

GOITRE

Iodine is an essential element for the proper functioning of the thyroid gland. If by any chance there is insufficient intake of iodine due to low levels in soil and water or, alternatively, if a chemical is present in which there is competition with iodine, then deficiency occurs and the thyroid gland becomes hyperactive in order to be able to produce thyroxine. This leads to a swelling of the gland or simple goitre. The former case occurs in high mountain regions of the world such as Alpine valleys, the Pyrenees, the Great Lakes basin between Canada and the United States, Derbyshire and the slopes of the Himalayas, etc., where the incidence of simple goitre was first

reported. The condition also occurs in the low-lying Netherlands, where it is believed to result from ingestion of goitrogens.

There were early theories about the aetiology of goitre, some of which have persisted to the present day. The oldest was that it was of dietary origin, being attributed, for example, to the eating of certain types of chestnut (Barton, 1800) or, alternatively, that it was due to the eating of coarse and unrefined foods and hence was only common amongst European peasants. Early experiments with rats showed that high protein diets, consisting of meat and especially liver, produced an enlarged thyroid and this could be increased by the supply of calcium-rich water (Tenabe, 1925). Thus, liver was incriminated in the aetiology of goitre. When the liver was extracted with alcohol the active component was removed and the size of the thyroid decreased from the original 41·3 mg/100 g body weight to above 10·9 mg (Horn, 1940). Fat was also implicated, especially pork fat (St Lager, 1867). This was confirmed by Mellanby & Mellanby (1921) who showed that butterfat was goitrogenic but cod liver oil was not. Later, Chesney *et al.* (1928) provided the clue to the problem through the accidental discovery that rabbits fed on cabbage develop goitre and that this was inhibited by iodine. The active agent can be destroyed on drying and so glycosides were suspected, especially as these were known to be common in the seeds of the Cruciferae family, particularly the *Brassica* species. Finally, the goitrogen was identified as L-5-vinyl-2-thiooxazolidone. The whole subject has been ably reviewed by Greer (1950).

We now know that there are two mechanisms leading to the formation of goitre. In the first example, complex ions of a similar molecular size to the iodide ion (e.g. ClO_4) competitively inhibit the active transport of I^-, thereby reducing the amount of iodine to the thyroid. Where iodine intake is already insufficient this interference could be sufficiencly marked to produce hyperthyroidism or goitre. In the second place there are several compounds (antithyroid agents) that interfere with thyroxine formation from the iodine ion, thereby causing a decrease in the levels of the circulating hormone. This leads to the activation of the thyroid stimulating-hormone and ultimately the thyroid enlargement of goitre. The most active of these compounds are believed to inhibit the transfer of iodide to the tyrosyl group in thyroglobulin.

The thiocyanate ion is of a similar size to the iodide ion and so can successfully compete with it in the uptake by the thyroid gland. This does not seem to pose a serious problem in the presence of sufficient iodine intake. Experiments conducted by Ekpechi (1964) with rats fed 100% cassava for 7 days, resulting in high thyroid weight and high thyroidal ^{131}I, cannot be regarded as conclusive, although they gave an idea that thiocyanate might be included. This was confirmed by De Lange (1973) who found that ^{131}I uptake was lowered in rats given cassava or a thiocyanate injection in proportion to the dose of thiocyanate administered. He found a decreased ^{131}I uptake in the northern people of Idjwi Island in the Republic of Zaire (where goitre was prevalent) who had taken cassava, compared with those who had

taken imported rice (71·9 and 86·8 %, respectively), but the ingestion of cassava did not modify the uptake of iodine by the thyroid of those in the non-goitrous areas of the south (73·5 and 74·8 %, respectively).

Tewe (1975) has shown that iodine deficiency had no marked effect when rats and pigs were fed balanced diets in the presence of 750 and 500 ppm cyanide.

Maner (1972) compared the effects of raw cassava (150 mg HCN/kg), dried cassava (5 mg HCN) and sucrose (control) on the performance, thyroid development and urinary excretion of SCN in rats and found a better performance in those on raw cassava, apart from significantly larger quantities of SCN excretion (37-fold) compared with the control. Autopsy showed no differences in thyroid size in any of the treatments. Even when cyanide was added to the diets at 0–2400 ppm, with or without methionine supplementation, rats fed the methionine-supplemented diets exhibited a significantly ($p < 0{\cdot}01$) faster rate of growth than those fed unsupplemented diets and also excreted more SCN as the level of cyanide increased. Again, autopsy showed no indications of enlarged thyroids. Maner & Gomez (1973) therefore concluded that, although the detoxication of cyanide-produced SCN exerted a goitrogenic effect on the body, in the presence of adequate methionine and iodine, cyanide was without measurable effect on goitre production or nerve degeneration.

RECENT THINKING ON CYANIDE TOXICITY

Over a number of generations the body has developed very efficient mechanisms for detoxifying cyanide. This subject has already been reviewed by Oke (1973). In general there seems to be a low β-glucosidase activity in the small intestine of rats which decreases to a minimum after a weaning age of 21 days (Matsumoto *et al.*, 1972). The small amount of hydrolysis which occurs when cyanogenic glycosides are ingested by rats is therefore mainly due to the normal intestinal flora and most of it is excreted unchanged in the urine (Spatz, 1968). If this is so it would explain why the parenteral LD_{50} in rats is several times higher than the oral LD_{50}. Recently, Zitnak *et al.* (1977) have established a method for the determination of linamarin in biological tissues and this has enabled Barret *et al.* (1977) to assess the fate of linamarin fed to rats. An oral dose of 50 mg was sufficient to kill, within 4 h, 7 out of 10 rats, each weighing 100 g. With a dose of 30 mg no linamarin was detected in the faeces or blood, but 5·65 mg was found in the urine, along with 0·743 mg of thiocyanate, which is equivalent to about 7·1 mg of linamarin. A total of 12·75 mg was therefore accounted for after 72 h. The remaining linamarin might have been absorbed completely intact or partially hydrolysed and the products absorbed and/or lost in the faeces, or else the quantity in the blood was too dilute to be detected by the method employed.

Spatz & Lacquer (1968) explored the importance of bacterial flora in promoting

hydrolysis of the cyanogenic glycoside, cycasin, by comparing the excretion pattern in germ-free rats with that of normal rats. They found that the germ-free rats excreted cycasin quantitatively (75 % in urine, 25 % in faeces), indicating that they have no β-glucosidase, while in the normal rats about 20–30 % was recovered in the urine and practically none in the faeces, the remainder presumably having been metabolised. All the same, there is a considerable variation in the percentage excretion of the glucoside, indicating that the bacterial flora could significantly influence the metabolism of the glycoside—and hence the toxicity. Hill (1977) reported that when a 25 mg linamarin dose was fed to rats, clinical signs of toxicity, including apnea, ataxia and paresis, were produced. These were very marked in the absence of methionine supplementation, in which case 50 % of the rats died within 4 h. In the presence of adequate methionine supplementation, 10 % of the rats died and about 40 % showed no signs of toxicity. The activity of Na^+K^+-dependent ATPase was reduced in much the same way as it was by the glucoside, digitalis, which may raise the possibility that linamarin may be acting through the same mechanism, independent of cyanide. On the other hand, when a dose of 9·4 mg linamarin was given daily for 5 weeks, Hill (1977) found that there was no difference in weight gained between the control and the methionine-adequate rats (161 g and 160 g, respectively) and no mortality. However, the lactate/pyruvate ratio was significantly increased by linamarin and cardiac cytochrome oxidase activity was significantly decreased. There was an increase in the urinary thiocyanate (0·5 and 1·9 μg/24 h, respectively), indicating that some hydrolysis was taking place. However, when 80 mg of linamarin was fed as a mixture with food no ill-effects were found except for a small but significant increase in plasma thiocyanate concentration (0·40 versus 0·82 mg) and a decrease in ^{131}I uptake by the thyroid (0·77 versus 0·36 %). This decrease is probably sufficient to implicate it as a goitrogen.

Kobayashi & Matsumoto (1965) injected rats intraperitoneally with an aqueous solution of 138·7 mg cycasin and determined the concentration of the cycasin in urine spectrophotometrically by the chromotropic acid method and in the faeces by chromatography. None was found in the faeces, but almost all the cycasin was excreted in the first 12 h (95–99 %) and only traces (0·5–2 %) in the following 12 h. All the rats gained weight normally with no signs of any gross toxicity or abnormal histology, even when the compound was given a second time, after a week, and then a third time. The remainder of the animals were observed on the normal diet for 180 days and still showed no signs of toxicity. However, when rats were force-fed with 150 mg cycasin, all died within 24 h. When the dose was reduced to 45–50 mg, again, like the injection, most of the cycasin was excreted in the urine (30–63 %) within the first 12 h, and only traces (0·7 %) in the following 24 h. The rats were generally affected and lost weight within 2–3 days but thereafter they recovered. In spite of the fact that the aglycone of cycasin (MAM) is a more potent poison in that the body has no way of detoxifying it, animals could still tolerate it for days, excreting 12–76 % of

the cycasin, although some succumbed, e.g. one rat died after 3 days and two after 10 and 13 days. The high recovery (almost quantitative) of intact cycasin in the urine showed that the tissue does not metabolise or retain the glucoside and that the small amount unaccounted for was probably lost in collecting and handling, rather than metabolised. If it is assumed that the quantity unaccounted for was hydrolysed, then each of the rats that died must have had a quantity exceeding 40 mg of MAM released in their tissue (with no known way of detoxifying it) and this far exceeds the 35 mg estimated to be the lethal dose for MAM. In spite of this the remainder of the animals were able to survive.

Hill (1977) found that rats could tolerate 600–2400 ppm cyanide as KCN in their diets for 8 weeks. Examination of sections of the central nervous system, including observations on myelin and axons and sections of the thyroid, revealed no differences among the group of rats which could be attributed to the diets. This indicates that the rats have a very efficient system of detoxifying cyanide without harmful effects, even at high levels, as long as it is ingested normally with the feed. This ability is not confined to rats alone as Hill (1977) showed that a casein diet containing 1500 ppm KCN with no methionine supplementation could not support growth (weight gain, 1·6 g; urinary SCN, 8·8 mg) whereas with 0·4% methionine supplementation the weight gain was increased to 44·6 g and the urinary SCN to 34·2 mg, while 0·46% K_2SO_4 gave 13·2 g and 16·1 mg, respectively, showing that although the latter can supply some sulphur for detoxification it is not an efficient source. This was clearly shown using 35 g labelled methionine and K_2SO_4, in which case 27·8% of the 35 g was recovered as SCN in the urine of methionine-supplemented rats while 5·2% was from K_2SO_4.

PRECAUTIONS AGAINST CYANIDE TOXICITY

There is no doubt that cyanide is one of the most potent cytotoxic poisons and every effort should be made to eliminate as much of it as possible from edible plants. In general, cyanogenic glucosides occur extensively in many plants which are eaten by man and animals—e.g. millet, sorghum, cassava, lima beans, bitter almond, etc.—and sometimes the concentration is very high. They are normally partially hydrolysed by the β-glucosidase in the intestine to yield sugar, HCN and acetone or benzaldehyde. However, both man and animals have developed extremely efficient systems for detoxifying the toxic HCN to SCN which can be excreted in the urine (Oke, 1969). As the digestive tube develops, the concentration of the β-glucosidase in its walls continues to fall, although the quantity is still sufficient to cause some hydrolysis (Matsumoto *et al.*, 1972). The HCN is converted to SCN while the intact glucoside is absorbed into the general circulatory system and excreted intact into the urine. If by chance some of the glucoside is hydrolysed in the liver, it will there meet with a high concentration of rhodanese and hydroxocobalamine, etc. which will

detoxify it efficiently. It appears from this, and from the results of Spatz *et al.* (1966), that germ-free rats excrete the glucoside quantitatively intact and that normal tissues are quiescent as far as β-glucosidase activity is concerned, especially as no SCN is excreted even in normal rats when injected with the glucoside. Even when taken orally the SCN formed is effectively cleared within 24 h. This suggests that there is no cumulative or chronic toxicity from ingestion of the glucoside. It is analogous to an explosion which is heard only if one is not killed. If there is no toxicity in the first instance then there will be no residual toxicity. Olusi & Oke (1979) have administered several times the lethal dose of cyanide as KCN to experimental animals with no necrosis, necrobiosis or other cellular or tissue damage, most of the glucoside being excreted intact in the urine as inert substances. With cyanogenic glucosides, Spatz *et al.* (1966) found that after 24 h there is no more intact glycoside and no further SCN excretion, indicating that it is not stored in tissue sites to be released subsequently at a slow rate over prolonged periods. The only situation in which the glucoside seems to be toxic is when it is force-fed to animals in high concentrations (rather than mixed with food) in which case so much more cyanide might be released that it will overwhelm the capacity of the detoxifying systems and so become fatal.

On the other hand, what happens to people who consume cassava products three times daily, with the result that they have high plasma SCN and excrete a lot of SCN in their urine? De Lange (1973) attributed the high plasma SCN of the inhabitants of the northern part of Idjwi Island, Zaire (1·1 mg/100 ml or four times that of the Belgian control (0·24 mg)) and the high SCN excretion (14·3 mg/24 h compared with 10·0 mg for the southern people) to the high intake of cassava in this region which he therefore felt to be the cause of the high prevalence of goitre (55% compared with 5% in the south where little cassava is taken). The SCN was therefore taken as evidence of a goitrogenous factor in the foodstuff. In the same way, Suk (1931) reported that the goitrous population of Carpathian Ruthenia consume large quantities of cabbage, especially between October and December when pickled or raw stored cabbage represent the staple diet taken three times daily, just as those in Nigeria suffering from TAN take cassava products three times a day. However, the fact that the foodstuff has been proved to be goitrogenous in animals does not prove that its consumption would necessarily lead to goitre. Wagelin (1935) stated that inhabitants of south and west Switzerland ate more cabbage than those of other parts of the country where goitre was much more common.

Strictly speaking, cyanide should not constitute a danger to people who eat cassava because it has been known for a long time that it contains toxic principles which they remove in different ways, e.g. by steeping in water, drying, fermenting, etc. To make 'gari' the cassava is steeped in water and allowed to ferment for 3 days. Finally, the starchy part is fried on open fires in an iron pot, either alone or with palm oil. In general, garis prepared by frying alone contain about 3–27 ppm of cyanide but those fried with palm oil are virtually devoid of cyanide (Olanrewaju &

Baszormenyi, 1975). In the preparation of several types of 'lafun' the cassava is dried in the sun for several days before being pounded into a fine powder. This type of product may contain up to 10–20 ppm cyanide. These figures might have been underestimated in view of the recent work of Cooke & Maduagwu (1978) in which it was shown that decreases in total cyanide content in fresh cassava chips during dehydration, boiling or soaking were smaller than indicated by previous workers. In their analysis, Cooke & Maduagwu (1978) used an enzymic assay which is more sensitive, more reproducible and faster than earlier quantitative methods.

If it is assumed that about 100–2000 mg is the lethal dose for man, then as much as 10–20 kg will have to be consumed at a sitting to produce toxicity. No doubt the body is able to cope with the small amount of cyanide that is released from the glycoside when ingested. If we assume, as does Hill (1977), that only about 25% of the ingested glycoside is hydrolysed (and probably about 20% excreted unchanged) then the amount needed to constitute a lethal dose will be much higher still. Krebs (1970) believes that most of the glycosides are excreted unchanged in urine and faeces and thus present no toxic problems and this has been confirmed by the work of Matsumoto *et al.* (1972). If, by chance, a sufficient amount of thiocyanate is produced from cyanide in this way, in the absence of sufficient protein and iodine intake this may reduce the thyroid iodine uptake and possibly result in goitre. Even then it is not very certain because of the insignificant increases in weight in the thyroid gland reported by several workers who fed rats with a high dose of KCN or linamarin for several weeks (Hill, 1977; Umoh & Oke, 1974).

The dietary requirement for iodine is very low (70 mg/day or limits of 40–120 mg/day, depending on individuals) (Wayne *et al.*, 1964) and this could easily be met except in places where the soil is poor in iodine and hence the plants contain low quantities. As this will be confined to villages which depend on locally produced food, this may account for the higher incidence of this disease amongst the lower socio-economic classes than those in urban areas who depend on food transported from various places and who tend to consume food rich in iodine (fish, milk, eggs, iodised salt, etc.). On the other hand, SCN, in addition to inhibiting I^- transport, is a competitive substrate for the postulated thyroidal iodide peroxidase, thereby inhibiting iodination (Maloof & Soodak, 1966). Since SCN is present in small quantities in the body fluid, it can be increased to a critical level through frequent ingestion of cassava products, so that it can potentially affect the thyroid. Could it be that there might be other goitrogens present in cassava or the diets of the people in the goitre-endemic areas reported by Ekpechi (1964) and De Lange (1973)? Can it be due to their diathesis? We know that not all forms of nutrient have equal activity and that, for unknown reasons, some individuals have greatly increased requirements while others are susceptible to toxicity from excess. Have these people lost the ability to dispose of the thiocyanate being formed or are they metabolising the glucoside at such an unusual rate that there is enough thiocyanate to compete with iodine, or is the problem simply one of iodine or protein deficiency? I pose these problems so that

none of the salient points will be overlooked in our subsequent interpretations of data to prove the exact causes of goitre and TAN.

CONCLUSIONS

It appears *a priori* that ingestion of foodstuffs such as cassava, rich in cyanogenic glucosides, may be the cause of TAN and goitre. Recent experiments using KCN and linamarin indicate that the relationship may be more complex. Lack of good quality protein—and hence methionine—may be responsible, to a large extent, in the aetiology of TAN in cassava-eating areas, while lack of iodine may be responsible for goitre.

With a strategy that will improve the socio-economic condition of the people, coupled with an integrated nutrition programme (public hygiene, better food, immunisation, family planning, health and improved water supply, improved transportation of foodstuffs), nutritional diseases such as tropical ataxic neuropathy and goitre will become history in Nigeria just as pellagra is now in the United States of America.

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Food Chemistry 6 (1980–81) 111–114

ANTITHIAMIN ACTIVITY OF TEA FRACTIONS

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ABSTRACT

Tea infusion liquor was separated into molecular weight fractions on various grades of Sephadex gels and the antithiamin activity of these fractions was determined. It was found that tea liquor contains three molecular weight fractions that affect thiamin recovery. The fraction containing the very high molecular weight components had a high level of antithiamin activity but three fractions of low to moderate molecular weight components appeared to protect thiamin from inactivation.

INTRODUCTION

Tea ranks as one of the world's most popular beverages and is consumed in virtually all countries. It is consumed mainly for its refreshment value but can be a minor source of certain vitamins and minerals (Stagg & Millin, 1975). It has, however, been found to possess anti-nutrient properties. Vimokesant *et al.* (1974) examined a group of people in Thailand with thiamin deficiency and attributed the cause to their substantial ingestion of tea. Hilker *et al.* (1971) examined the antithiamin activity of a range of types of tea and concluded that this activity was due to the tannins which comprise 30–50% of the total soluble solids (Millin *et al.*, 1969*a*). Many workers (e.g. Berueter & Somogyi, 1967; Hilker, 1968; Somogyi & Bonicke, 1969; Sarkar & Chaudhuri, 1976) have found that a wide range of monomeric phenolic acids possess antithiamin activity and have established the belief that these polyphenols are responsible for the antithiamin activity of various plants. The tannins in fresh tea leaves are mainly low molecular weight polyphenols (Bokuchava & Skobeleva, 1969) but these compounds polymerise extensively during the processes that result in an aqueous infusion of black tea (Millin *et al.*, 1969*a*, *b*). In the study reported in this paper we have separated a tea infusion into molecular weight fractions by exclusion chromatography on Sephadex gels and determined the antithiamin activity of each fraction.

Food Chemistry 0308-8146/80/0006-0111/$02·50

MATERIALS AND METHODS

Black tea leaves (1 g) were added to boiling distilled water (100 ml). The mixture was allowed to cool to ambient temperature with occasional stirring, then filtered and an aliquot (25 ml) of the tea liquor was diluted to 250 ml with distilled water.

Sephadex gels (Pharmacia Fine Chemicals) of various grades from G-10 to G-200 were prepared in distilled water with a packing bed volume of 30–40 ml for each column. Blue dextran 2000 (Pharmacia Fine Chemicals), a water-soluble dye of high molecular weight, was used to determine the void volume and the excluded volume of each column (MacLeod, 1973). The excluded fraction from each column contained all the components that were too large to be delayed in their passage through that particular column. An aliquot (2 ml) of the diluted tea liquor was pipetted onto each of the Sephadex columns. Distilled water was passed through each column and the excluded fractions were collected. A solution (8 ml) of thiamin hydrochloride (5 μg/ml) was added to each fraction and the mixtures were allowed to stand at 30 °C for 90 min. The pH of the mixtures was 2·3, which is a stable pH for thiamin (Dwivedi & Arnold, 1973).

Analysis of the thiamin remaining in solution was by the AOAC method (AOAC, 1975). This involved purification by adsorption on—and subsequent release from—a base exchange resin (Decalso) and conversion to thiochrome which was assayed by fluorimetry.

RESULTS AND DISCUSSION

The data in Table 1 show that 22 % of the thiamin added to the total tea infusion liquor was destroyed or inactivated when the solution of tea plus thiamin was allowed to stand for 90 min at 30 °C. However, when the tea liquor was separated into molecular weight fractions, a higher percentage of the thiamin added to each fraction was found to be inactivated. The components in the tea liquor large enough to pass unimpeded through the Sephadex G-200 column had an antithiamin activity of 68 %. The unimpeded components collected from the G-150, G-100, G-75 and G-50 columns had a similar antithiamin activity. Since the compounds that passed through the G-200 column were also eluted from all other columns, the antithiamin activity in the G-50 to G-150 fractions would be due to the high molecular weight compounds collected from the G-200 column. The solution obtained from the G-25 column had an antithiamin activity of 49 % which is significantly less than that exhibited by the G-50 to G-200 fractions. This suggests that compounds of a molecular weight low enough to be impeded by their passage through the G-50 column but which passed unimpeded through the G-25 column have some ability to prevent the inactivation of thiamin. The antithiamin activity of the G-15 fraction was not significantly different from that of the G-25 fraction, indicating that the

TABLE 1
ANTITHIAMIN ACTIVITY OF TEA LIQUOR SEPARATED INTO MOLECULAR WEIGHT FRACTIONS BY EXCLUSION CHROMATOGRAPHY WITH SEPHADEX

Fraction	*Molecular weight of compounds in fraction*†	*Percent of recovery of thiamin added*††	*Antithiamin activity* (%)
Water	Nil	100[d]	0
Total tea liquor	All	78·5[a]	22
Eluant from G-200	$>2 \times 10^5$	32·5[c]	68
Eluant from G-150	$>1{\cdot}5 \times 10^5$	32·0[c]	68
Eluant from G-100	$>10^5$	34·2[c]	66
Eluant from G-75	$>5 \times 10^4$	34·2[c]	66
Eluant from G-50	$>10^4$	34·6[c]	65
Eluant from G-25	>5000	50·6[b]	49
Eluant from G-15	>1500	58·2[b]	42
Eluant from G-10	>700	75·0[a]	25

† Molecular weight limits of the fractions excluded from the Sephadex gels are those reported for dextran polymers (Anon., 1977).
†† Values are means of six determinations. Different alphabetical superscripts indicate that values are significantly different at $P = 0{\cdot}05$ by Duncan's Multiple Range test (Little & Hills, 1978).

newly eluted compounds did not greatly affect thiamin activity. The eluate from the G-10 column, however, had a further reduced antithiamin activity (25%), suggesting that additional compounds which prevented thiamin inactivation were eluted from this column. The G-10 eluate had a similar antithiamin activity to the whole tea liquor.

Table 1 also gives the molecular weight limits of dextran carbohydrate polymers excluded from the Sephadex gels. These values can only be used as an indication of the molecular weight ranges of compounds likely to be found in the fractions of interest. The polymers in tea liquor have been shown to be comprised of carbohydrate and protein, as well as polyphenol polymers (Millin *et al.*, 1969*a*, *b*). It is well recognised that different types of polymer have different affinities for the gel phase relative to the aqueous phase; in particular, that aromatic substances are retarded on a column compared with aliphatic compounds of similar molecular weight (Anon, 1977). The values shown in Table 1, however, give some indication of the size of the polymers. Of the three molecular weight fractions of tea liquor that affect thiamin recovery, the antithiamin activity is contained in the very high molecular weight polymers, possibly of the order of >200,000 molecular weight. The two lower molecular weight polymer fractions, possibly within the range 700–10,000 molecular weight, appear to be able to protect thiamin from inactivation.

Many polyphenol monomers have been found to possess antithiamin activity in a wide range of plants but our results suggest that polyphenol monomers are not active in brewed tea liquor as the eluant from the G-10 column, which would contain all compounds except those of low molecular weight (possibly <700), had a similar

thiamin recovery to whole tea liquor. If polyphenols are the active antithiamin compounds in tea, they must be present as end groups or side chains on the high molecular weight polymers.

The thiamin-protective polymers in tea could be proteins. Dwivedi & Arnold (1973), in a review of thiamin degradation, reported that in model systems various proteins such as albumin and casein have been found to protect thiamin from oxidation by the formation of a stable but reversible complex between thiamin and the protein. Leichter & Joslyn (1969) have reported that soluble starch can protect thiamin. This raises the possibility that some of the carbohydrate polymers in the tea liquor could also be protecting thiamin.

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Food Chemistry 6 (1980–81) 115–122

THE CATALYSIS OF THE *N*-NITROSATION OF SECONDARY AMINES BY NITROSOPHENOLS

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ABSTRACT

p-Nitrosophenols and the structurally related compounds, p-nitroso-N-alkylanilines and p-nitroso-N-dialkylanilines, have been found to catalyse the N-nitrosation of pyrrolidine and morpholine. The p-nitroso-o-cresol catalysed N-nitrosation of pyrrolidine at pH 5 was found to be first order with respect to each of nitrosocresol, pyrrolidine and nitrite. The third order rate constant for the reaction has a value of 2·08 mole^{-2} litre^{2} min^{-1}. The corresponding reaction with morpholine follows similar kinetics and has a third order rate constant of 18·47 mole^{-2} litre^{2} min^{-1}. p-Nitroso-o-cresol has no effect on the N-nitrosation of N-methylaniline. A mechanism is proposed in which the nitrosophenol reacts, via its quinonemonoxime tautomer, with nitrous acid (or species in equilibrium with nitrous acid) to produce an intermediate nitrosating agent which undergoes attack by the amine to produce the N-nitrosamine and regenerate the catalyst.

INTRODUCTION

The effects of phenolic compounds on the rate of N-nitrosation of secondary amines have been the subject of some confusion. Challis (1973) has calculated that the C-nitrosation of phenol should be faster than the N-nitrosation of dimethylamine by a factor of about 10^4 at pH 1·5 and 25 °C. This reasonably leads to the expectation that the presence of phenols would inhibit the N-nitrosation of secondary amines. Tannins inhibit *N*-nitrosamine formation (Bogovski *et al.*, 1972) and inhibition of the N-nitrosation of morpholine at pH 3 was observed in the presence of some phenolic constituents of smoked foods (Issenberg & Virk, 1974). However, gallic acid has been reported to catalyse the N-nitrosation of diethylamine (Walker *et al.*,

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1975) and 4-methylcatechol and chlorogenic acid to act similarly on the N-nitrosation of piperidine at gastric pH (Challis & Bartlett, 1975). In this laboratory we studied the competitive nitrosations of pyrrolidine and *p*-cresol and found that, under certain conditions, the presence of *p*-cresol enhanced *N*-nitrosopyrrolidine formation (Davies *et al.*, 1978*b*).

In an attempt to resolve this confusing situation we examined the effects of the products of the C-nitrosation of phenols on the N-nitrosation of secondary amines. The initial product of phenol nitrosation is a *C*-nitrosophenol. Many nitrosophenols are unstable under aerobic conditions and are oxidised to the corresponding *C*-nitrophenol. Nitrophenols have no effect on the N-nitrosation of secondary amines. However, we have recently found that *p*-nitroso-*o*-cresol catalyses the N-nitrosation of pyrrolidine at pH 5 (Davies & McWeeny, 1977; Davies *et al.*, 1978*a*). In this paper we describe the results of our further studies on the catalytic effect of nitrosophenols on *N*-nitrosamine formation.

RESULTS AND DISCUSSION

N-Nitrosation of pyrrolidine and morpholine in the presence of p-*nitroso*-o-*cresol*

At pH 5·0 and 37 °C, in the absence of *p*-nitroso-*o*-cresol, the rate of N-nitrosation was found to be dependent upon the square of the nitrite concentration and on the amine concentration, as shown by eqn. (1), using the methods of initial rates.

$$\text{Rate} = {}^{1}\bar{k}_3[\text{amine}][NO_2^-]^2 \qquad (1)$$

Table 1 lists values of ${}^{1}\bar{k}_3$ corresponding to a range of initial nitrite concentrations at pH 5·0.

The rate of reaction is enhanced in the presence of *p*-nitroso-*o*-cresol at pH 5·0 and 37 °C. As shown in Fig. 1, the initial rate of *N*-nitrosopyrrolidine (NPYR) formation at pH 5·0 is proportional to *p*-nitroso-*o*-cresol concentration. For the N-nitrosation reaction in the presence of *p*-nitroso-*o*-cresol the rate equation (eqn. (1)) needs to be modified to include an additional term, as shown by eqn. (2).

$$\text{Rate} = {}^{1}\bar{k}_3[\text{amine}][NO_2^-]^2 + F[p\text{-nitroso-}o\text{-cresol}] \qquad (2)$$

To obtain information concerning the mechanism of the N-nitrosation reaction in the presence of *p*-nitroso-*o*-cresol, it is necessary to perform two sets of kinetic runs under equivalent conditions. One set is carried out in the absence of the nitrosocresol, these being the control reactions, and the other set is carried out in the presence of nitrosocresol and we have called these total reactions. Subtraction of the rates of nitrosamine formation determined in the control reactions from those found in the corresponding total reactions gives the rates of nitrosamine formation solely by the mechanism involving nitrosocresol. By varying the concentrations of one of the reactants in turn and holding constant the concentrations of the other two, the

TABLE 1

VALUES OF $^1\bar{k}_3$ FOR THE REACTION OF (i) PYRROLIDINE (0·05 MOLE LITRE^{-1}) WITH NITRITE AND (ii) MORPHOLINE (0·005 MOLE LITRE^{-1}) WITH NITRITE; VALUES OF $^2\bar{k}_3$ FOR THE REACTION OF (iii) PYRROLIDINE (0·05 MOLE LITRE^{-1}) WITH NITRITE CATALYSED BY *p*-NITROSO-*o*-CRESOL ($0{\cdot}5 \times 10^{-3}$ MOLE LITRE^{-1}) AND OF (iv) MORPHOLINE (0·005 MOLE LITRE^{-1}) WITH NITRITE CATALYSED BY *p*-NITROSO-*o*-CRESOL ($1{\cdot}0 \times 10^{-3}$ MOLE LITRE^{-1}) AT pH 5, 37°C

$[NO_2^-]$ (*mole litre*$^{-1}$)	$^1\bar{k}_3$ (*pyrrolidine*) (*mole*$^{-2}$ *litre*^{2} *min*$^{-1}$)	$^2\bar{k}_3$ (*pyrrolidine*) (*mole*$^{-2}$ *litre*^{2} *min*$^{-1}$)
0·025	$2{\cdot}77 \times 10^{-3}$	2·14
0·05	$3{\cdot}81 \times 10^{-3}$	2·07
0·075	$4{\cdot}14 \times 10^{-3}$	2·03
0·100	$3{\cdot}99 \times 10^{-3}$	2·08
0·125	$3{\cdot}95 \times 10^{-3}$	2·08
$[NO_2^-]$ (*mole litre*$^{-1}$)	$^1\bar{k}_3$ (*morpholine*) (*mole*$^{-2}$ *litre*^{2} *min*$^{-1}$)	$^2\bar{k}_3$ (*morpholine*) (*mole*$^{-2}$ *litre*^{2} *min*$^{-1}$)
0·005	2·13	17·2
0·010	2·17	18·6
0·015	2·21	19·5
0·020	2·10	18·9
0·025	2·04	18·1

order of the nitrosocresol-enhanced N-nitrosation reaction was determined with respect to each reactant, using the method of initial rates. Thus, a plot of log (initial rate) against each of log [amine], log[nitrite] and log[*p*-nitroso-*o*-cresol] in turn was linear with unit slope, demonstrating the first order dependency of the reaction on each reactant. Equation (2), describing the rate of the total reaction, can therefore be re-written as eqn. (3):

$$\text{Rate} = {}^1\bar{k}_3(\text{amine})(NO_2^-)^2 + {}^2\bar{k}_3(\text{amine})(NO_2^-)(p\text{-nitroso-}o\text{-cresol}) \qquad (3)$$

The nitrosocresol-enhanced N-nitrosations of pyrrolidine and morpholine therefore differ from the control reaction in their order with respect to nitrite. The second order dependency of the control reaction suggests that N_2O_3 is the effective nitrosating agent, whereas the first order dependency of the nitrosocresol-enhanced reaction suggests the nitrosating agent to be HNO_2, NO^+, H_2ONO^+ or NO-citrate buffer. The high reaction pH tends to favour either nitrous acid or NO-citrate. Nitrous acid has been reported to effect the nitrosation of phenols (Schmid *et al.*, 1966); however, Challis & Butler (1968) have subsequently suggested that this is unlikely in view of the poor electrophilic nature of this species in diazotisation (Challis & Lawson, 1971). Our present results do not permit exact identification of the nitrosating agent which we have therefore termed NOX.

Table 1 shows values of $^2\bar{k}_3$ corresponding to varying initial concentrations of nitrite. The values are constant, and similarly constant values of $^2\bar{k}_3$ have been

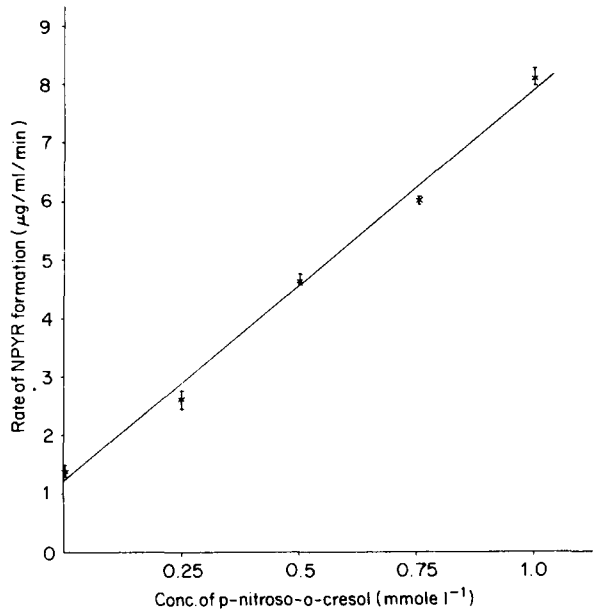

Fig. 1. Dependence of the initial rate of NPYR formation in the reaction of pyrrolidine (0·15 mole litre^{-1}) with nitrite (0·15 mole litre^{-1}) at pH 5·0, 37 °C on the concentration of *p*-nitroso-*o*-cresol.

obtained corresponding to varying initial amine and *p*-nitroso-*o*-cresol concentrations. The averages obtained for $^{2}\bar{k}_3$ were 2·08 mole^{-2} litre^2 min^{-1} and 18·47 mole^{-2} litre^2 min^{-1} for pyrrolidine and morpholine, respectively. These are approximately 0·5 × 10^3 and 8·5 times greater than the corresponding values of $^{1}\bar{k}_3$, the rate constants for the control reactions at pH 5·0.

The pH-rate profile for the nitrosocresol-enhanced N-nitrosation of pyrrolidine is shown in Fig. 2. For comparison the pH-rate profile of the control reaction is included. The nitrosocresol-enhanced reaction has its maximum rate at about pH 5·0 and this demonstrates clearly that the reaction proceeds by a different mechanism to the control reaction, which has its maximum rate at about pH 3·0.

N-Nitrosation of N-*methylaniline in the presence of* p-*nitroso*-o-*cresol*

It was found that *p*-nitroso-*o*-cresol (0·5 mmole litre^{-1}) had no observable effect on the rate of reaction between *N*-methylaniline (1 mmole litre^{-1}) and nitrite (1 mmole litre^{-1}) at pH 5·0, 37 °C.

Related compounds capable of enhancing the N-nitrosation of pyrrolidine

In order to obtain further information regarding the role of the nitrosocresol in the enhanced N-nitrosation reaction, other compounds were tested for their ability

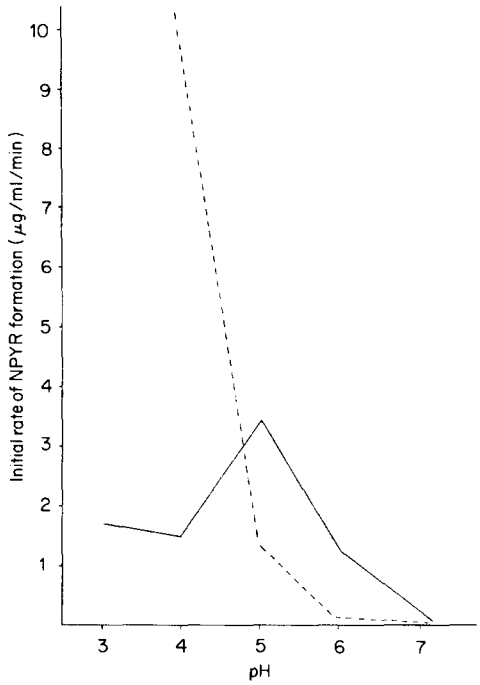

Fig. 2. pH-initial rate profile for the reaction of pyrrolidine (0·15 mole litre^{-1}) with nitrite (0·15 mole litre^{-1}) at 37 °C in the presence and absence of *p*-nitroso-*o*-cresol (0·5 × 10^{-3} mole litre^{-1}). ——, Catalysed reaction; – – –, uncatalysed reaction.

to enhance the N-nitrosation of pyrrolidine at pH 5·0. Table 2 lists all the compounds tested and divides them into two categories: those that do and those that do not enhance the N-nitrosation reaction. An examination of the structures of those compounds which do enhance N-nitrosation shows that all are capable of tautomerism to quinonemonoximes or quinonemonoxime imines. None of the compounds which do not enhance N-nitrosation is capable of such tautomerism.

TABLE 2

COMPOUNDS TESTED AT 0·5 × 10^{-3} MOLE LITRE^{-1} CONCENTRATION FOR ANY CATALYTIC EFFECT ON THE REACTION OF PYRROLIDINE (0·15 MOLE LITRE^{-1}) WITH NITRITE (0·15 MOLE LITRE^{-1}) AT pH 5, 37 °C

Catalysts	*Non-Catalysts*
p-Nitroso-*o*-cresol	Nitrosobenzene
p-Nitrosophenol	*p*-Nitrosophenazone
p-Nitrosodiethylaniline	Acetaldoxime
p-Nitroso-*N*-methylaniline	*p*,*N*-Dinitroso-*N*-methylaniline
p-Nitrosothymol	
1-Nitroso-2-naphthol	
2,4-Dinitrosoresorcinol	

The ability to enhance the N-nitrosation reaction therefore correlates well with the ability to undergo this tautomeric change. The probable reason for *p*-nitrosophenazone not being able to enhance the N-nitrosation reaction is that the molecule is prevented from attaining the planar configuration about the nitrogen atom necessary for tautomeric change by the constraint of the nitrogen being part of a five-membered ring.

It has been established that *p*-nitroso-*N*,*N*-diethylaniline exerts its enhancing effect in its own right and that under the experimental conditions it is not hydrolysed to *p*-nitrosophenol. All the compounds which did enhance N-nitrosation gave a constant rate of NPYR formation over the period of 30 min for which each reaction was studied, with the exception of *p*-nitroso-*N*-methylaniline. In the presence of this compound the rate decreased with time and this was found to be due to the formation of *p*,*N*-dinitroso-*N*-methylaniline which has no effect on the rate of the N-nitrosation reaction.

The effect of *p*-nitroso-*o*-cresol on the N-nitrosation of pyrrolidine was found to be a genuine catalytic effect in that the nitrosocresol remained unchanged at the end of the reaction. It was also established that no reaction between pyrrolidine and *p*-nitroso-*o*-cresol occurs under the conditions in which these experiments were carried out.

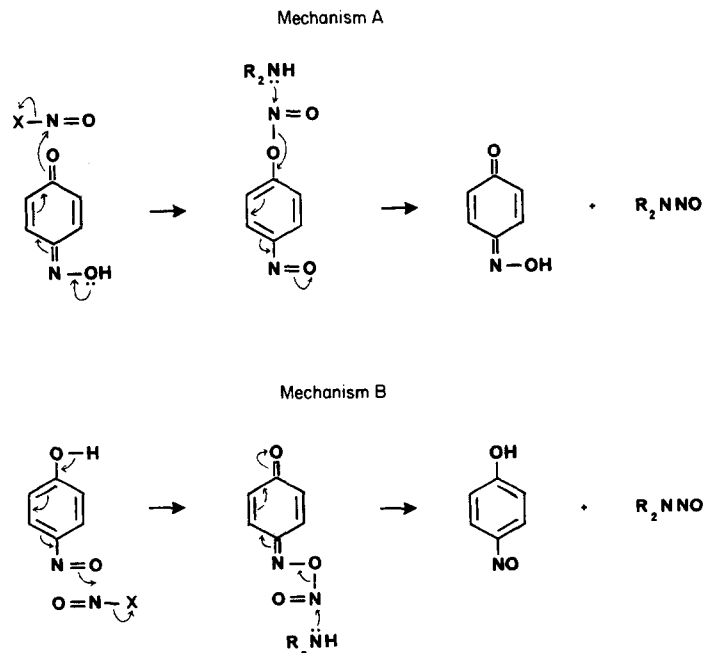

Fig. 3. Possible mechanisms for the nitrosophenol catalysed N-nitrosation of pyrrolidine.

Mechanism

The form of eqn. (3) implies that nitrosation of the amine is the rate-limiting step in the catalysed reaction. The rate of this reaction is proportional to the concentrations of the unprotonated amine, the catalyst and nitrous acid (or species NOX in equilibrium with nitrous acid). Two mechanisms which correlate with eqn. (3) are shown in Fig. 3. In either case the mechanism can be thought of as an initial reversible reaction between the nitrosophenol and NOX to form an intermediate nitrosating agent. This is followed by a slower reaction in which the amine attacks the intermediate, resulting in the formation of the nitrosamine and the regeneration of the nitrosophenol.

As regards the choice between the two mechanisms, A and B, it is useful to consider the N-nitrosation reaction catalysed by *p*-nitroso-*N*-methylaniline. If mechanism A were the correct one the intermediate nitrosating agent formed would be *p*,*N*-dinitroso-*N*-methylaniline. However, this compound is listed in Table 2 as one which has no effect on the N-nitrosation reaction. For this reason we believe that mechanism B is to be preferred.

EXPERIMENTAL

All reagents were analytical grade. Dichloromethane was distilled once prior to use.

Kinetics of the N-nitrosation in the presence of catalyst

The initial reagent concentrations were as described in Table 1. Buffer solutions in the pH range of 3·0 to 7·0 were prepared by mixing appropriate volumes of 0·1 M citric acid and 0·1 M tri-sodium citrate solutions. In preliminary tests, sodium nitrite was dissolved in 90 ml buffer solution at reaction pH and the volume (x ml) of perchloric acid or 5% Na_2CO_3 solution required to readjust the pH of the solution was noted.

The amine was dissolved in 40 ml of buffer and the pH readjusted with perchloric acid, a further x ml of perchloric acid or 5% Na_2CO_3 solution, as appropriate, was added and the volume made up to 50 ml with buffer. The catalyst was separately dissolved in 50 ml of buffer and both solutions were incubated at 37 °C. Reactions were initiated by the addition of these solutions to solid sodium nitrite. At the appropriate time a 10-ml aliquot of the reaction solution was quenched with 5 ml of 2 N H_2SO_4 containing 0·25 g of ammonium sulphamate, extracted with 3 × 3 ml of dichloromethane, and a 1 ml solution of the internal standard added.

A Pye 104 gas chromatograph equipped with a glass column (1·5 m × 6·36 mm outside diameter) of 5% Carbowax 20 M-TPA on Diatomite CLQ 100-120 mesh and a flame ionisation detector was used for the analyses of NPYR and *N*-nitrosomorpholine. The chromatograph was operated isothermally and with a nitrogen carrier gas flow rate of 60 ml min^{-1}. At 145 °C NPYR and the internal

standard (*N*-nitrosopiperidine) eluted after 5·8 and 5·2 min, respectively; at 150 °C *N*-nitrosomorpholine and *N*-nitrosopiperidine eluted after 5·5 and 4·0 min, respectively. Peak areas were measured using an Infotronics CRS 309 integrator and the identities of the nitrosamines were checked periodically by gas chromatography–mass spectrometry.

N-Nitroso-*N*-methylaniline was analysed by reverse phase hplc using a Partisil ODS column. The eluent, 30/70 ethanol/water, was pumped at 1·5 ml min^{-1} and the uv detector was operated at 270 nm.

To determine whether p-*nitrosodiethylaniline is hydrolysed to* p-*nitrosophenol*

A solution of *p*-nitrosodiethylaniline (8·9 mg) in 0·1 M citrate buffer, pH 5·0, (100 ml) was left overnight at 37 °C. Analysis by reverse phase hplc showed no decrease in the concentration of *p*-nitrosodiethylaniline and no trace of *p*-nitrosophenol.

To determine whether p-*nitroso-o-cresol is consumed in the N-nitrosation of pyrrolidine*

A reaction solution containing nitrite, pyrrolidine and *p*-nitroso-*o*-cresol was made up in 0·1 M citrate buffer, pH 5·0, as described above. Analysis of the sample by reverse phase hplc after 0 and 30 min showed no decrease in the concentration of the catalyst.

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Food Chemistry 6 (1980–81) 123–131

ON THE COLOUR REACTIONS OF POTATO GLYCOALKALOIDS IN STRONG ACIDS IN THE PRESENCE OF PARAFORMALDEHYDE

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ABSTRACT

The colour reaction of potato glycoalkaloids, containing solanidine as steroid, with strong acids and paraformaldehyde was examined to elucidate the kinetics and mutual relationship of the 'Clarke' and 'Marquis' reactions. It was found that the influence of time of addition of paraformaldehyde was the major factor in deciding the type of colour changes encountered. This factor and the specific absorption kinetics have important consequences for the use of these types of colour reactions as a quantitative assay. According to the structural similarity of the steroidal part of potato glycoalkaloids and cholesterol, colour reactions of both have been compared, indicating that both steroids react in strong acids in the presence of an oxidator, provided that the more lipophilic character of cholesterol is taken into account. Therefore a similar mechanism of a serial oxidation of carbonium ions as in the case of cholesterol, is suggested as the basis for these specific colour reactions of potato glycoalkaloids.

INTRODUCTION

Because of their poisonous nature, potato glycoalkaloids have been investigated starting from their discovery in 1820 by the pharmacist Desfosses up to the present time. Quantitative determination of the glycoalkaloids from the cultivated potato (*Solanum tuberosum* L.) has been thoroughly explored and methods have been changed from gravimetric to colour reactions with strong acids (Jadhav & Salunkhe, 1975). In addition, polarographic (Pierzchalski & Mrozowska, 1968), titrimetric (Fitzpatrick & Osman, 1974; Gyenes, 1961) and gas–chromatographic (Herb *et al.*, 1975; Roosen-Runge & Schneider, 1977; Siegfried, 1976) methods are known. It must be emphasised that most of these methods are especially adapted to analysis of potato samples and only some of them are suited for toxicological analysis.

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The main glycoalkaloids of the cultivated potato are α-solanine and α-chaconine. Both contain the same steroidal part, solanidine, and differ regarding the covalently bonded sugar molecules: for α-solanine the sugar chain is composed of O-β-D-glucopyranosyl-(1→3)-O-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranose and for α-chaconine of O-α-L-rhamnopyranosyl-(1→4)-O-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranose. Besides these main glycoalkaloids, potatoes may contain β and γ forms of solanine and chaconine with shorter sugar chains or the aglycone solanidine alone (Kuhn & Löw, 1954). Quantitative methods are based on the nature of the aglycone solanidine, which is a characteristic organic steroidal base and has a close relationship to the structure of cholesterol.

Three colour reactions have been used extensively for quantitative assay of these potato glycoalkaloids: 1, reaction of the potato glycoalkaloids with concentrated sulphuric acid followed by the addition of an aqueous solution of formaldehyde (Alberti, 1932; Pfankuch, 1938; Baker *et al.*, 1955; Schreiber *et al.*, 1961); 2, reaction of the potato glycoalkaloids with a mixture of concentrated phosphoric acid and paraformaldehyde (Clarke, 1958; Schreiber *et al.*, 1961; Sachse & Bachmann, 1969; Wang *et al.*, 1972); 3, reaction of the potato glycoalkaloids with a mixture of concentrated hydrochloric acid and antimony trichloride (Wierzchowski & Wierzchowska, 1961; Bretzloff, 1971).

These methods are based on time-dependent colour reactions of the steroidal part of the potato glycoalkaloids and have been used with several modifications. Little is known about their specific mechanisms or mutual relationships and therefore confusion exists about their value for quantitative assay. In this paper evidence for the nature of the mutual relationship of the first two colour reactions is given and the consequence of this for quantitative assay is discussed. The latter antimonychloride/hydrochloric acid reaction is not considered here, although data on this may also be important. In addition, a possible mechanism is suggested by analogy and comparison with colour reactions of cholesterol.

MATERIAL AND METHODS

Reagents

Sulphuric acid 95–97% PA; phosphoric acid 85% PA; paraformaldehyde from Merck. Reference α-solanine from USDA ARS Eastern Regional Research Center (Porter, 1972); cholesterol standard for chromatography from Sigma.

Procedures for colour reactions of potato glycoalkaloids

1. 'Marquis' reaction: 0·4 ml of potato glycoalkaloids (1 mg/ml*) (dissolved in 7% (w/w) H_3PO_4), is mixed thoroughly with 4 ml of 70% (w/w) of aqueous sulphuric

* Weight relative to α-solanine.

acid. A typical yellow colour develops and five minutes after the initial mixing 1·2 mg paraformaldehyde (0·03 % w/v) is added and the mixture again thoroughly mixed. Colour development: the yellow colour changes to orange, then to red and finally to a violet red.

2. 'Clarke' reaction: 0·4 ml of a solution of potato glycoalkaloids (1 mg/ml), dissolved in 7 % (w/w) H_3PO_4 is thoroughly mixed with 4 ml of 85 % (w/w) phosphoric acid, containing 1·2 mg paraformaldehyde (0·03 % w/v). Colour development: a blue colour develops, reaching a maximum after 30–40 min and then slowly fades.

Spectral analysis of colour reactions

Spectra of coloured glycoalkaloid solutions were taken from 700–200 nm at a speed of 100 nm/min with a Beckman model 26 scanning spectrophotometer. Time studies at a fixed wavelength were recorded at a paper speed of 0·1 inch/min. Colour reactions have been carried out at room temperature.

RESULTS AND DISCUSSION

Reaction of potato glycoalkaloids with strong acids

When the reaction of potato glycoalkaloids with strong acids is followed in 66 % and 80 % H_3PO_4 a yellow colour develops in time with a characteristic absorbance maximum at 405 nm (see Figs 1 and 2). In sulphuric acid there is a very rapid increase in a few minutes and then a gradual decline, while in phosphoric acid this yellow intermediate develops more gradually, reaching a maximum after 40–50 min and then declines gradually.

Reaction of potato glycoalkaloids with sulphuric acid followed by paraformaldehyde

If paraformaldehyde is added after development of the yellow intermediate (formaldehyde has the same effect) a typical colour change is initiated by a rapid decrease of the 405 nm maximum followed by a rapid increase of two absorbance maxima at 470 nm and 510 nm. These latter maxima in turn decrease and a new maximum at 570 nm arises (see Figs. 3 and 4), resulting in an isobestic point at 530 nm. These colour changes are typical for the 'Marquis' reaction of potato glycoalkaloids and a continuously increasing absorbance maximum at 205 nm is also observed.

Reaction of potato glycoalkaloids with phosphoric acid in the presence of paraformaldehyde

In the reaction of potato glycoalkaloids in 80 % H_3PO_4 containing 0·03 % (w/v) paraformaldehyde, a typical blue colour develops with an absorbance maximum at 600 nm together with a minor absorbance maximum at 510 nm and an absorbance

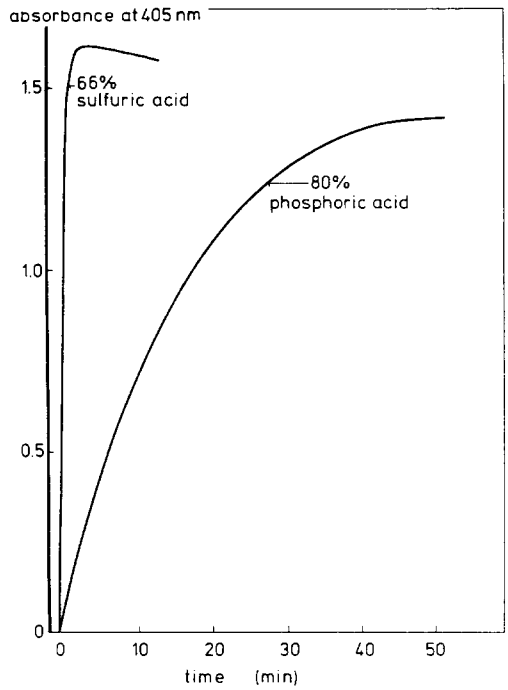

Fig. 1. Colour reaction of potato glycoalkaloids in 66% (w/w) sulphuric acid and 80% (w/w) phosphoric acid. Kinetics of absorbance at the fixed wavelength 405 nm.

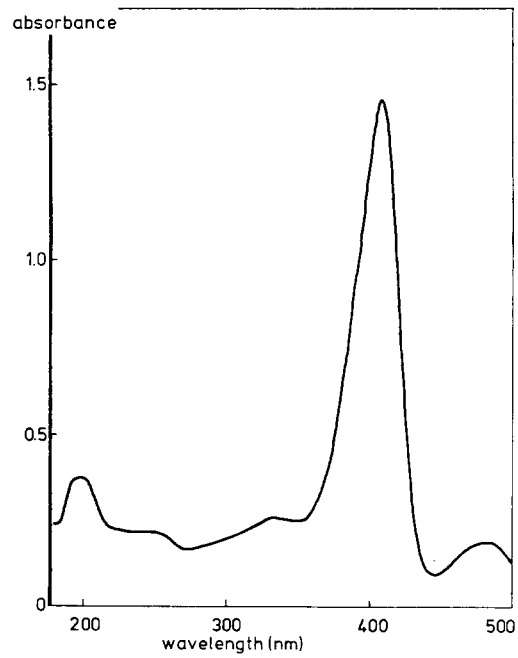

Fig. 2. Colour reaction of potato glycoalkaloids in sulphuric and phosphoric acid. Spectrum obtained with both acids, when maximum absorbance at 405 nm is reached.

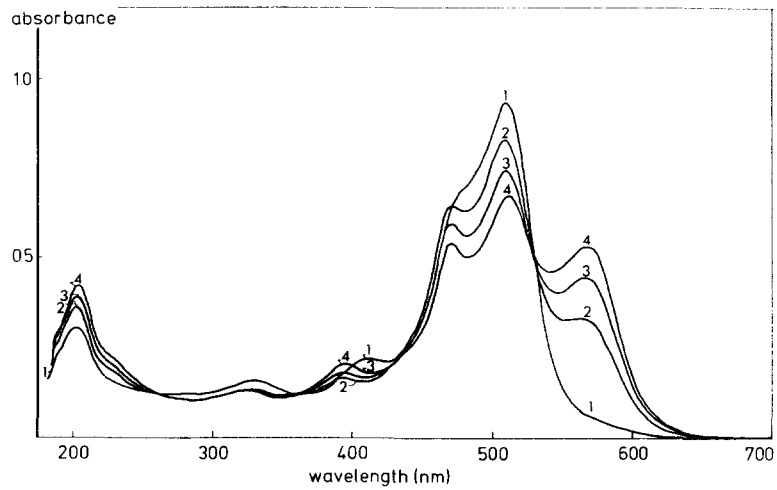

Fig. 3. 'Marquis' colour reaction. Spectra of glycoalkaloids in sulphuric acid/paraformaldehyde taken at 1–5, 15–20, 40–45 and 80–85 min, respectively. The numbers 1, 2, 3 and 4 refer to the spectra recorded at 1–5, 20–25, 40–45 and 80–85 min, respectively.

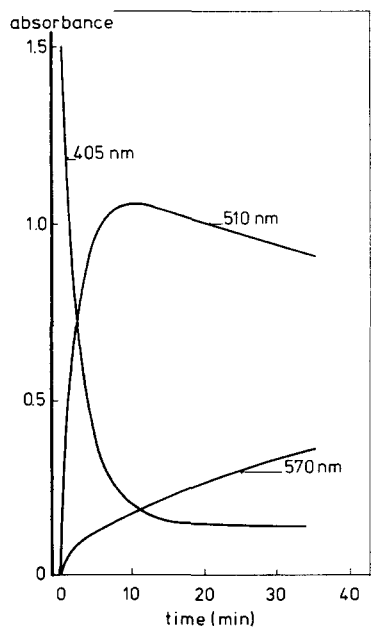

Fig. 4. 'Marquis' colour reaction. Kinetics of absorbance at fixed wavelengths: 405 nm, 510 nm and 570 nm.

maximum at 660 nm, which continues to increase after fading of the 600 nm absorbance maximum resulting in an isobestic point at 630 nm (see Fig. 5). These colour changes are typical for the 'Clarke' reaction and moreover a very pronounced continuously increasing absorbance maximum at 205 nm is observed.

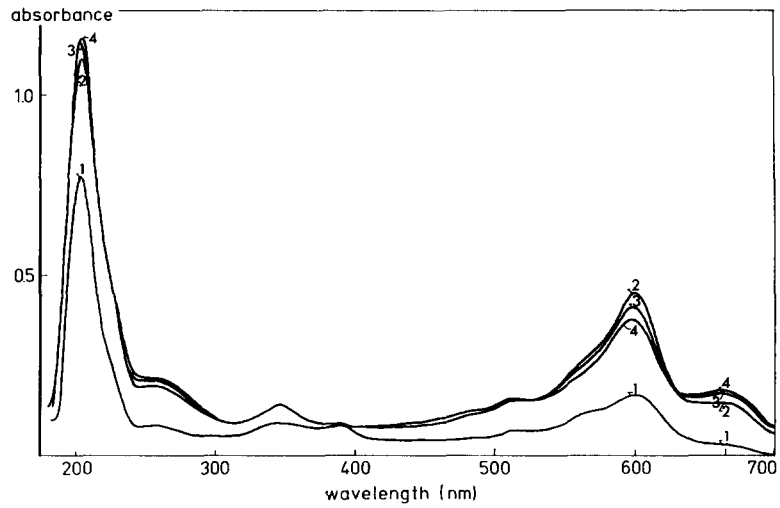

Fig. 5. 'Clarke' colour reaction. Spectra of glycoalkaloids in phosphoric acid/paraformaldehyde taken at 1–5, 20–25, 40–45 and 60–65 min. The numbers 1, 2, 3 and 4 refer to the spectra recorded at 1–5, 20–25, 40–45 and 60–65 min, respectively.

Atypical colour reaction of 'Marquis' and 'Clarke' reactions by changing the addition time of paraformaldehyde

A rather different picture arises, when in the case of the 'Marquis' reaction of potato glycoalkaloids in 66% aqueous sulphuric acid, the alkaloid is added to the acid in the presence of paraformaldehyde, without prior development of the yellow intermediate at 405 nm. A blue colour is rapidly developed and also rapidly decreases with absorbance maxima at 600 and 660 nm and in addition, a continuously increasing maximum at 205 nm is observed. In the case of the 'Clarke' reaction the situation can be equally shifted by changing the time of addition of paraformaldehyde. If paraformaldehyde is added after a foregoing development of the yellow intermediate (405 nm), then a rather 'Marquis' like reaction takes place accompanied by the characteristic colour shift to orange, red and finally purple red.

Colour reactions at low molar ratios of glycoalkaloids to paraformaldehyde

At molar ratios of paraformaldehyde to glycoalkaloids less than 5, the 'Clarke' and 'Marquis' reactions behave differently. In the case of the 'Marquis' reaction there is hardly any colour shift besides that of the yellow intermediate, while the

'Clarke' reaction proceeds at low molar ratio only partly and in this case the absorbance maximum at 510 nm is more pronounced than at 600 nm, whilst the absorbance maximum at 660 nm is hardly detectable.

The influence of the time of addition of paraformaldehyde is evidently a major factor in deciding the type of colour changes of the 'Clarke' or 'Marquis' type colour reaction. The influence of acid or the concentration of paraformaldehyde, when added with the acid, is mainly on the reaction speed (see Figs 1 and 6).

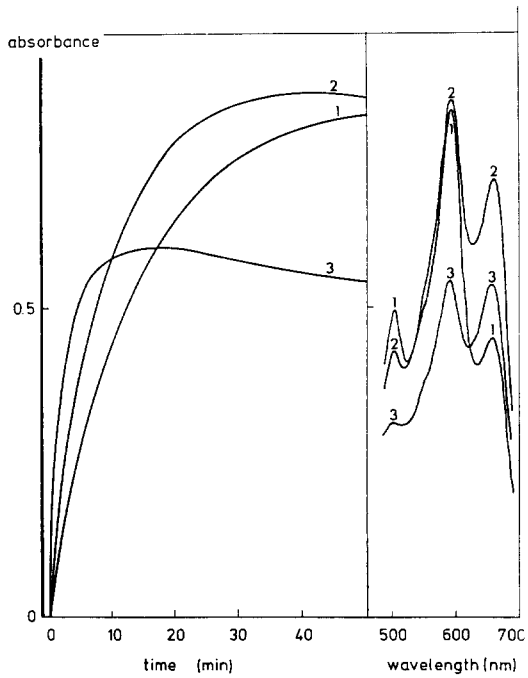

Fig. 6. Kinetics of 'Clarke' colour reaction at several paraformaldehyde concentrations. 1, 2 and 3 represent the absorbance kinetics of this reaction at 600 nm for paraformaldehyde concentrations 0·01; 0·03 and 0·1 %(w/v) respectively and their spectral composition after 60 min.

Particularly, the serial nature of the colour shifts encountered in the 'Marquis' type of colour reaction explains the criticism of several authors using this reaction as a quantitative assay for potato glycoalkaloids. In this respect the 'Clarke' reaction seems better suited as a quantitative assay, although the molar extinction coefficient is slightly lower.

It is known that these colour reactions of potato glycoalkaloids are rather specific and that the double bond at C_5 of the steroid nucleus is required (Schreiber *et al.*, 1961) (see Fig. 7). No suggestions for the mechanism of these reactions have been made so far. However, rather similar colour reactions have been used for the quantitative assay of cholesterol.

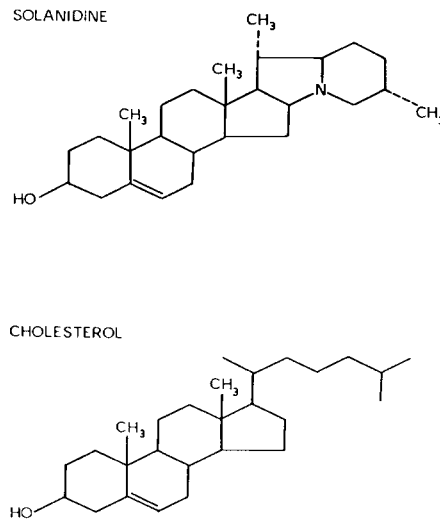

Fig. 7. Chemical structures of solanidine and cholesterol.

Investigations of the mechanism suggest a serial oxidation of steroidal carbonium ions (Brieskorn & Hofman, 1964; Burke *et al.*, 1974; Kurasawa *et al.*, 1976, 1978). Because of the structural similarity of cholesterol and solanidine (see Fig. 7) and the rather comparable requirements of strong acid and an oxidising agent, both 'Clarke' and 'Marquis' reactions were attempted with cholesterol. However, there was no reaction with cholesterol (Schreiber, 1961). The reason for this failure is clearly the lack of solubility of this rather lipophilic compound compared with the potato glycoalkaloids. By changing from aqueous sulphuric acid to mixtures of acetic acid and sulphuric acid (2:1 v/v) both potato glycoalkaloids and cholesterol reacted with the addition of paraformaldehyde, although with different colour shifts. Moreover in the 'Zak' reaction of cholesterol (colour reaction of cholesterol in acetic acid/sulphuric acid mixture with $FeCl_3$ as an oxidising agent (Boutwell, 1964)), potato glycoalkaloids are also reactive. Therefore it is suggested that 'Marquis' and 'Clarke' reactions have similar mechanisms and form, under conditions of strong acid and an oxidising agent, specific series of oxidised carbonium ions.

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Food Chemistry 6 (1980–81) 133–137

BIOLOGICAL EVALUATION OF IMPROVED LOCAL MAIZE HYBRIDS USING RATS

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ABSTRACT

The incorporation of the opaque-2 gene into local maize hybrids results in an increase in the levels of lysine and tryptophan and an improved biological value. The average weight gain of rats fed diets containing opaque-2 maize, normal maize and normal maize supplemented with synthetic lysine was 22·6 g, 12·2 g and 18·4 g respectively. Using other parameters: Efficiency of Food Conversion (EFC), Net Protein Utilisation (NPU), Protein Efficiency Ratio (PER) and Biological Value (BV), opaque-2 hybrid was superior to normal maize or normal maize supplemented with lysine.

Supplementation of opaque-2 maize with lysine alone had no added beneficial effect but supplementation of normal maize with lysine and tryptophan gave an additional improvement in its biological value.

INTRODUCTION

In tropical countries, maize accounts for between 80 and 90 % of the total cereals consumed. In Nigeria, for example, maize is consumed in various processed forms. A common form is the 'ogi' for which maize is steeped in water for 2–3 days, milled, sieved and fermented for about 48 h. The final product is a white mash which when boiled produces a thin gruel called ogi. This ogi is used as a weaning food for infants and as breakfast meal for adults. Studies by some workers (Akinrele, 1966; Ekpenyong *et al.*, 1974) have shown that due to the loss in nutrients occurring during processing, the nutritive value of ogi is so low that it cannot support growth in rats.

Maize proteins are generally low in two essential amino acids, lysine and tryptophan, thus rendering maize proteins of low biological value for human beings and monogastric animals. However, the notable discovery by Mertz *et al.* (1964) and

Food Chemistry 0308-8146/80/0006-0133/$02·50

Nelson *et al.* (1965), that the endosperm of maize kernels homozygous to the opaque-2 mutant gene has a substantially higher lysine content than normal maize, raised hopes among the maize-consuming populations. The recessive mutant gene blocks the synthesis of zein and modifies the endosperm quality by increasing the lysine level by 70–100% and tryptophan by 66%.

This paper attempts to highlight the results of research on locally produced opaque hybrids fed to weanling, albino rats and makes a comparison between this, normal maize, and normal maize supplemented with lysine, in the light of measured growth rate and other nutritional parameters.

MATERIALS AND METHODS

Analyses of the air-dried samples were done in duplicate and the results expressed on a dry matter basis after correcting for the residual moisture. Determinations of moisture, crude protein (6·25 × N), crude fat, crude fibre and ash were carried out using the methods of the AOAC (1970). For the lysine determination, maize endosperm samples were prepared by the method of Mertz & Bressani (1957). The endosperms were defatted by extraction with petroleum ether for 8 h. The tryptophan content was estimated as described by Hernandez & Bates (1969). The regression equation used to obtain the lysine content was:

$$y = 0{\cdot}3601 + 4{\cdot}0745x$$

where y = % lysine in protein, and x = % tryptophan in protein.

For the biological evaluation of the test diets, a ten-day experiment was conducted using weanling male albino rats weighing 50–60 g. The rats were distributed at random by weight and litter and housed in individual cages in three replications. One group of rats was fed a nitrogen-free diet. Water and feed were provided *ad lib.* The composition of the test diets is shown in Table 1. Weights of the rats were taken individually. Daily records were kept of food consumption and body weight of the individual rats.

TABLE 1
COMPOSITION OF TEST DIETS

Composition (%)	*Diet 1*	*Diet 2*	*Diet 3*
Opaque-2 maize	79·49	—	—
Normal maize	—	85·47	85·47
DL. Lysine	—	—	0·50
Groundnut oil	4·00	4·00	4·00
Non nutritive cellulose	4·00	4·00	4·00
Vitamin mix	2·50	2·50	2·50
Dicalcium phosphate	1·50	1·50	1·50
Oyster shell	0·50	0·50	0·50
Maize starch	8·01	2·03	1·53

At the end of the experiment, the rats were weighed and killed with chloroform. Incisions were made into the skull, thoracic and body cavities to enhance uniform drying. The carcasses were laid on a tray and dried in a hot-air oven at 100 °C for 4–6 days until a constant weight was reached.

The oven-dried carcasses were run through a domestic mincer and carcass nitrogen determination was carried out as described by Njike *et al.* (1974).

RESULTS AND DISCUSSION

The results of the chemical composition of the two maize varieties are summarised in Table 2. The data in this table show that opaque-2 maize had higher values for protein, fat and the two essential amino acids, lysine and tryptophan. Maize is deficient in these two amino acids and any improvement in its content would suggest an improvement in nutritive value, since lysine and tryptophan affect biological

TABLE 2
CHEMICAL COMPOSITION OF NORMAL AND OPAQUE-2 MAIZE

	Normal	*Opaque-2*
Crude protein (%)	9·1	10·3
Fat (%)	3·6	4·7
Carbohydrate (%)	74·1	68·4
Crude fibre (%)	1·7	2·2
Ash (%)	1·3	1·5
Moisture (%)	10·7	13·6
Lysine (g/100 g protein)	2·3	3·9
Tryptophan (g/100 g protein)	0·8	1·4

value in maize. The data also show that opaque-2 corn contains about 70% more lysine than the normal maize. From these results it can be seen that more oil can be extracted (weight by weight) from opaque-2 maize than from normal maize. Opaque-2 maize yields a lower amount of flour than normal maize. This lower yield of flour by opaque-2 maize is due to the fact that the opaque-2 maize contains less endosperm in the kernel whose greater portion is occupied by the germ. The fibre content, higher in opaque-2 maize than in normal is also of significance in the nutrition of monogastric animals. In Table 3 is presented the performance of weanling male rats fed on the different maize diets. At the same protein level (10%), rats fed on opaque-2 maize gained twice as much weight as those fed on normal maize (22·6 *vs* 12·2 g). There was an improvement when the deficient amino acid lysine was added to normal maize. Evidence, however, has shown that lysine when added to normal maize does appear to increase the weight gain. The PER was highest in the opaque-2 diet followed by normal maize supplemented with synthetic lysine. The data suggest that opaque-2 maize has a PER value which is 67% greater than that of normal maize. Since the addition of lysine appeared to improve the PER

TABLE 3
BIOLOGICAL EVALUATION OF THE DIFFERENT MAIZE DIETS FED TO RATS FOR 10 DAYS

Mean values	*Normal*	*Opaque-2*	*Normal ± lysine*
Weight gain (g)	12·2	22·6	18·4
Daily weight gain (g)	1·2	2·3	1·8
EFC	7·6	4·7	5·8
NPU	27·3	58·2	54·4
PER	1·5	2·5	1·9
BV	32·8	66·9	62·4

$$\text{EFC} = \text{Efficiency of Feed Conversion} = \frac{\text{food eaten (g)}}{\text{gain in body weight (g)}}$$

$$\text{NPU} = \text{Net Protein Utilisation} = \frac{\text{retained N (g)}}{\text{intake N (g)}}$$

$$\text{PER} = \text{Protein Efficiency Ratio} = \frac{\text{gain in body weight (g)}}{\text{N intake (g)}}$$

$$\text{BV} = \text{Biological Value} = \frac{\text{retained N (g)}}{\text{absorbed N (g)}}$$

a critical look was taken when this amino acid was added to opaque-2 maize. Unpublished results by the author indicate that lysine alone when added to opaque-2 maize appears to improve the PER slightly but has no effect on weight gain. This result agrees with that of Mertz (1966) who also indicated that the addition of the limiting amino acids, lysine and tryptophan, to normal maize not only increases the PER but also increases weight gain by more than 300%.

The efficiency of food conversion was consistently better with opaque-2 maize followed by normal maize supplemented with lysine than with normal maize alone. Our results also indicate that the higher the feed consumed at this protein level, the better the efficiency of food conversion. The NPU values also show the same trend with highly significant values between opaque-2 maize diet and normal maize diet. There were, however, no significant differences between opaque-2 maize diet and normal maize supplemented with lysine. The BV of opaque-2 maize diet was about 106% and 7·3% higher than that of normal maize diet and normal maize diet supplemented with lysine respectively. Highly significant differences were obtained between opaque-2 maize diet and normal maize diet.

CONCLUSION

Despite the known agronomic disadvantages of opaque-2 maize, certain intrinsic characteristics such as higher lysine and tryptophan values may promote its wide use in animal feeds and human foods especially baby foods. In view of the great nutritional potential of the opaque-2 maize and the successful incorporation of the

opaque-2 gene into local maize hybrids, methods avoiding losses occurring during the wet processing of maize should be encouraged and a study of processing techniques to overcome these losses is needed.

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Food Chemistry 6 (1980–81) 139–151

THE NEUTRAL VOLATILE COMPONENTS OF CIDER APPLE JUICES

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ABSTRACT

Examination of the volatile compounds from juices of four cultivars of cider apple ('Bramley's Seedling', 'Sweet Coppin', 'Kingston Black' and 'Yarlington Mill') has led to the definite identification of eighteen alcohols, forty-eight esters, five carbonyls, eight acetals, four ethers, one lactone, one hydrocarbon and three halogenated compounds, tentative evidence being presented for a further 51 components. Fifty-five of the reported compounds have not previously been found in whole apples or juices. Of these cis *and* trans *linalool oxide and the presence of 5-hexen-1-ol, rather than 2-hexen-1-ol, in the juice of the cultivar 'Kingston Black', are of particular interest. Differences between cultivars were largely quantitative and due to variations in the amounts of components with boiling points in excess of that of hexanol.*

INTRODUCTION

The volatile components of apple juices and the whole fruit have been investigated many times since the advent of gas chromatography (Van Straten *et al.*, 1977), but few of these investigations have been concerned with cultivars specifically used for cider manufacture. Pompei (1968) examined blends of juice obtained from several cultivars used for cider making in Bretagne and Perche; similarly, Bermejo & Saura Calixto (1975; 1976) published data on the juices of the cultivars 'Clara', 'Teorica' and 'Prieta', used in northern Spain. Volatiles from juices for apple wine production were investigated by MacGregor *et al.* (1964) and, since the commencement of the current project, Mikeladse & Kereselidse (1977) and Schreier *et al.* (1978*a*, *b*) have also followed the development of volatiles from juice production to the final apple wine or brandy; the latter workers used both conventional juice preparation procedures and HTST heating of the mash. Both groups of workers used as their starting material cultivars principally grown for purposes other than cider and wine manufacture. Many of the apple cultivars used for cider making are completely

Food Chemistry 0308-8146/80/0006-0139/$02·50

different in flavour character and suitability for processing from those normally eaten as fresh fruit, although some do have outlets for culinary purposes. Cider apple cultivars are selected for their ability to give a high yield of juice and for the specific flavour characters they impart to the finished product. Such fruit is traditionally classified by the English cider industry into four types; bittersweets, bittersharps, sharps and sweets. Bittersweets have a high content of polyphenols and are low in acids, bittersharps have a high content of polyphenols and of acids, whereas the other two groups are both low in polyphenols but possess high and low levels of acidity, respectively.

As part of our Division's interest in the origin of cider volatiles, the aroma components found in juices prepared from four apple cultivars, each from one of the four categories described above, have been investigated. A preliminary report on this project has been made already (Williams, 1974). The present paper discusses in detail the results of the gas chromatographic–mass spectrometric examination of these four juices.

EXPERIMENTAL

Reagents

Reagents and their purification were as outlined previously (Williams & Tucknott, 1978).

Fruit and preparation of juices

Apples of the four cultivars, 'Sweet Coppin' (sweet), 'Bramley's Seedling' (sharp), 'Kingston Black' (bittersweet) and 'Yarlington Mill' (bittersharp) were obtained from commercial orchards over the years 1971–74. Fruit from each cultivar (approximately 220 kg) was milled and pressed using a pilot-scale mill and press (H. Beare & Sons Ltd) to yield approximately 95 litres of juice. This was then stored at 0 °C until subjected to further processing.

Examination of neutral constituents

The volatiles were removed from the bulk of the juices within 24 h of processing using a pilot plant Kestner evaporator (Williams & Tucknott, 1971) operating under vacuum (6300 N m^{-2}), approximately 20 % of the juice being removed as distillate. Distillates were then stored at −20 °C and subsequently extracted with trichlorofluoromethane using an 8·5-litre pilot plant continuous liquid–liquid extractor. Acids were removed and the remaining material concentrated to a volume of 2 ml, as described for ciders (Williams & Tucknott, 1978).

Samples of the non-acid fraction were examined by both gas chromatography and coupled gas chromatography–mass spectrometry. Gas chromatographic examinations were carried out using a Hewlett Packard 7620 or 5710 gas chromatograph

fitted with flame ionisation detectors and using the following columns and conditions:

(i) 6·1 m × 3·2 mm Outside diameter glass column packed with 10% Carbowax 20M on 60:80 mesh Chromosorb W programmed from 65 to 210 °C at 8 °C/min with a nitrogen flow of 30 ml/min.

(ii) 150 m × 0·76 mm Inside diameters stainless steel capillary column coated with Carbowax 20M, held isothermally at 65 °C for 15 min and then programmed from 65 to 110 °C at 2 °C/min, 110 to 145 °C at 4 °C/min and 145 to 210 °C at 2 °C/min. Nitrogen flow was 3 ml/min.

(iii) 150 m × 0·76 mm Inside diameter stainless steel capillary column coated with SP1200 (Supelco Ltd.) held isothermally at 65 °C for 15 min and then programmed from 65 to 210 °C at 3 °C/min with a nitrogen flow rate of 5 ml/min.

In all cases injection ports and detectors were held at 250 °C.

Mass spectra of separated components were obtained using an LKB 9000 coupled gas chromatograph–mass spectrometer operating at 70 eV. Oven dimensions restricted column lengths to 90 m, otherwise column and operating conditions were as described in (i) and (ii) above. Spectra were recorded both on uv charts and on magnetic tape for processing off-line at the Food Research Institute, Norwich (Johnson *et al.*, 1973).

Compounds in the extracts were identified by comparison of their mass spectra and retention times with published data and with that obtained from authentic samples as previously described (Williams & Tucknott, 1978).

Quantitative information on major components was obtained both by direct injection of the juices after adding 4-methyl-1-pentanol (15 ppm) and by extracting 100 ml of the juices (to which sodium chloride, 20 g, had been added) with 10 ml of ether and concentrating the extract to 1 ml. In both cases quantities were estimated by comparison of peak heights with those obtained from a standard mixture of known composition. Amounts of minor components, which could only be detected in the samples obtained when using the climbing film evaporator, were estimated by comparison of their peak heights with the chromatographically nearest major component of similar chemical class whose concentration had been estimated by direct injection or simple extractions.

RESULTS AND DISCUSSIONS

The results of the various examinations conducted on the extracts of the juices obtained from the four cultivars are summarised in Table 1. Good evidence is presented for the identification of eighty-eight components comprising eighteen alcohols, forty-eight esters, five carbonyls, eight acetals, four ethers, one lactone,

TABLE 1

AROMA COMPONENTS IN JUICES PREPARED FROM APPLES USED FOR CIDER MAKING

Compound	*'Bramley's Seedling'*			*'Sweet Coppin'*			*'Kingston Black'*			*'Yarlington Mill'*		
	Amount (μg/litre)	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*
Alcohols:												
Ethanol	4000	1	a	1000	1	a	5000	1	a	2500	1	a
n-Propanol	3000	1	a	600	5	c	6200	1	a	7000	1	a
n-Butanol	8000	1	a	16000	1	a	40000	1	a	35000	1	a
Isobutanol	2000	1	a	1000	1	a	500	1	a	tr	1	b
n-Pentanol	100	1	a	200	1	a	500	1	a	50	2	b
2- and/or 3-Methylbutanol	8000	1	a	1000	1	b	9000	1	a	2000	1	a
2-Pentanol	4	2	b	3	6	b	10*	2	b	18	2	b
3-Pentanol	tr	6	c	5	6	c	4	3	c	tr	6	c
2-Methyl-2-butanol	0	—	—	0	—	—	10*	3	c	0	—	—
4-Pentenol	10	7	c	1	7	c	tr	4	c	1	7	c
Hexanol	6000	1	a	3000	1	a	11000	1	a	4100	1	a
2-Methylpentanol	1	2	a	1	6	c	0	—	—	3	3	b
3-Methylpentanol	0	—	—	0	—	—	0	—	—	5	3	c
4-Methylpentanol	0	—	—	0	—	—	tr	3	c	0	—	—
A branched hexanol	0	—	—	5	6	c	0	—	—	0	—	—
cis-3-Hexenol	50	1	a	1200	1	a	10	2	b	20	1	a
trans-2-Hexenol }	200	2	a	1000	2	a	0	—	—	1000	2	a
5-Hexenol }	0	—	—	0	—	—	100	2	b	0	—	—
Heptanol	0	—	—	tr	3	c	20	3	c	0	—	—
n-Octanol	5	1	a	12	2	b	20	2	b	12	1	b
3-Octanol	tr	4	c	0	—	—	0	—	—	6	4	c
An ethylhexanol	0	—	—	0	—	—	0	—	—	tr	3	c
Nonanol	0	—	—	0	—	—	0	—	—	tr	3	c
6-Methyl-5-heptenol	30	1	b	20	2	b	40	1	b	21	1	b
3-Octenol	0	—	—	tr	3	c	0	—	—	0	—	—
Benzyl alcohol	5	2	b	3	2	b	2	2	b	70	2	b
2-Phenethanol	2	2	a	6	1	a	tr	2	b	16*	2	b
Terpinen-4-ol	5	1	a	50	1	a	20	2	a	24	1	a
α-Terpineol	6	1	a	2*	2	b	1*	2	b	18	1	a
Isoborneol	0	—	—	tr	4	c	0	—	—	0	—	—
Citronellol	0	—	—	tr	4	c	0	—	—	0	—	—
Total	31418			25108			72437			51864		
Esters (formates):												
n-Butyl formate	0	—	—	0	—	—	tr	3	c	tr*	3	c
2- and/or 3-Methylbutyl formate	tr	6	c	0	—	—	0	—	—	tr	3	c
n-Hexyl formate	tr	6	c	tr	6	c	tr	6	c	tr	3	c
A hexenyl formate	0	—	—	tr	6	c	0	—	—	tr	3	c
Total	tr			tr			tr			tr		

Esters (acetates):												
Methyl acetate	0	—	—	tr	6	c	tr	6	c	tr	3	b
Ethyl acetate	2000	1	a	1000	1	a	1000	1	a	2000	1	a
n-Propyl acetate	20	2	b	10	6	c	100	2	b	300	1	a
n-Butyl acetate	30	1	a	300	1	a	500	1	a	2200	1	a
Isobutyl acetate	0	—	—	0	—	—	tr	2	b	tr	2	b
n-Pentyl acetate	33	1	a	100	2	a	20	1	a	12	1	a
2- and/or 3-Methylbutyl acetate	100	1	a	20	1	a	200	1	a	160	1	a
n-Hexyl acetate	300	1	a	300	1	a	200	1	a	600	1	a
n-Heptyl acetate	0	—	—	0	—	—	0	—	—	tr	3	c
n-Octyl acetate	3	2	b	tr	5	c	tr	2	b	2	1	a
Benzyl acetate	10	2	c	tr	5	c	tr	5	c	14*	2	b
cis-3-Hexenyl acetate	9	5	b	20	2	b	2	5	c	7	1	a
trans-2-Hexenyl acetate	20	1	a	30	1	a	0	—	—	20	1	a
A hexenyl acetate	0	—	—	0	—	—	20	5	b	0	—	—
Total	2525			1780			2042			5315		
Esters (*n*-propionates):												
Ethyl *n*-propionate	200	2	b	10	6	c	30	3	b	100	1	a
n-Propyl *n*-propionate	tr	6	c	12	6	c	2	6	c	4	2	b
n-Butyl *n*-propionate	tr	2	b	0	—	—	tr	3	c	tr	1	b
2- and/or 3-Methylbutyl propionate	3	2	b	0	—	—	tr	3	b	tr	1	a
n-Hexyl *n*-propionate	20*	2	b	5*	2	b	5*	2	b	6*	1	b
A hexenyl *n*-propionate	0	—	—	0	—	—	tr	6	c	tr	3	c
Total	223			27			37			110		
Esters (*n*-butyrates):												
Ethyl *n*-butyrate	300	1	a	10	5	c	200	1	a	300	1	a
n-Propyl *n*-butyrate	tr	2	b	tr	5	c	tr	5	c	tr	2	c
n-Butyl *n*-butyrate	20	2	b	7	2	b	50	2	b	12	1	a
Isobutyl *n*-butyrate	0	—	—	0	—	—	tr	2	c	0	—	—
n-Pentyl *n*-butyrate	tr	2	b	0	—	—	tr	1	b	7	3	c
2- and/or 3-methylbutyl *n*-butyrate	10	1	a	tr	2	b	tr	1	a	1	1	a
n-Hexyl *n*-butyrate	70	1	a	tr*	1	a	tr	1	a	10	1	a
Cinnamyl *n*-butyrate	tr	2	c	0	—	—	0	—	—	0	—	—
Total	400			17			250			330		
Esters (isobutyrates):												
Ethyl isobutyrate	8	1	b	6	5	c	20	2	c	21	1	a
n-Butyl isobutyrate	0	—	—	0	—	—	tr	2	c	0	—	—
n-Hexyl isobutyrate	tr	2	b	0	—	—	tr	2	b	0	—	—
Total	8			6			20			21		

TABLE 1—*contd.*

Compound	*'Bramley's Seedling'*			*'Sweet Coppin'*			*'Kingston Black'*			*'Yarlington Mill'*		
	Amount (μg/litre)	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*	*Amount (μg/litre)*	*Method of identification*	*Certainty*
Esters (*n*-butenoates):												
Ethyl *trans*-2-butenoate	tr	3	c	0	—	—	0	—	—	0	—	—
Total	tr			0			0			0		
Esters (*n*-pentanoates):												
Ethyl *n*-pentanoate	tr	2	c	0	—	—	0	—	—	0	—	—
n-Butyl *n*-pentanoate	tr	3	c	0	—	—	tr	2	c	tr	3	c
n-Pentyl *n*-pentanoate	tr	2	c	0	—	—	0	—	—	0	—	—
n-Hexyl *n*-pentanoate	2*	1	a	tr	6	c	tr	3	b	2*	2	c
Total	2			tr			tr			2		
Esters (2-methylbutyrates):												
Ethyl 2-methylbutyrate	40	1	a	1	2	b	10	1	b	5	1	b
n-Propyl 2-methylbutyrate	tr	6	c	0	—	—	tr	2	c	tr	2	b
n-Butyl 2-methylbutyrate	tr	1	b	tr	2	b	tr	2	c	tr	2	b
Isobutyl 2-methylbutyrate	tr	6	c	tr	6	c	0	—	—	tr	3	c
n-Hexyl 2-methylbutyrate	40*	1	a	0	—	—	5*	1	a	24*	1	b
Total	80			1			15			29		
Esters (*n*-hexanoates):												
Ethyl *n*-hexanoate	tr	2	b	0	—	—	3	6	c	tr	1	a
n-Propyl *n*-hexanoate	tr	2	c	tr	2	b	20	2	b	12	1	a
n-Butyl *n*-hexanoate	70*	2	b	tr*	3	b	tr*	3	b	10*	3	b
Isobutyl *n*-hexanoate	tr	2	b	0	—	—	0	—	—	tr	2	b
n-Pentyl *n*-hexanoate	2*	2	c	0	—	—	0	—	—	2	3	b
2 and/or 3-Methylbutyl *n*-hexanoate	tr	3	c	tr	5	c	tr	1	b	1	2	b
n-Hexyl *n*-hexanoate	10	1	a	5*	1	a	tr	1	a	6*	1	a
Total	82			5			23			31		
Esters (*n*-hexenoates):												
n-Butyl *trans*-2-hexenoate	tr	5	c	4	2	b	2	5	c	13	5	c
Total	tr			4			2			13		
Esters (*n*-heptanoates):												
n-Propyl *n*-heptanoate	0	—	—	tr*	2	c	0	—	—	0	—	—
n-Butyl *n*-heptanoate	tr	5	c	1	2	c	tr	2	c	5	2	c
Total	tr			1			tr			5		

Esters (*n*-octanoates):												
Ethyl *n*-octanoate	0	—	—	0	—	—	0	—	—	3*	2	b
n-Propyl *n*-octanoate	1*	3	c	tr*	3	c	tr*	3	c	18*	3	c
n-Butyl *n*-octanoate	0	—	—	5	3	c	0	—	—	6*	3	c
Isobutyl *n*-octanoate	0	—	—	0	—	—	0	—	—	3*	3	c
n-Pentyl *n*-octanoate	tr	3	c	1	6	c	1	3	c	69	2	c
2- and/or 3-Methylbutyl *n*-octanoate	tr	1	a	tr	5	b	tr	2	b	tr	2	b
n-Hexyl *n*-octanoate	1	2	b	tr	6	c	tr	2	b	tr	3	c
Total	2			6			1			99		
Esters (*n*-decanoates)												
Ethyl *n*-decanoate	0	—	—	tr	6	c	tr	6	c	1	3	c
n-Butyl *n*-decanoate	0	—	—	0	—	—	0	—	—	tr	3	c
Isobutyl *n*-decanoate	tr	6	c	tr	6	c	0	—	—	12	3	b
n-Hexyl *n*-decanoate	tr	2	c	0	—	—	0	—	—	0	—	—
Total	tr			tr			tr			13		
Esters (*n*-dodecanoates):												
Ethyl *n*-dodecanoate	0	—	—	tr	6	c	0	—	—	12	2	b
n-Butyl *n*-dodecanoate	0	—	—	2	2	b	0	—	—	5	3	c
n-Hexyl *n*-dodecanoate	tr	3	c	0	—	—	0	—	—	0	—	—
Total	tr			2			0			17		
Esters (phthalates):												
Dimethylphthalate	tr	6	c	tr	2	b	0	—	—	tr	6	c
Diethylphthalate	tr	3	b	tr	2	b	tr	3	b	tr	2	b
Dipropylphthalate	0	—	—	tr	3	b	0	—	—	0	—	—
Total	tr			tr			tr			tr		
Carbonyls:												
n-Propanal	tr	6	c	tr	6	c	0	—	—	tr	3	b
2-Propanone	200	2	a	6	1	a	100	1	a	132	1	a
n-Butanal	tr	6	c	tr	6	c	tr	3	c	tr	3	c
2-Butanone	0	—	—	30	3	c	0	—	—	0	—	—
2-Pentanone	50	1	a	27	2	b	5	1	b	tr	3	c
3-Pentanone	0	—	—	tr	3	c	0	—	—	0	—	—
n-Hexanal	tr	5	c	tr	5	c	tr	5	c	20	1	a
trans-2-Hexenal	4	6	c	2	6	c	10	6	c	600	1	a
Total	254			65			115			752		

TABLE 1—*contd.*

Compound	*'Bramley's Seedling'*			*'Sweet Coppin'*			*'Kingston Black'*			*'Yarlington Mill'*		
	Amount (µg/litre)	*Method of identification*	*Certainty*	*Amount (µg/litre)*	*Method of identification*	*Certainty*	*Amount (µg/litre)*	*Method of identification*	*Certainty*	*Amount (µg/litre)*	*Method of identification*	*Certainty*
Acetals:												
Diethoxymethane	tr	3	c	0	—	—	0	—	—	0	—	—
Dibutoxymethane	0	—	—	tr	3	c	tr	3	c	0	—	—
Dihexoxymethane	tr	3	c	tr	3	c	0	—	—	0	—	—
1-Ethoxy 1-*n*-hexoxyethane	tr	3	b	0	—	—	0	—	—	0	—	—
1-Ethoxy 1-*n*-octoxyethane	0	—	—	tr	3	b	0	—	—	tr	6	c
1,1-di-*n*-butoxyethane	tr	3	b	tr	3	c	tr	6	c	tr	3	b
1,1-Diisobutoxyethane	0	—	—	tr	3	c	0	—	—	0	—	—
1-*n*-Butoxy 1-2-methylbutoxy ethane	tr	3	b	0	—	—	tr	3	c	0	—	—
1-*n*-Butoxy 1-*n*-hexoxyethane	tr	3	b	0	—	—	tr	3	c	0	—	—
1,1-di-2-Methylbutoxyethane	1	2	b	1	6	c	4	2	c	tr	3	c
1,2-Methylbutoxy 1-*n*-hexoxy-ethane	tr	3	b	0	—	—	0	—	—	0	—	—
1,1-di-*n*-Hexoxyethane	tr	3	b	0	—	—	tr	6	c	0	—	—
Total	1			1			4			tr		
Ethers:												
Diethyl ether	0	—	—	0	—	—	0	—	—	tr	3	b
Methyl *n*-propyl ether	tr*	6	c	5*	6	c	0	—	—	tr	3	c
Di-*n*-butyl ether	0	—	—	0	—	—	tr	3	c	0	—	—
2- and/or 3-Methylbutyl ether	0	—	—	0	—	—	0	—	—	tr	3	c
Di-*n*-hexyl ether	0	—	—	0	—	—	tr	3	c	0	—	—
Methyl phenyl ether	0	—	—	tr	3	c	0	—	—	0	—	—
4-Methoxyallylbenzene	7	2	b	4	5	c	1	5	c	28	1	b
cis-Linalool oxide	50*	2	b	7	1	a	30*	1	a	21	1	a
trans-Linalool oxide	1	2	b	3	1	a	10	1	a	10	1	a
Total	58			19			41			59		

Lactones:												
γ-Hexalactone	tr*	1	b	4*	2	b	5	1	b	60	2	b
γ-Undecalactone	0	—	—	tr	3	c	0	—	—	tr	6	c
Total	tr			4			5			60		
Hydrocarbons:												
Styrene	tr	3	b	0	—	—	0	—	—	0	—	—
A substituted benzene (molecular weight, 120)	0	—	—	0	—	—	tr	3	c	0	—	—
A substituted styrene (molecular weight, 132)	0	—	—	0	—	—	0	—	—	tr	3	c
Total	tr			0			tr			tr		
Halogen compounds:												
Chloroform	30	1	a	50	1	a	5	2	b	18	1	a
Trichlorofluoromethane	Solvent	1	a	Solvent	1	a	Solvent	1	a	Solvent	1	a
1,2-Dichloro-1,2-difluoroethylene	0	—	—	0	—	—	0	—	—	tr	3	b

Key:
* = Mixed peak, contains another component.
tr = Trace.
— = No data.
Method of identification:
1 = Mass spectra and retention times on two columns.
2 = Mass spectrum on one column; retention times on two columns.
3 = Mass spectrum on one column; retention time on one column.
4 = Mass spectrum only.
5 = Retention times on two columns.
6 = Retention time on one column.
7 = Comparison of retention time with peak identified by 4 in juices from other cultivars.
Certainty:
a = Definite identification.
b = Good evidence but not as certain as a.
c = Tentative.

one hydrocarbon and three halogen-containing compounds in the extracts, tentative evidence being presented for a further twelve alcohols, twenty-four esters, three carbonyls, four acetals, five ethers, one lactone and two hydrocarbons. Excluding the halogenated compounds which probably came from the trichlorofluoromethane used during the extraction, sixty-four were unreported in apples in the most recent survey of food volatiles from the Central Institute of Nutrition and Food Research (Van Straten *et al.*, 1977), although recent work since the completion of the current examination (Schreier *et al.*, 1978*a*, *b*) has provided evidence for nine of these. Despite the allocation of 134 identities, a number of compounds, particularly amongst those eluting after the hexenols, still elude identification. It is suspected that many of these are derivatives of terpenes, acetals or esters based on unsaturated alcohols or acids. When comparing the chromatograms of the four extracts, whether on Carbowax 20M or SP1200, it is in this region of the chromatograms, too, that many of the differences occur, the two cultivars high in phenolics ('Kingston Black' and 'Yarlington Mill') being more complex than the other two. Some of this is obviously due to quantitative differences, particularly amongst the higher esters, but in many cases spectra were recorded from some extracts for which nothing comparable could be found in others.

Quantitatively, 'Kingston Black' and 'Yarlington Mill' possess a higher concentration of aroma components than the 'Bramley's Seedlings' or 'Sweet Coppin' juices, this being largely accounted for by the alcohols which made up the bulk of the extracts from all the juices. As enzymic cleavage of esters is known to occur on disruption of apple tissue (Drawert *et al.*, 1965) this is not unexpected. Esters are the next most abundant class of compounds, their amounts in relation to the alcohols being greatest in the 'Bramley's Seedlings' and 'Yarlington Mill' cultivars. Although acetates dominate this group of compounds in all four juices the relative amounts of the other esters vary from juice to juice.

The virtual absence of *n*-hexanal and trans-2-hexenal in juices from all cultivars except 'Yarlington Mill' is difficult to explain as both these compounds are usually produced if apple tissue is ruptured. Enzymatic or microbial reduction at the juice stage (Drawert *et al.*, 1973) is a possible explanation, but, as all juices were stripped of volatiles within 24 h of preparation, it is possible that microbial or chemical changes in either the distillates or the extracts prior to gas chromatographic–mass spectrometric examination may account for this situation.

Of the previously unreported compounds in apple juices the two linalool oxides are of interest. These two compounds are normally considered to be the result of chemical or enzymic oxidation resulting from the disruption or heating of the fruit tissue or juice (Stevens *et al.*, 1966; Tang & Jennings, 1968). Temperatures did not exceed 40 °C at any stage during the handling of the juices; if produced as a consequence of processing their formation is therefore most likely to be enzymatic. Both these oxides have, however, been found in extracts obtained from the

headspace of intact apples (Williams & Lewis, unpublished results) so the possibility that they are natural constituents of the fruit cannot be overlooked.

The reporting of 5-hexenol in 'Kingston Black' juice is also worthy of comment, particularly as it appears to replace the *trans*-2-hexenol in this fruit and has not previously been reported in fruits or vegetables (Van Straten, *et al.*, 1977). *trans*-2-Hexenol and 5-hexenol have very similar retention times on the Carbowax 20M column and both occur in the tail of the relatively poorly resolved hexanol peak on the SP1200 column (Table 2); hence they could well be confused on retention data alone. The mass spectra of the two components are, however, completely different. Figure 1 compares the spectra of the peak at the retention time of these two components, obtained from the 'Sweet Coppin' juice extract and 'Kingston Black' juice extract, with authentic spectra of the two alcohols (Stenhagen *et al.*, 1974). The

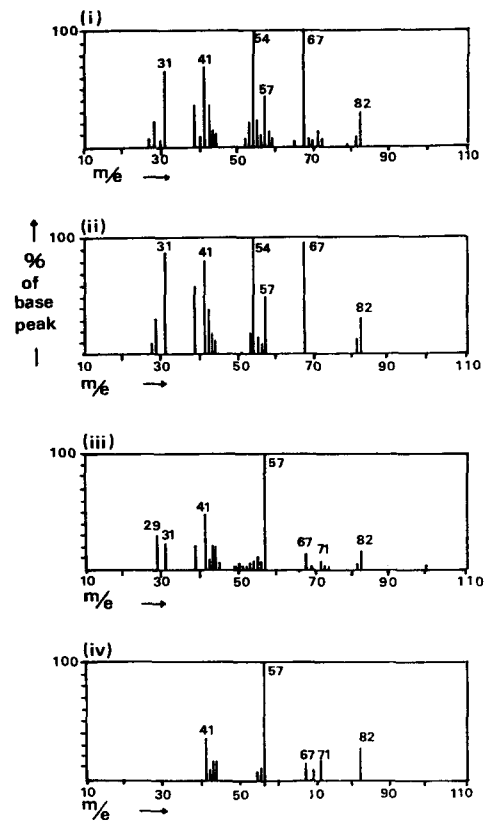

Fig. 1. Comparison of spectra of hexenols from 'Kingston Black' and 'Sweet Coppin' juice with authentic 5-hexenol and *trans*-2-hexenol. (i) Spectra obtained from 'Kingston Black' juice. (ii) Authentic 5-hexenol. (iii) Spectra obtained from 'Sweet Coppin' juice. (iv) Authentic *trans*-2-hexenol.

TABLE 2
COMPARISON OF RETENTION DATA FOR HEXENOLS ON CARBOWAX 20M AND SP1200. DATA GIVEN RELATIVE TO *n*-HEXANOL

	Carbowax 20M	*SP1200*
n-Hexanol	1·000	1·000
trans-2-Hexenol	1·036	1·001
cis-3-Hexenol	1·023	0·972
5-Hexenol	1·040	1·014

presence of the *m/e* 54 ion and the larger amount of the *m/e* 67 ion in the spectrum for the 'Kingston Black' juice extract leads one to believe that it is 5-hexenol, rather than 2-hexenol, which is present in this fruit.

Of the other unreported components the terpene alcohols, 6-methyl-5-heptenol and the isobutyl esters probably contribute to the aroma of these juices. The acetals, however, could have arisen during the distillation stage as has been reported for ciders (Williams & Tucknott, 1978) and apple brandies (Schreier *et al.*, 1978).

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Food Chemistry 6 (1980–81) 153–161

CARBOHYDRATE COMPOSITION OF DIFFERENT VARIETIES OF COWPEA (*VIGNA UNGUICULATA*)

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ABSTRACT

Twenty varieties of cowpea with the following proximate composition: dry matter, 87–94%; crude protein, 24–33% ether extract, 1–2%; crude fibre, 2–5% and ash, 2–5% were analysed for sugar contents, starch, cell wall carbohydrates and lignin. The legume seeds exhibited a total carbohydrate content ranging from 56% to 68%, the major constituent being starch. Starch values as high as 45% to 48% were obtained for some varieties although most values ranged between 37% and 42%. Ethanol-soluble sugars were verbascose, stachyose, sucrose and raffinose in varying amounts but there were only traces of fructose and glucose. Samples had total soluble sugar contents of 6% to 13%. Values for unavailable carbohydrates for most samples were 11% to 13%. Lignin was very low and ranged from 0·6% to 1·8%.

INTRODUCTION

The chemical composition of several leguminous seeds such as horsebeans (*Vicia faba*), peas (*Pisum sativum*), lupins (*Lupinus*), field beans (*Vicia faba*), broad beans (*Vicia faba*) and groundnut (*Arachis hypogea*), which serve as protein sources for Man and animals has been studied in some detail by several workers including French (1954), Clarke (1970), Courtois & Percheron (1971), Pritchard *et al.* (1973), Cerning *et al.* (1975), Tharanathan *et al.* (1975) and Cerning-Beroard & Filiatre (1976). Most of these seeds are known to contain soluble sugars, particularly oligosaccharides related to raffinose (French, 1954, Courtois & Percheron, 1971), and also to have fairly high starch contents. Their hemicelluloses are characterised by polysaccharides containing arabinose, xylose and galactose. The carbohydrate composition has, however, been shown to vary considerably between varieties (Pritchard *et al.*, 1973; Cerning *et al.*, 1975).

The cowpea (*Vigna unguiculata*) is another leguminous seed which grows best in rich, loamy soils. It is indigenous to Africa but has been introduced to other parts of

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the world. It is the most widely used plant protein in Nigeria. Its mineral, vitamin, fatty acid and amino acid composition have been reported by Johnson & Raymond (1964) and by Oyenuga (1968). The free sugars of the cowpea have also been quantified by Nigam & Giri (1961) and the carbohydrate content was determined by difference as 57–58% by Johnson & Raymond (1964).

The present study deals with the soluble sugars, starch and cell wall carbohydrates of cowpeas of different origin.

MATERIALS AND METHODS

Materials

Twenty varieties of cowpea (*Vigna unguiculata*) of different origin were grown at the National Cereal Research Institute, Ibadan, Nigeria. The samples were variable in growth habit and also in colour, size and texture (Table 1). The seeds were ground in a Cross-Beater laboratory mill to a particle size of 0·5 mm.

Methods

Proximate composition was determined according to AOAC methods (AOAC, 1970). Ethanol-soluble sugars were exhaustively extracted from samples with

TABLE 1
ORIGIN AND APPEARANCE OF COWPEA VARIETIES

Variety	*Country of origin*	*Colour*	*Dimensions*[a] *(mm)*	*Seed coat texture*	*Seed shape*
Adzuki	United States of America	Maroon	7·3 × 4·3 × 5·5	Smooth	Ovoid
New Era	South Africa	Dark brown	9·2 × 4·5 × 5·9	Smooth	Ovoid
Nig. A95	Nigeria	White	9·5 × 5·2 × 7·3	Rough	Ovoid
Farin Juda C	Nigeria	Cream with mottled brown back	8·7 × 5·3 × 7·0	Rough	Ovoid
West bred	Nigeria	Cream	7·9 × 4·0 × 5·9	Smooth, wrinkled	Ovoid
Nig. B4	Nigeria	Brown	8·8 × 5·7 × 7·3	Rough	Oblong
Mala	Nigeria	Cream	8·4 × 4·9 × 6·5	Rough	Ovoid
Nig. A104	Nigeria	White	9·2 × 5·0 × 8·4	Rough	Oblong
Nig. B7	Nigeria	Brown	8·4 × 4·6 × 7·3	Rough	Ovoid
Prima	Nigeria	Cream	9·5 × 4·6 × 5·9	Smooth, wrinkled	Oblong
Kano 2479	Nigeria	White	20 × 6·3 × 7·7	Rough	Ovoid
Kano 1696	Nigeria	White	17 × 6·3 × 8·6	Rough	Oblong
Line 7	Senegal	Cream	8·8 × 5·1 × 6·1	Smooth, wrinkled	Ovoid
Anna	Nigeria	White	9·3 × 5·0 × 6·3	Rough	Oblong
Ima	Nigeria	Cream	8·9 × 5·5 × 6·5	Rough	Oblong
Kudi	Nigeria	White	8·6 × 4·8 × 6·9	Rough	Oblong
Ayi	Nigeria	Cream	13·5 × 5·8 × 6·4	Rough	Ovoid
Ife Brown	Nigeria	Brown	8·3 × 4·4 × 6·8	Rough	Ovoid
FARV 13	Nigeria	Dark maroon	16·6 × 3·7 × 5·0	Smooth	Flat
TVu 4557	Nigeria	Cream	8·3 × 4·2 × 5·4	Rough	Oblong

[a] Length × width × thickness.

80% v/v ethanol. Starch was estimated by the glucoamylase method of Thivend *et al.* (1972) while cell wall constituents were estimated after eliminating starch and soluble sugars, by successive hydrolysis in sulphuric acid. The residue left after hydrolysis was regarded as crude lignin. The hydrolysates of the cell wall fractions were neutralised with barium carbonate and excess barium carbonate was removed with Dowex 50 (H^+) resin. Neutralised solutions for chromatography were concentrated at reduced pressure by using a rotary film evaporator with water bath temperatures not exceeding 40°C.

Sugars were identified by paper chromatography on a Whatman No. 1 filter paper using ethylacetate–pyridine–water (8:2:1 v/v) as solvent for neutral sugars, *n*-propanol ethanol–water (7:1:2 v/v) and *n*-butanol–ethanol–water (10:1:2 v/v) for oligosaccharides and *n*-butanol–acetic acid–water (4:1:5, upper phase) for uronic acids. Sugars on paper chromatograms were detected with *p*-anisidine hydrochloride spray reagent (Mukherjee & Strivastava, 1952). Total sugars, reducing sugars and pentose contents of ethanol extracts or hydrolysates were determined by the phenol–sulphuric acid (Dubois, *et al.*, 1956), copper sulphate–arsenomolybdate (Hodge & Hofreiter, 1962) and orcinol (Albaum & Umbreit, 1947) methods, respectively. Glucose, fructose and sucrose were estimated by the enzymic and chemical methods of Johnson *et al.* (1964). Galactose was determined with galactose dehydrogenase and uronic acid by the carbazole method of Dische (1955). Oligosaccharides separated on chromatograms using *n*-butanol–ethanol–water as solvent were estimated by the phenol–sulphuric acid method of Dubois *et al.* (1956).

RESULTS AND DISCUSSION

Proximate composition

The proximate composition is shown in Table 2. Average dry matter content is 89·6%. Crude protein (N × 6·25) varied from 24·6% for FARV 13 to 33·1% for New Era, a variety originating from South Africa. Varieties of Nigerian origin of comparable crude protein content with New Era include Anna and Farin Juda C with values of 31·4% and 30·3%, respectively. The average crude protein of cowpea of 28% is higher than the values of 23·6% to 24·6% reported by Johnson & Raymond (1964) and Oyenuga (1968) but lower than the 32·5% reported for horsebean by Cerning *et al.* (1975). Mean crude fibre, ash and ether extract were 3·1%, 3·8% and 1·9%, respectively. The nitrogen-free extract was inversely related to the crude protein content (Table 2).

Available carbohydrate

The sum of free sugars plus starch is presented in Table 3. Values ranged from 37·2% to 57%.

TABLE 2
PROXIMATE CHEMICAL COMPOSITION OF TWENTY VARIETIES OF COWPEA IN g/100 g SAMPLE (DRY MATTER BASIS)

Variety	*Crude protein*	*Ether extract*	*Ash*	*Crude fibre*	*Nitrogen-free extract (NFE)*	*Dry matter*
Adzuki	29·9	2·0	3·9	3·7	60·5	93·8
New Era	33·1	1·6	2·3	3·3	59·7	91·3
Nig. A95	27·8	1·9	3·1	3·6	63·7	89·5
Farin Juda C	30·3	2·2	3·1	3·5	60·9	92·5
West bred	25·8	1·7	3·3	3·0	66·2	90·6
Nig. B4	29·0	2·1	3·1	2·0	63·8	88·5
Mala	27·0	2·0	3·6	3·9	63·6	87·8
Nig. A104	26·1	2·0	3·4	2·9	65·6	88·1
Nig. B7	28·3	2·0	4·3	2·1	63·6	89·7
Prima	26·5	2·4	3·0	1·7	66·3	87·4
Kano 2479	28·3	1·5	4·5	3·6	62·1	88·2
Kano 1696	29·3	1·7	4·9	3·2	60·9	87·4
Line 7	28·1	1·3	4·1	3·5	63·1	93·5
Anna	31·4	1·5	5·3	3·4	58·5	87·3
Ima	26·4	1·9	5·2	3·1	63·5	89·4
Kudi	26·4	2·1	3·6	3·2	64·7	90·4
Ayi	28·1	1·9	3·7	3·4	63·1	88·0
Ife Brown	27·4	2·0	3·5	3·0	63·5	90·0
FARV 13	24·6	1·8	4·4	2·7	66·4	89·9
TVu 4557	26·7	1·9	4·1	3·9	63·5	89·8
Mean	28·0 ± 4·5	1·9 ± 0·3	3·8 ± 0·8	3·1 ± 0·6	63·1 ± 2·2	89·6 ± 2·0

Ethanol-soluble sugars

The 80% ethanol extract from the cowpea varieties indicated sucrose, fructose and glucose. The last two sugars are only present as traces but higher levels of sucrose were obtained. The extracts were also abundant in soluble oligosaccharides —raffinose, stachyose and verbascose. The presence of an oligosaccharide higher than verbascose cannot be precluded, as is shown by the value for total sugars and by a tiny spot which did not move from the origin when sugars were examined chromatographically. Sucrose values ranged from 0·7% for Anna to 2·3% for FARV 13. Raffinose was present at lowest concentrations amongst the oligosaccharides and for most samples the proportion of verbascose was higher than stachyose. The oligosaccharide content was lowest for TVu 4557 (Table 3).

Starch

The starch content is shown in Table 3. Generally, this carbohydrate fraction was the most abundant single carbohydrate fraction in cowpea and ranged from 25·5% in Adzuki to 48% in Prima and West bred. Apart from Adzuki from the USA, New Era from South Africa and a Nigerian variety, FARV 13, which were relatively lower in starch, the starch contents were higher than values reported for spring and winter field beans by Pritchard *et al.* (1973) and wrinkled peas (Cerning-Beroard & Filiatre, 1976). The values obtained for most varieties are, however, close to those of Cerning-Beroard & Filiatre (1976) for horse beans and peas.

TABLE 3
AVAILABLE CARBOHYDRATES IN g/100 g SAMPLE (DRY MATTER BASIS)

Variety	*Glucose*	*Fructose*	*Sucrose*	*Raffinose*	*Stachyose*	*Verbascose*	*Other galactosides*	*Starch glucose*	*Available carbohydrate*
Adzuki	0·1	0·4	2·1	0·6	3·8	4·7	0·04	25·5	37·2
New Era	0·2	0·3	1·5	0·9	2·9	4·7	0·10	31·6	42·1
Nig. A95	0·3	0·4	1·8	1·5	2·9	4·0	0·09	37·7	48·7
Farin Juda C	0·2	0·5	1·5	1·3	1·1	2·4	0·05	37·9	44·9
West bred	0·1	0·4	1·5	0·9	1·9	2·2	0·03	48·0	54·9
Nig. B4	0·4	0·4	1·4	0·6	2·2	3·3	0·03	42·4	50·8
Mala	0·2	0·5	1·6	0·6	3·3	4·5	0·14	40·6	51·2
Nig. A104	0·2	0·4	1·9	0·5	3·4	4·8	0·07	39·1	50·2
Nig. B7	0·2	0·5	1·5	0·6	2·7	3·7	0·04	40·9	50·0
Prima	0·1	0·3	1·5	0·6	2·6	3·6	0·04	48·3	57·0
Kano 2479	0·1	0·3	1·1	0·3	2·4	3·1	0·09	42·0	49·4
Kano 1696	0·1	0·4	1·7	1·4	3·0	4·8	0·09	45·4	56·8
Line 7	0·1	0·3	1·6	1·5	3·1	3·0	0·08	46·1	55·8
Anna	0·1	0·2	0·7	0·7	2·3	2·4	0·05	39·0	45·4
Ima	0·1	0·4	1·4	0·7	2·4	2·9	0·04	40·8	48·7
Kudi	0·1	0·4	2·0	0·6	3·7	3·7	0·05	45·6	56·2
Ayi	0·2	0·3	1·4	0·6	3·6	2·7	0·04	46·1	54·9
Ife Brown	0·3	0·4	2·1	0·2	3·1	4·9	0·05	37·8	48·8
FARV 13	0·1	0·5	2·3	0·4	4·0	5·2	0·07	30·4	42·9
TVu 4557	0·1	0·4	1·9	0·5	0·7	2·3	0·12	47·5	53·5
Mean	0·2 ± 0·1	0·4 ± 0·1	1·6 ± 0·4	0·7 ± 0·9	2·7 ± 0·9	3·6 ± 1·0	0·07 ± 0·03	40·6 ± 6·1	50·0 ± 5·4

Unavailable carbohydrates

The water-soluble fraction, hemicellulose and cellulose (including lignin) have been fractionated as unavailable carbohydrates. Values are presented in Table 4. Generally, samples with high available carbohydrate contents were notably lower in unavailable carbohydrates. A considerable portion of the cell wall fraction was soluble in water even though cellulose constituted an appreciable proportion of the unavailable carbohydrates. The varieties from the USA and South Africa (Adzuki and New Era, respectively) and FARV 13 from Nigeria were highest in unavailable carbohydrates (16·8 %–19·9 %). The seeds of these varieties are notably smooth in texture.

Water-soluble carbohydrates

Hydrolysates of this fraction were found to contain glucose, galactose, arabinose and xylose although very slight traces of rhamnose were also detected. Glucose found in this fraction was higher than that observed for horsebeans by Cerning *et al.* (1975) and, according to their suggestion, the glucose must have originated from water-soluble glucose-containing polymers which are mostly located in the hulls. Xylose was the predominant pentose, as observed chromatographically, and arabinose, like galactose, was only present in trace amounts. Uronic acids which were not identified by Cerning-Beroard & Filiatre (1976) in horsebeans, peas and lupins, were detected, although in variable amounts with values ranging from 0·7 % to 1·9 %. The presence of uronic acids has been previously reported in field beans by Pritchard *et al.* (1973). Pectic substances are the most probable source of uronic acids, the presence of which has been correlated with cooking time in dry beans (*Phaseolus vulgaris*) by Kon (1968). The difference measured between total reducing sugars and individual sugars would most probably represent rhamnose.

Hemicellulose

Glucose, xylose, arabinose and galactose were the products of hydrolysis of the water-soluble residue and their composition is shown in Table 4. The pentoses present in this fraction are much lower than those found in the water-soluble fraction which is an indication that a great proportion of the pentosans present in cowpea are water-soluble. Although both xylose and arabinose have been measured together as pentoses, chromatographic investigation indicates only traces of arabinose. Glucose is also present in highest concentration in this fraction and this sugar may also have originated from non-cellulosic glucans, as reported for field beans (*Vicia faba*) by Pritchard *et al.* (1973), for mung bean (*Phaseolus aureus*) by Buchala & Franz (1974) and for wrinkled peas (*Pisum sativum*) by Cerning-Beroard & Filiatre (1976).

Cellulose

Glucose was the only sugar detected on paper chromatograms. The cellulose contents were slightly variable and values ranged from 2·5 % in Prima to 6·9 % in

TABLE 4

UNAVAILABLE CARBOHYDRATES IN g/100 g SAMPLE (DRY MATTER BASIS) AND THEIR HYDROLYSIS PRODUCTS

Variety	*Total reducing sugars*	*Water-soluble fraction*				*Hemicellulose*			*Cellulose*	*Lignin*	*Unavailable carbohydrate*
		Pentoses	*Glucose*	*Galactose*	*Uronic acid*	*Pentoses*	*Glucose*	*Galactose*			
Adzuki	7·0	2·5	2·1	0·5	1·8	1·5	2·6	0·5	5·7	1·8	19·0
New Era	7·8	2·4	3·9	0·3	1·2	1·4	1·6	0·8	6·9	1·4	19·9
Nig. A95	7·3	2·6	2·2	1·0	1·5	0·6	0·3	0·6	4·0	1·0	13·7
Farin Juda C	6·6	1·2	2·9	0·3	1·9	0·6	0·7	0·4	4·3	0·6	12·8
West bred	3·9	1·4	1·3	0·4	0·8	0·3	0·8	0·2	5·0	0·9	11·0
Nig. B4	5·6	1·6	2·9	0·1	1·0	0·7	1·0	0·5	4·9	0·9	13·4
Mala	6·1	2·4	2·1	0·3	0·8	0·6	1·0	0·4	5·5	1·0	14·2
Nig. A104	4·9	1·1	2·6	0·6	0·5	0·5	1·1	0·4	4·2	1·4	12·3
Nig. B7	4·6	1·6	1·7	0·2	0·5	0·5	1·3	0·3	5·0	0·8	12·0
Prima	4·7	2·1	1·2	0·5	0·9	0·5	1·8	1·1	2·5	0·6	11·0
Kano 2479	6·0	2·8	2·1	0·3	0·7	0·5	0·8	1·0	4·5	0·7	13·2
Kano 1696	5·2	1·8	1·4	0·2	1·0	0·5	0·9	0·3	4·5	0·6	11·3
Line 7	5·0	1·7	1·8	0·3	1·0	0·3	0·7	0·8	5·1	0·7	12·4
Anna	4·5	1·5	1·6	0·6	0·8	0·8	0·9	0·2	6·4	1·0	13·7
Ima	5·8	2·0	2·0	0·5	1·1	0·3	0·6	0·5	6·1	1·0	14·0
Kudi	4·8	1·5	1·8	0·2	1·2	0·3	0·7	0·8	5·0	0·9	12·4
Ayi	5·1	1·2	2·6	0·1	1·4	0·5	1·4	0·7	5·6	0·8	14·0
Ife Brown	5·0	1·6	2·0	0·5	0·8	0·5	0·5	0·1	4·6	0·8	11·4
FARV 13	6·3	1·9	3·2	0·1	1·0	0·6	0·9	0·5	6·5	1·1	16·8
TVu 4557	3·8	2·0	0·9	0·2	0·7	0·3	1·9	0·9	4·6	1·1	12·6
Mean	5·5 ± 1·1	1·8 ± 0·5	2·1 ± 0·7	0·3 ± 0·2	1·0 ± 0·4	0·6 ± 0·4	1·1 ± 0·5	0·6 ± 0·3	5·0 ± 1·0	0·9 ± 0·3	13·6 ± 2·4

New Era. Values of 5·3% and 8·5% have been reported for smooth and wrinkled peas, respectively by Cerning-Beroard & Filiatre (1976). Most values obtained for cowpeas fall between 4% and 5%.

Lignin

The crude lignin obtained after ignition of the sulphuric acid insoluble residue is shown in Table 4. In no variety did the value of crude lignin exceed 1·8%.

The results obtained in this investigation indicate that, apart from varietal differences existing in the physical characteristics of the cowpea, there are also differences in chemical composition. Since the different varieties, even though of different origins, were grown at the same station under similar climatic conditions, variation in carbohydrate composition may largely be attributed to genetic variability. Further studies on the same varieties are, however, necessary to establish such variation. It may eventually be possible to identify (and probably select) agronomically acceptable varieties for particular traits such as starch or fibre content or a single soluble sugar.

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ANNOUNCEMENT

'PROGRESS IN FOOD ENGINEERING'—A EUROPEAN SYMPOSIUM

Milan, Italy, will be the venue for a European symposium on 'Progress in Food Engineering' to take place there from the 3rd to the 5th of June, 1981.

The symposium, jointly organised by the Italian Association of Food Technology and the Italian Association of Chemical Engineering, with the sponsorship of the Italian National Research Council, the International Union of Food Science and Technology and the International Commission of Food Industries, will be composed of three simultaneous sessions on 'Solid Extraction', 'Isolation and Purification Techniques' and 'Texturisation and Modification of Rheological Properties'.

Individual subject areas to be discussed during these sessions include solid–liquid extraction and mechanical extraction processes and their applications to the extraction of lipids, sugars, proteins, etc., ultrafiltration, ion exchange and fractional distillation and their applications to lipids, pigments and flavour extracts and the extrusion, extrusion-cooking, spinning, rolling and flaking of liquid and solid foods.

Further information may be obtained from 'Symposium: Progress in Food Engineering', Istituto di tecnologie Alimentari, Via Celoria 2, 20133, Milan, Italy.

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Food Chemistry 6 (1980–81) 165–166

BOOK REVIEW

Developments in Food Carbohydrates—2. Edited by C. K. Lee. Applied Science Publishers Ltd, London. 1980. 397 pp. Price: £30·00

The increasing interest in the use and potential use of carbohydrates and their derivatives in the food industry has made detailed descriptions of their chemistry an essential requirement for further research. *Developments in Food Carbohydrates—2* has made a significant contribution in this area. While there is not as much discussion of the use of these sugars in foods as the title implies, the book nonetheless provides an excellent compilation of the chemistry of some of the less common food sugars. The extensive bibliography and table of derivatives with which each chapter ends are particularly noteworthy qualities of this book.

The opening chapter by the Editor, C. K. Lee, reviews the recent advances made in the structure determination and chemistry of the disaccharide, trehalose. The author points out that while this sugar has not been considered of dietary significance *per se*, the development of new fungal protein foods has stimulated research on the chemistry and metabolic effects of trehalose.

Although sucrose is used almost universally in the food industry as a sweetening agent, Chapter 2 also describes some of the research into the chemistry of sucrose which has led to the preparation of certain derivatives having potential agricultural and pharmaceutical applications. Following a discussion of one of the chemical transformations of sucrose, the author goes on to review structure–sweetness relationships with particular reference to partially methylated and chlorinated derivatives of sucrose, the extreme sweetness of some of the latter derivatives being a recent discovery.

The trisaccharides raffinose and melezitose are the subject of Chapter 3, wherein the author, E. B. Rathbone, has not only reviewed their chemistry but has also

Food Chemistry (6) (1980–81)—
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considered their biochemical synthesis and degradation. Sections 4 and 5 of this chapter deal with the use of raffinose in foods. The former describes its limited use in food manufacture due to its side-effects such as flatulence, whilst the latter compares its sweetness with that of sucrose.

Chapter 4, on maltose, confines itself almost exclusively to the chemistry of this disaccharide, with little reference to its application or occurrence in foods. In view of the chemical bias of this chapter, a more detailed review of the preparation of derivatives would have been worth while.

Chapters 5 and 6 are devoted to the chemistry of cellobiose and lactose, respectively. Chapter 5 is again almost exclusively chemically oriented but provides a very comprehensive review. Chapter 6, on the other hand, combines, with the chemistry of lactose, an interesting section on its commercial source and application. However, it is a pity that the derivatives of lactose were not tabulated in the manner of the preceding chapters, but in a more complicated format. As lactose and cellobiose are C4′ epimers, a chapter combining the chemistry and application of both these sugars would have provided extra information by way of comparative review.

Chapter 7, written by J. R. Hurford, provides an interesting and concisely written review of several aspects of surface active agents derived from disaccharides. Following a description of the manufacture and structure of disaccharide fatty acid esters, the author goes on to discuss their properties. The chapter concludes with a summary of the applications of these esters which include their use in the food, pharmaceutical and cosmetic industries.

The final chapter deals with the relatively new application of ^{13}C-nmr analysis to food-related di- and trisaccharides. In Sections 2 to 4 of this chapter, the author discusses the spectral data of ^{13}C-nmr. The information is both detailed and explicit and would be of value to those readers unfamiliar with this technique.

This book has succeeded in bringing together in one volume a review of the chemistry of some of the carbohydrates which are prominent in current research and thus provides an excellent reference text for both students and research workers.

AVA RAY

Food Chemistry **6** (1980–81) 167

EDITORIAL

On 17 October 1979, the Food Chemistry Group of The Royal Society of Chemistry held a symposium entitled 'Some Non-nutritional Nitrogenous Constituents of Foods'. It is the privilege of *Food Chemistry* to present these papers as a fourth in this series.

To those chemists not closely associated with food, whose first thought about nitrogen in food is 'protein', it may come as a surprise that there is sufficient material related to non-nutritional nitrogenous constituents to make up a symposium. However, after the papers have been read, it will be realised that there is more yet to be discovered than has already been elucidated!

Many interesting facts are revealed: although the naturally occurring nitrogenous complexes present fascinating areas of study, there are others developed during processing of considerable interest; the Maillard reaction plays an important role, but is still by no means fully understood; a realisation that Nature has its own specialised way of dealing with microbial and insect attacks; some of the complexes may have specialised nutritional and therapeutic value; compounds which give certain foods their particular piquancy may play other important roles; that a number of human illnesses can be traced to the toxic effects of some of these nitrogen-complexes—to recite but a few of the fascinating data given in these papers.

Once or twice the Editor has noticed the suggestion that some of these constituents have been evolved for specific purposes; no credence is given to the possibility of a beneficent Creator. The latter thought raises an interesting debate in relation to such syndromes as Lathyrism and the so-called 'Cheese Reaction'. When all is said and done, the reader is left with a sense of wonder that the human body manages, usually, to cope with such a variety of intake and to realise that as yet we only 'know in part'.

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AMINES IN FOOD

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ABSTRACT

The role of aromatic amines is emphasised, as these may be toxic. Those considered are tyrosine, involved in the so-called 'cheese reaction', which may cause headache, brain haemorrhage or heart failure; those caused by bacterial action in cheese, milk, yeast extract, alcoholic drinks, meat products, sauerkraut, and fish; those from various plants and plant products including citrus fruits, apples, beer, coffee, wheat, chocolate (with a discussion on migraine) and tea.

1. INTRODUCTION

Amines are basic nitrogenous compounds in which one, two or three atoms of hydrogen in ammonia are replaced by alkyl or aryl groups. Aryl-substituted amines are uncommon in nature, and the carcinogenic property of many synthetic aryl amines may explain their rarity. The simple aliphatic monoamines are widespread. These amines are usually formed by decarboxylation of amino acids in bacteria, but in plants aldehyde amination is a common mode of formation (see Section 4C). Methylamine and ethanolamine are probably ubiquitous, but together with di- and trimethylamine they may also be easily formed as artefacts by degradative processes in the course of isolation (Lerch & Stegemann, 1966; Hartmann *et al.*, 1972).

The diamine putrescine and the polyamines spermidine and spermine (Table 1) probably occur universally in animals and plants, and at least putrescine and spermidine are found in most bacteria. These amines are important in the regulation of nucleic acid function and protein synthesis, and probably also in the stabilisation of membranes. In all organisms spermidine and spermine are formed from putrescine by successive donation of one or two aminopropyl groups, respectively,

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from decarboxylated *S*-adenosylmethionine (Jänne *et al.*, 1978; Smith, 1977*a*, 1980*a*).

Certain classes of amines, the catecholamines, indoleamines and histamine, fulfil important metabolic functions in man, especially in the nervous system and in the control of blood pressure. These amines occur widely in animals, plants and bacteria, and they are frequently found in high concentrations in food, especially in that which has been subjected to deliberate or accidental bacterial contamination.

TABLE 1
DI- AND POLYAMINES OCCURRING IN FOODS

Putrescine	$NH_2(CH_2)_4NH_2$
Cadaverine	$NH_2(CH_2)_5NH_2$
Agmatine	$NH_2(CH_2)_4NH{-}\overset{\overset{NH}{\|}}{C}{-}NH_2$
Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

Amino acid decarboxylation is the most common mode of synthesis of these amines, the structures of which are shown in Table 2. In the present review, emphasis will be placed on the occurrence of the aromatic amines since these may render a food toxic.

Phenethylamine and the catecholamines, like tyramine, cause a rise in blood pressure by constricting the vascular system and increasing the heart rate and force of contraction of the heart. These are known as the pressor amines. By contrast, histamine reduces the blood pressure by causing vasodilatation. Phenethylamine, tyramine and histamine are found in animals, plants and bacteria. Adrenaline, also known as epinephrine, is formed only by animals, but noradrenaline is formed by animals and plants. Bacteria do not appear to produce noradrenaline and adrenaline.

Under normal circumstances in man exogenous amines absorbed from food are rapidly detoxified by amine oxidases or by conjugation. Histamine is oxidised by a diamine oxidase while the catecholamines are oxidised by a different multiple enzyme system known as monoamine oxidase (MAO). For instance after oxidation by MAO, tyramine is excreted as *p*-hydroxyphenylacetic acid.

2. THE 'CHEESE REACTION'

When MAO inhibitors were first used in the treatment of tuberculosis and subsequently for depressive illness, a number of patients developed severe hypertension and these attacks were traced to the accumulation in the body of high concentrations of pressor amines like tyramine derived from foods, notably cheese (Table 3). The parent amino acid, tyrosine, occurs at high concentrations in cheese

TABLE 2
STRUCTURES OF SOME AMINES AND RELATED COMPOUNDS FOUND IN FOODS

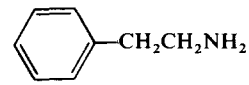
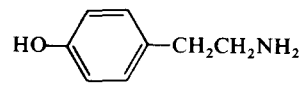
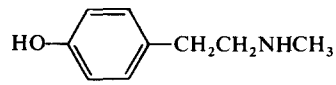
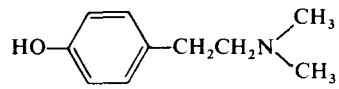
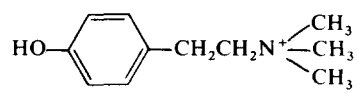
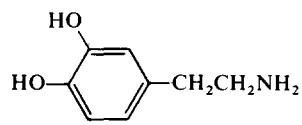
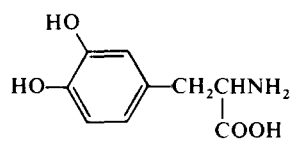
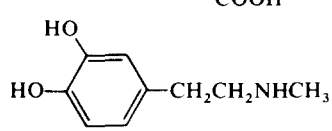
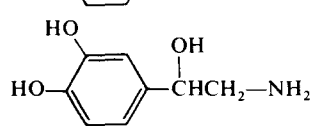
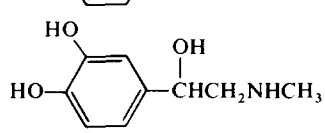
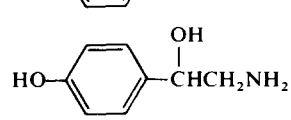
Amine	*Abbreviation in Table 10*	*Structure*
Phenethylamine	Pe	
Tyramine	Tyr	
Methyltyramine	Me-Tyr	
Hordenine	Hord	
Candicine	Cand	
Dihydroxyphenylethylamine (Dopamine)	Dop	
Dihydroxyphenylalanine	DopA	
Epinine	Epin	
Noradrenaline	Noradr	
Adrenaline		
Octopamine	Oct	

TABLE 2—*contd.*

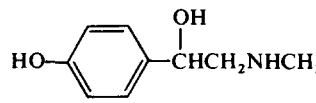
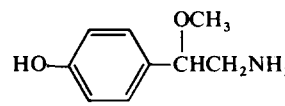
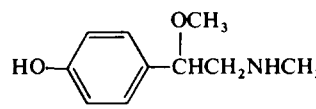
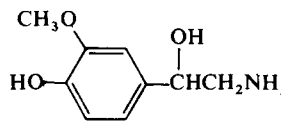
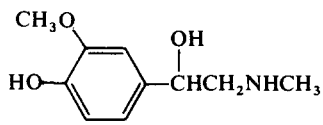
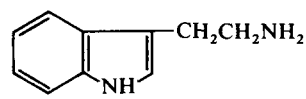
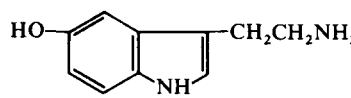
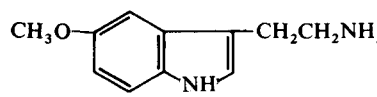
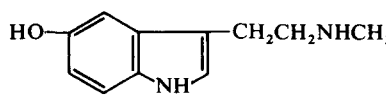
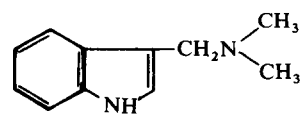
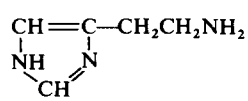
Amine	*Abbreviation in Table 10*	*Structure*
Synephrine	Syn	
Methoxyoctopamine	MeO-Oct	
Methoxysynephrine	MeO-Syn	
Normetanephrine	Normetaneph	
Metanephrine	Metaneph	
Tryptamine	Try	
Serotonin	Ser	
Methoxytryptamine	MeOTry	
Methylserotonin	Me-Ser	
Gramine	Gra	
Histamine	Hist	

TABLE 3
AMINES IN CHEESE (μg/g)

	Tyramine	*Phenethylamine*	*Tryptamine*	*Histamine*	*Ref.*
Cheddar	136–1500	—	—	—	Sen (1969)
Cheddar	<10–700	—	<10–300	<50–1300	Voigt *et al.* (1974)
Cheddar	350–1085	—	—	37–265	de Vuyst *et al.* (1976)
Cheddar (medium)	28–490	ND–140	ND–2	—	Koehler & Eitenmiller (1978)
Cheddar (mature)	775	—	—	—	Kaplan *et al.* (1974)
Roquefort	656	—	—	—	Kaplan *et al.* (1974)
Roquefort	50–1100	—	<10–1100	<50–2300	Voigt *et al.* (1974)
Gouda	80–670	—	<10–200	<50–450	Voigt *et al.* (1974)
Camembert	70–210	—	<10–60	<50–480	Voigt *et al.* (1974)
Bleu de Bresse	tr–313	—	—	tr–4100	de Vuyst *et al.* (1976)
Sap-Sago	520	—	150	2600	Voigt *et al.* (1974)
Swiss	—	—	—	4–2500	Chambers & Staruszkiewicz (1978)
Provolone	—	—	—	20–235	Chambers & Staruszkiewicz (1978)
Cottage	6–13	—	—	—	Kaplan *et al.* (1974)
Processed	11–72	ND–155	ND–1	—	Koehler & Eitenmiller (1978)

ND = not detectable.
tr = trace.

and it was from this source that tyrosine was first isolated by Liebig, who named it after 'tyros' the Greek word for cheese.

Some pressor amines, like noradrenaline, may increase blood pressure directly by constricting the vascular system and stimulating the heart muscle. However, tyramine does this indirectly by causing the release of noradrenaline from the sympathetic nervous system (Stockley, 1973). The increase in blood pressure, now known as the 'cheese reaction', can cause severe headache and may induce a brain haemorrhage or heart failure. One of the functions of MAO in the intestine and liver is apparently to destroy potentially harmful amines derived from food before they reach the blood. Use of the MAO inhibitor drugs eliminates this detoxication mechanism (Fig. 1).

The rapidity of the reaction, in some cases within 5 min, suggested that absorption of at least part of the amine may take place through the oral mucosa, bypassing the intestinal and hepatic MAO. Inhibition of the neuronal MAO would therefore render the subject very sensitive to amines entering by this route. The absorption of amines in this way is dependent on alkaline pH and on the length of time the food is retained in the buccal cavity (Price & Smith, 1971).

In order to prevent these hypertensive attacks it has been necessary to warn patients to avoid foods containing pressor amines, and standard cards listing these

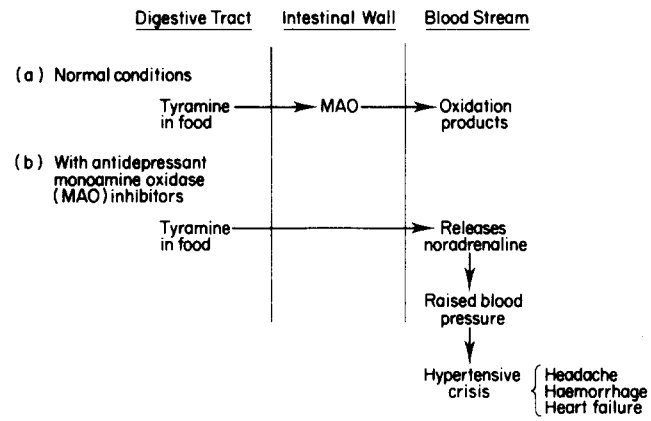
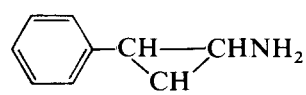


Fig. 1. The mechanism of the 'cheese reaction'. Tyramine in food is normally oxidised by monoamine oxidase present in the liver and intestinal wall to form inactive aldehydes (a). In patients taking monoamine oxidase inhibitors this 'detoxication' mechanism is rendered inoperative and the tyramine enters the blood stream causing a hypertensive crisis (cheese reaction) (b).

foods are now issued to such patients. Since the inhibition is often irreversible, such attacks may take place as long as three weeks after drug withdrawal, the time taken to restore the MAO activity by enzyme synthesis. The early work on this subject has been reviewed by Marley & Blackwell (1970), Stockley (1973), Lovenberg (1973), Rice *et al.* (1976) and Lindner (1979). The reactions to foods by patients on MAO inhibitors are cited by Stewart (1976) and in *Martindale* (Anon., 1977). Especially comprehensive surveys of amines in foods are given by Askar (1976, 1979) and Maga (1978).

Most of the early attacks occurred in patients eating cheese after being given the antidepressant tranylcypromine and in at least one of these incidents the patient died as a consequence of the hypertension (Cuthill *et al.*, 1964). Many similar MAOI



Tranylcypromine

drugs are now used in the treatment of depression. The antihypertensive drug pargyline, the cytotoxic agent procarbazine, and the antibacterial drug furazolidone are also MAO inhibitors (Stewart, 1976). Although reaction to a food is very variable between individuals, other foods were soon shown to cause similar hypertensive attacks. Yeast extract (Section 3C, Table 4) (Blackwell *et al.*, 1965*a*,*b*), pickled herring (Nuessle *et al.*, 1965) (containing 3 mg tyramine per gram) and beef (Boulton *et al.*, 1970) and chicken liver (Hedberg *et al.*, 1966) (see Section 3E) were shown to induce similar hypertensive crises attributable to tyramine. An attack after

ingestion of broad bean pods was apparently due to the high concentration of dihydroxyphenylalanine (DopA) in this material (Hodge *et al.*, 1964) (see Section 4).

3. AMINES FORMED BY BACTERIAL ACTIVITY

A. *Cheese*

Tyramine, phenethylamine, tryptamine and histamine (for structures see Table 2) are found in high concentrations in various cheeses, especially near the rind and in well-matured cheeses like Cheddar (Table 3). However, appearance is no guide to tyramine content and mild tasting cheese may contain a considerable amount of tyramine. The tyramine is probably formed by aerobic bacteria and not by the *Lactobacilli* added for cheese manufacture, which are anaerobes or facultative anaerobes (Price & Smith, 1971). Cottage cheese has a relatively low concentration of amines. Phenethylamine occurs in variable concentrations in Cheshire and Edam cheeses.

Histamine in cheese may cause a reaction even in the absence of MAO inhibitors. Gouda cheese, found to contain 850 μg/g, induced a severe occipital headache and generalised flushing in a sensitive subject. However, feeding the same cheese to a control subject elicited no symptoms (Doeglas *et al.*, 1967) (see also Sections 3D, 3F and 3G).

B. *Milk*

The amine concentration in fresh milk is quite small. The mean tyramine and histamine concentrations for 170 samples of dried milk products were respectively 1·31 and 0·42 μg/g (Grove & Terplan, 1975). Other amines present in milk include propylamine and hexylamine (Cole *et al.*, 1961), and cadaverine, putrescine, spermidine and spermine (Sanguansermsri *et al.*, 1974) (Table 1). The last three amines of this group are found in most organisms (Jänne *et al.*, 1978; Smith, 1977*a*, 1980*a*) and hence they occur ubiquitously in foods.

After storage for 2 days at 25 °C before freeze drying, unpasteurised milk powder contained 94 and 2·8 μg/g of tyramine and histamine respectively and it is therefore unlikely that these amines would constitute a hazard in this food (Grove & Terplan, 1975). In yogurt, the tyramine concentration was less than 0·2 μg/ml (Horwitz *et al.*, 1964).

C. *Yeast extract*

Plasmolysis of brewer's yeast followed by autolysis in saline solution at 36 °C for 24 h (pH 6·3–6·6) is used in the preparation of yeast extracts (Blackwell *et al.*, 1969). Decarboxylation of the amino acids to form tyramine and histamine may be effected either by the yeast enzyme or more likely by contaminating bacteria. Hypertension

TABLE 4
AMINES IN YEAST EXTRACT (μg/g)

	Tyramine	*Histamine*	*Ref.*
Manufacturer A sample 1	1640	2830	Blackwell *et al.* (1969)
Manufacturer A sample 2	1440	1950	Blackwell *et al.* (1969)
Manufacturer A sample 3	1090	980	Blackwell *et al.* (1969)
Manufacturer A salt free	190	1660	Blackwell *et al.* (1969)
Manufacturer B	510	1340	Blackwell *et al.* (1969)
Manufacturer C	420	270	Blackwell *et al.* (1969)
Manufacturer D	150	210	Blackwell *et al.* (1969)
Manufacturer E	100	260	Blackwell *et al.* (1969)
English yeast extract	2100	—	Sen (1969)
Canadian yeast extract	75	—	Sen (1969)

can be induced in patients taking MAO inhibitors by about 4 g of yeast extract (i.e. about 6 mg of tyramine) (Table 4).

D. *Alcoholic drinks*

Tyramine and histamine are found in wine and beer (Table 5; see also Section 4D). The concentrations are quite variable but Chianti may contain relatively high concentrations of tyramine (Horwitz *et al.*, 1964). Alcohol may potentiate the effect of these amines present in wine or food by directly or indirectly inhibiting the amine oxidase (Maynard & Schenker, 1962; Marquardt & Werringloer, 1965*a*,*b*). An upper limit of 2 μg/ml histamine has been proposed by Marquardt & Werringloer (1965*a*). In order to achieve this concentration, bentonite may be added to remove the amines (Hernandez, 1975). Early work had suggested that on average red wines have a greater histamine concentration than white wines (Mayer *et al.*, 1971). Mayer *et al.* (1971) consider that histamine is formed by lactic acid cocci during malolactic fermentation. *Leuconostoc oenos*, occasional long rods (bacilli) and acetic acid bacteria were not implicated. They suggest that histamine formation may be

TABLE 5
AMINES IN ALCOHOLIC DRINK (μg/ml)

	Tyramine	*Histamine*	*Ref.*
Beer	8–11	—	Sen (1969)
Ale	9	—	Sen (1969)
Chianti	2–12	—	Sen (1969)
Chianti	25	—	Horwitz *et al.* (1964)
California wines	—	0·3–11	Ough (1971)
Bourgogne	—	0·4–21	Lafon-Lafourcade (1975)
White wine	—	16	Jakob (1978)
Red wine	—	10	Jakob (1978)
Red and white wines	0·3–0·6	—	Gonzalo *et al.* (1979)
Bordeaux	—	15	Quevauviller & Mazière (1969)
Chambertin	—	30	Quevauviller & Mazière (1969)

prevented by monitoring during malolactic fermentation and control of the bacterial flora in inoculum wines.

Flushing of the face and neck induced by alcohol is especially common in women, and may be prevented by a combination of H_1 and H_2 histamine antagonists (Tan *et al.*, 1979). However, no significant correlation between histamine concentration of wine and the incidence of flushing could be found (Granerus *et al.*, 1969). It is possible that certain wines contain components which cause the release of histamine in sensitive subjects. Indeed this may also occur with other foods where an apparent histamine reaction is induced (see also Sections 3F and 3G).

E. *Meat products*

Physiologically active amines may accumulate in meat products due to bacterial activity. Usually only low concentrations of histamine are found, but tyramine can occur at over 300 μg/g (Table 6). Several meat products would provide sufficient

TABLE 6
AMINES IN MEAT PRODUCTS (μg/g)

	Tyramine	*Phenethyl-amine*	*Tryptamine*	*Histamine*	*Ref.*
Chicken liver	94–113	—	—	—	Hedberg *et al.* (1966)
Chicken skin	—	—	—	10–140	Henry (1960)
Meat extract	95–304	—	—	—	Sen (1969)
Dry sausage	ND–1240	—	—	0·74–7·8	Rice *et al.* (1975)
Country cured ham	ND	—	—	0·82–2·7	Rice *et al.* (1975)
Salami	263	68	ND	—	Koehler & Eitenmiller (1978)
Cotte salami	103	696	ND	—	Koehler & Eitenmiller (1978)
Pork	11–22	—	19–48	11–45	Lakritz *et al.* (1975)
Ham	2–8	—	8–67	4–5	Lakritz *et al.* (1975)
Beef liver	274	—	—	65	Boulton *et al.* (1970)

ND = not detected.

tyramine in normal servings to induce a pressor response in patients taking MAO inhibitors. Chicken liver which caused a hypertensive crisis contained about 100 μg/g, though normally such liver would contain only 0·5 μg/g (Hedberg *et al.*, 1966). Histamine of physiological origin occurs in quite high concentrations (140 μg/g) in the skin of birds (Henry, 1960).

In a study of the formation of volatile amines in uncured pork during storage at −20 °C to +20 °C, amine formation was independent of bacterial growth until the count reached $10^9/cm^2$. The meat was spoiled before dimethylamine was formed in amounts sufficient for the detection of *N*-nitrosodimethylamine (Patterson & Edwards, 1975).

In pork stored for 16 days at 4 °C, no change was detected in the polyamine concentrations, which remained below 100 μg/g (Nakamura *et al.*, 1977).

F. *Sauerkraut*

The effect of the fermentation conditions on histamine formation in sauerkraut has been studied by Mayer *et al.* (1974). They found that histamine could increase to 160 μg/g after fermentation for 10 weeks. Taylor *et al.* (1978) found histamine in concentrations of up to 130 μg/g in 50 samples of sauerkraut. Although Mayer & Pause (1972) attributed a severe headache to the consumption of 200 g of sauerkraut, it is now considered that the histamine in sauerkraut is unlikely to reach hazardous concentrations (Taylor *et al.*, 1978).

G. *Fish*

It is well known that fish in the family Scombridae, which includes tuna and mackerel, are liable to cause a characteristic food poisoning. Tuna fish may be unsuitable for consumption for this reason even before spoilage can be detected by organoleptic examination (Ienistea, 1972). Recent literature on this subject has been reviewed by Arnold & Brown (1978), and Sinell (1978). Vomiting, abdominal pain, facial flushing and headache are the most notable effects of scombroid fish poisoning. Onset is rapid, but recovery is usually complete within 8 h (Cruickshank & Williams, 1978). Some of these symptoms closely resemble those of a histamine reaction, and it was not surprising to find high concentrations of histamine in suspect food (Table 7). Unlike tyramine, histamine is a powerful vasodilator, and this accounts for the facial flushing in histamine toxicity. Fish with a histamine content above 1 mg/g is usually toxic (Cruickshank & Williams, 1978). Early reports of a separate toxin named 'saurine' have proved to be attributable to histamine (Foo, 1976). The histamine is formed by bacterial decarboxylation of the amino acid histidine, which is a major component of muscle in the fish causing this reaction. Histidine decarboxylase is an inducible enzyme, and it occurs in many species of bacteria. Early work on the occurrence of histamine in fish and other food is reviewed by Ienistea (1972). The apparent high incidence of scombroid fish

TABLE 7
HISTAMINE IN FISH (μg/g)

Skipjack tuna	spoiled	7140	Omura *et al.* (1978)
Tuna	canned	20	Kim & Bjeldanes (1979)
Tuna	canned, decomposed	1180	Kim & Bjeldanes (1979)
Tuna	canned	260	Karmas & Mietz (1978)
Chunk light tuna	processed	12–280	Taylor *et al.* (1977)
Mackerel		10–315	Taylor *et al.* (1977)
Mackerel	stored 48 h at 25 °C	300	Edmunds & Eitenmiller (1975)
Mackerel	stored 20 days at 10 °C	1800	Fernández-Salguero & Mackie (1979)
Mackerel	smoked	1480	Cruickshank & Williams (1978)
Anchovy fillets		375–937	Peeters (1963)
Various	marinades	30–2400	Fücker *et al.* (1974)
Herring roes	smoked	12–350	Quevauviller & Van Hoa (1965)

poisoning in hospitals could be related to the availability of the means for diagnosis and may indicate that such symptoms are fairly common in the general population (Popovich *et al.*, 1960).

Sardines have also been implicated in apparent histamine poisoning episodes. Popovich *et al.* (1960) report that out of 88 patients who ate sardines, 44 showed symptoms which included hyperemia of the conjunctiva, a subjective feeling of warmth, headache and urticaria. Schulze *et al.* (1979) reported burning of the lips, swelling of the mouth mucosa and vomiting on eating 80 g of sardines containing histamine at 500 μg/g.

Fish kept under sterile conditions does not form histamine (reviewed by Arnold & Brown, 1978). A strain of *Klebsiella pneumonii* isolated from a sample of tuna implicated in an outbreak of scombroid fish poisoning was capable of forming large concentrations of histamine (Taylor *et al.*, 1979). Other bacteria isolated from tuna and mackerel include *Proteus morganii*, which is very active in forming histamine, and the less active *Hafnia alvei* (Omura *et al.*, 1978). It is possible to ingest as much as 180 mg of histamine free base in the absence of food without any serious ill effects (reviewed by Arnold & Brown, 1978) though individuals may differ in sensitivity. The presence of histamine in food may lead to false positive scratch tests in the diagnosis of food hypersensitivity (Doeglas & Nater, 1968).

In many cases of scombroid fish poisoning the concentrations of histamine in the suspect food would be insufficient to account for the symptoms observed on ingestion. For instance, Edmunds & Eitenmiller (1975) and Hardy & Smith (1976) conclude that histamine intoxication from mackerel is unlikely since the mackerel muscle would have to reach an advanced state of decomposition before the toxic concentration was reached. Arnold & Brown (1978) believe that it is extremely unlikely that histamine acting alone is the sole factor for scombroid poisoning. However, there is a strong positive correlation between toxicity and histamine.

Quail chicks fed with chick starter meal showed a significantly depressed growth on addition of histamine at 3·6 g/kg. However, good tuna meal consistently gave better growth than meal from spoiled tuna, despite the addition of histamine to a final concentration of 3·6 g/kg (Blonz & Olcott, 1978).

In order to explain the histamine reaction found when the histamine in the food does not achieve a high concentration, it has been suggested that other substances found in the food may be synergistic, enabling absorption of amounts of histamine larger than could be achieved in the absence of food. In man, histamine is normally detoxified by a diamine oxidase found in the intestinal wall, and the high concentrations of the diamines putrescine and cadaverine in spoiled fish (Karmas & Mietz, 1978) may depress histamine oxidation and act as synergistic factors (Parrott & Nicot, 1966; Arnold & Brown, 1978; Bjeldanes *et al.*, 1978). However, the presence of an alternative toxin cannot yet be eliminated. The relative proportions of putrescine, cadaverine, spermidine, spermine (Table 1) and histamine may be useful as quality indicators of fish, lobster and shrimps (Karmas & Mietz, 1978; Mietz & Karmas, 1978).

Trimethylamine-*N*-oxide (TMAO) is formed in salt water fish and crustacea as an innocuous excretion compound for excess nitrogen (Visek, 1974; Kostuch & Sikorski, 1977). Reduction of TMAO by bacteria appears to be the source of the trimethylamine in dead fish. Dimethylamine, together with formaldehyde, arises as a result of the activity of an endogenous enzyme on TMAO, and not from bacterial action (Visek, 1974; Kostuch & Sikorski, 1977). These amines, which give fish its characteristic smell, change extensively during storage. There are great inter-species differences and the figures quoted in Table 8 are examples of what has been observed and should not be regarded as too rigidly representative.

TABLE 8
METHYLAMINES IN FISH (μg/g)

		Dimethylamine	*Trimethylamine*	*Ref.*
Hake	frozen 750 days	>400	30	Miller *et al.* (1972)
Cod	frozen	740	—	Singer & Lijinsky (1976)
Cod	frozen 150 days	75	12	Miller *et al.* (1972)
Cod	12 days on ice	65	550	Keay & Hardy (1972)
Salmon	good	3·4–6·6	3·6–6·6	Gruger (1972)
Sable fish	good	ND	147	Gruger (1972)
Sable fish	spoiled	ND	1164	Gruger (1972)

ND = not detected.

Trimethylamine-*N*-oxide concentration is highest in the elasmobranchs like the dogfish, and least in the flatfish. In general, arctic fish have more than those from the North Sea, and there is more in older than younger fish. The content varies with season. It is twice as high in herring caught in February than in those caught in July. Trimethylamine-*N*-oxide may cause corrosion of tin cans, and therefore can be commercially important. The trimethylamine content in fish stored under identical conditions but caught at different times is not constant, and is not related in any simple manner to the loss of trimethylamine-*N*-oxide. It is therefore not possible to use the trimethylamine content as a method of estimating spoilage (Shewan, 1951; Hornstein *et al.*, 1963; Smith *et al.*, 1980).

Estimation of secondary amines in food is important in view of the possible formation of nitrosamines by reaction with nitrous acid (Fig. 2). Dimethylnitrosamine is known to be a potent carcinogen, and it might be unwise to consume fish together with food containing nitrite as a preservative (e.g. ham, pork pies, etc.).

Other nitrosatable secondary amines include agmatine (Table 1) which occurs widely in fish, meat (Kawabata *et al.*, 1978) and vegetable products (Smith *et al.*, 1979; Smith, 1980*b*) and the ubiquitous polyamines spermidine and spermine (Lakritz *et al.*, 1975; Hildrum & Scanlan, 1977; Correa *et al.*, 1978; Jänne *et al.*, 1978; Nakamura *et al.*, 1979; Smith *et al.*, 1979; Walters & Walker, 1979; Smith,

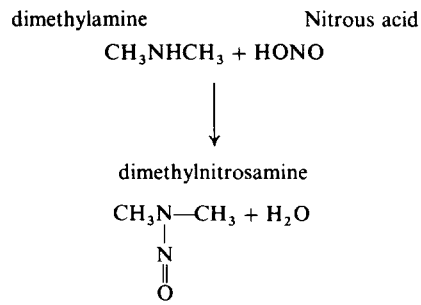


Fig. 2. Formation of dimethylnitrosamine

1980*b*). The distribution of secondary amines in food has been reviewed by Neurath & Schreiber (1973), Singer & Lijinsky (1976) and Neurath *et al.* (1977).

4. AMINES IN PLANT PRODUCTS

The simple aliphatic monoamines found in food plants are shown in Table 9. In cooked foods some of these amines can be formed by thermal decomposition of amino acids (Velíšek & Davídek, 1974). Little is known of the contribution of these amines to flavour in food. The distribution of these amines has been reviewed by Askar (1973).

TABLE 9
ALIPHATIC MONOAMINES FOUND IN EDIBLE PLANT PRODUCTS

Species	*Common name*	*Amines present*[a,b]	*Ref.*
Avena sativa	oat (instant oat flakes)	DM, E, P, iP, B, iB, A	Hrdlička & Janiček (1964)
Coffea arabica	coffee	DM, EM, E, P, iB	Maga (1978); Neurath & Schreiber (1973)
Glycine max	soya	DM, TM, E	Maga (1978)
Hordeum vulgare	barley	DM, E, iB, iA	Smith (1980*b*)
Malus spp.	apple	DM, E, P, B, HX, iP, iB, iA, OC	Smith (1980*b*); Hrdlička & Curda (1971)
Musa sapientum	banana	DM, E, iB, iA, PR	Askar *et al.* (1972)
Solanum tuberosum	potato	DE, P, iB	Schormüller & Weder (1966)
Thea sinensis	tea	DM, TM, E, P, iB, iA	Maga (1978)
Theobroma cacao	cocoa	DM, TM, E, TE, B, iB, iA	Maga (1978)

[a] *Key:* DM, dimethylamine; TM, trimethylamine; E, ethylamine; EM, ethylmethylamine; DE, diethylamine; TE, triethylamine; P, propylamine; B, butylamine; A, amylamine; HX, hexylamine; OC, octylamine; iP, isopropylamine; iB, isobutylamine; iA, isoamylamine; PR, propanolamine.
[b] The occurrence of methylamine and ethanolamine is not surveyed since these amines may be formed as artefacts of extraction (see text).

TABLE 10
PHENETHYLAMINES, TRYPTAMINES AND HISTAMINE IN PLANT PRODUCTS
(Figures in brackets are weight in μg/ml or per gram)

Species	*Common name*	*Plant part*	*Amines present*[a]	*Ref.*
Ananas sativus	pineapple	juice, slices	Tyr (0·4), Ser (19–65)	Bruce (1960); Foy & Parratt (1961); Sen (1969); Bancher (1975); Regula (1977*a*)
Beta vulgaris	beet	leaf	Dop, Hist (5·2)	Werle (1955); Gardner *et al.* (1967)
Carica papaya	pawpaw	fruit	Ser (1–2)	Foy & Parratt (1960)
Citrullus vulgaris	water-melon	fruit	Try	Dannenberg & Liverman (1957)
Citrus limon	lemon	leaf, juice	Tyr (25), Me-Tyr, Syn (2–20), Oct (4)	Stewart & Wheaton (1964); Wheaton & Stewart (1969)
Citrus mitis	calamondin	juice	Syn (9)	Stewart & Wheaton (1964)
Citrus medica × *sinensis*	citrange	juice	Tyr (7), Syn (1), Oct (2)	Stewart & Wheaton (1964)
Citrus reticulata	tangerine	fruit	Tyr (1), Me-Tyr (15–58), Hord (7), Oct (1–2), Syn (60–280)	Wheaton & Stewart (1970)
Citrus reticulata × *sinensis*	temple	juice	Tyr (1), Oct, Syn (27–43)	Stewart & Wheaton (1964)
Citrus sinensis	orange	fruit, juice	Tyr (1–10), Me-Tyr (1), Oct, Syn (19–50), Noradr, Try, MeO-Oct, MeO-Syn	Wheaton & Stewart (1970); Udenfriend *et al.* (1959) Coffin (1969)
Coffea arabica	coffee	bean	Ser conjugate	Folstar *et al.* (1979)
Colocasia antiquorum	taro	leaf	Tyr (34)	Wheaton & Stewart (1970)
Ficus carica	fig	fruit	Ser (12)	Stachelberger *et al.* (1977)
Hordeum vulgare	barley	seedlings	Tyr (10), Me-Tyr (48), Cand (15), Hord (80), Try (0·5), Ser (0·2), Gra (623), Me-Ser (0·4)	Rabitzch (1959); Schneider & Wightman (1974)
Juglans regia	walnut	embryo	Ser (550)	Bergmann *et al.* (1970)
Lycopersicon esculentum	tomato	fruit	Tyr (4), Try (4), Ser (12), Hist (0·3–1)	Udenfriend *et al.* (1959) Holtz & Janisch (1937); West (1959); Regula (1977*b*); Villanueva & Adlakha (1978); Haartmann *et al.* (1966)
Musa sapientum	banana	pulp	Tyr, Dop, Noradr, Ser (see Table 11) Pe, Metaneph, Normetaneph, Oct, Try, MeO-Try, Hist	Foy & Parratt (1960); Udenfriend *et al.* (1959); Stachelberger *et al.* (1977); Deacon & Marsh (1971); Askar *et al.* (1972); Vettorazzi (1974); Riggin *et al.* (1976); Kenyhercz & Kissinger (1978)
Passiflora foetida	passion fruit	fruit	Ser (1–4)	Foy & Parratt (1960)

TABLE 10—*contd*

Species	*Common name*	*Plant part*	*Amines present*[a]	*Ref.*
Panicum miliaceum	millet	seed	Tyr, Me-Tyr, Hord	Brady & Tyler (1958); Sato *et al.* (1970)
Persea americana	avocado	fruit	Tyr (23), Dop (4–5), Ser (10)	Udenfriend *et al.* (1959)
Phaseolus radiatus	greengram	seed	Tyr	Kasai & Sakamura (1973)
Phoenix dactylifera	date	fruit	Ser (9)	Stachelberger *et al.* (1977)
Portulaca oleracea	purslane	whole plant	Dop (2500), Noradr (2500), DopA	Feng *et al.* (1961)
Prunus amygdalus	almond	oil	Pe	*Merck Index* (1968)
Prunus domestica	plum	fruit	Tyr (6), Noradr, Try (2), Ser (10)	Udenfriend *et al.* (1959)
Rubus idaeus	raspberry	fruit, jam	Tyr (8–93)	Coffin (1970)
Solanum melongena	egg plant	fruit	Tyr (3), Try (0·5–3), Ser (2)	Udenfriend *et al.* (1959)
Solanum tuberosum	potato	tuber	Tyr (1), Dop (0·2), Noradr (2)	Udenfriend *et al.* (1959); Bygdeman (1960)
Spinacia oleracea	spinach	leaf	Tyr (1), Dop (100), Hist (400), *N*-dimethylHist, *N*-acetylHist	Werle (1955); Gardner *et al.* (1967); Udenfriend *et al.* (1959); Haartmann *et al.* (1966); Appel & Werle (1959); Gewitz & Völker (1961)
Thea sinensis	tea	seed	Pe	Serenkov (1959)
Vicia faba	broad bean	pods, germinating seeds	DopA, Epin (600)	Piccinelli (1955); Hodge *et al.* (1964)
Zea mays	maize	seed	Tyr (1), Hist (1), Pe (4)	Neumark (1964)

[a] For abbreviations see Table 2.

The phenethylamines, tryptamines and histamine content of various fruit and vegetables is shown in Table 10. The fruits of lemon, avocado and raspberry contain tyramine in moderate amounts. There is a high concentration of dopamine and noradrenaline in *Portulaca oleracea* (purslane), used in salads in sub-tropical regions. In this plant, the noradrenaline content is even greater than that found in the suprarenal glands of mammals. Spinach contains histamine and dopamine in high concentrations, and serotonin accumulates in the embryo of walnuts.

Broad bean pods which are commonly consumed in New Zealand (McQueen, 1975) have been known to cause a hypertensive crisis in patients (Hodge *et al.*, 1964). This is attributable to the presence of DopA which may be converted to dopamine by enzymes in the body.

The occurrence of phenethylamines and tryptamines in plants is reviewed by Smith (1977*b*,*c*).

In plants arginine is decarboxylated to agmatine, and putrescine is formed from agmatine via *N*-carbamylputrescine (Smith, 1977*a*, 1980*a*). In conditions of potassium and magnesium deficiency and especially with high ammonium concentrations in the nutrient medium, agmatine and putrescine accumulate in plants (Basso & Smith, 1974; Le Rudulier & Goas, 1975; Klein *et al.*, 1979). Little is known of the diamine content of the edible parts of plants, but in some leaves the putrescine (free base) concentration can be in excess of 350 μg/g fresh weight and agmatine may be 82 μg/g fresh weight (Smith, 1968, 1977*a*).

On feeding a series of amines including benzylamine, phenethylamine, trimethylamine, piperidine, agmatine, putrescine, cadaverine, tyramine and histamine in non-toxic doses to rats for prolonged periods, the amounts of these amines accumulated in the liver were estimated. Trimethylamine was detectable by its odour, but at most only traces of benzylamine, tyramine, phenethylamine and histamine were found. Of the diamines, the greatest accumulation (to 20 μg/g) occurred on feeding 2·3 g agmatine over 60 days. This amine is apparently only relatively slowly detoxified in the rat (Laurent *et al.*, 1956).

A. *Bananas*

In the banana fruit several amines are found which are of physiological importance in animals. These include dopamine, noradrenaline and serotonin. The presence of serotonin in bananas was discovered by accident. At the conclusion of a study on the excretion of 5-hydroxyindoleacetic acid (5-HIAA) in monkeys fed with monkey chow diet, a banana was given as a reward for satisfactory performance. The final estimate of 5-HIAA in the urine was increased 24-fold (Anderson *et al.*, 1958). Subsequently it was shown by Waalkes *et al.* (1958) that the increase of 5-HIAA was due to oxidation of serotonin after ingestion of the banana. Serotonin is similarly oxidised in man to 5-HIAA, and excretion of 5-HIAA is used in the clinical diagnosis of carcinoid tumour (argentaffinoma), a rare tumour of the small intestine characterised by the production of high concentrations of serotonin (Crawford, 1962). Plant products like banana, pineapple, fig, walnut and tomato (Table 10) should be excluded from the diet before attempting to diagnose carcinoid tumour.

Ingestion of large amounts of serotonin may be harmful to humans. The staple diet of certain African tribes is matoke (banana species) usually eaten green in meals of 400–800 g of pulp (Crawford, 1962). The high incidence of endomyocardial fibrosis in this area may be related to the high consumption of these bananas. This connection is further substantiated by the increased incidence of heart lesions in cases of carcinoid tumour, a condition in which the concentration of serotonin in blood is raised considerably (Foy & Parratt, 1962).

Serotonin inhibits gastric secretion and stimulates smooth muscle (Haverback *et al.*, 1957) and bananas have been used in the prevention and treatment of experimental peptic ulcers induced by phenylbutazone in guinea pigs (Sanyal *et al.*, 1963). Serotonin is a powerful vasoconstrictor and vasopressor when 1 mg is

injected, but an oral dose of 20 mg has no effect (Waalkes *et al.*, 1958). Although harmful effects of consuming bananas by patients taking MAO inhibitors have been reported only rarely (Blackwell & Taylor, 1969), the simultaneous consumption of foods like banana containing high concentrations of serotonin with MAO inhibitors may presumably predispose to endomyocardial fibrosis.

Banana ingestion by humans not only increases urinary 5-HIAA, but also causes excretion of conjugates of noradrenaline and dopamine (Richter, 1940). Diagnosis of phaeochromocytoma (adrenal neoplasm) may be made by estimation of urinary catecholamine. This tumour secretes large amounts of adrenaline and noradrenaline, and its diagnosis depends on a diet deficient in foods like bananas which contain these amines (Crout & Sjoerdsma, 1959).

The amount of serotonin in the pulp of *Musa cavendishii* decreases during maturation whereas that in the peel increases (Vettorazzi, 1974). Little is known of the enzymes involved in serotonin synthesis.

An enzyme has been isolated from banana pulp which oxidises tyramine to dopamine. At least one other product, possibly noradrenaline, was also formed in fresh extracts (Deacon & Marsh, 1971).

In the banana fruit dopamine may also be conjugated with endogenous acetaldehyde to form the alkaloid salsolinol (Fig. 3) (Riggin *et al.*, 1976). This alkaloid increases with ageing in parallel with the increase in acetaldehyde. The post climacteric banana pulp may contain 40 μg/g of salsolinol.

Other amines found in bananas include (with concentrations in μg/g) a series of

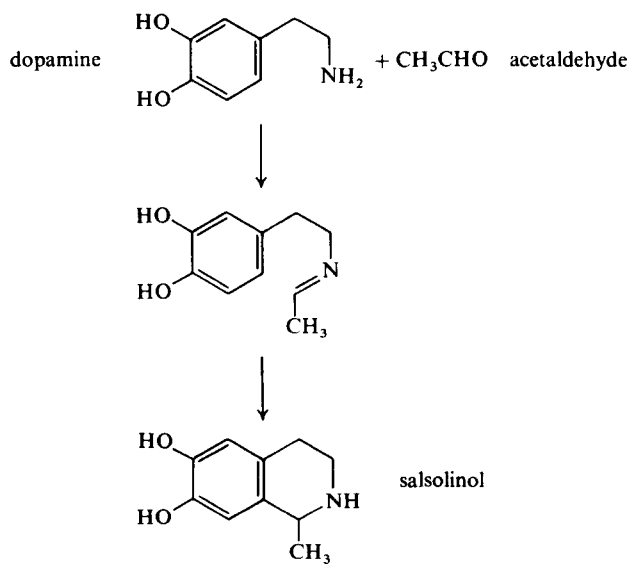


Fig. 3. Formation of salsolinol in bananas

aliphatic monoamines, histamine, phenethylamine (Askar *et al.*, 1972), metanephrine (1), normetanephrine (18), octopamine (4), tryptamine (1) and methoxytryptamine (1) (Kenyhercz & Kissinger, 1978).

The concentrations of tyramine, dopamine, noradrenaline and serotonin in banana pulp are shown in Table 11. The reason for the considerable variation in the estimates obtained by various workers may lie in the changes in the fruit associated with maturation (Vettorazzi, 1974).

TABLE 11
AMINES IN BANANA PULP (μg/g)

Tyramine	*Dopamine*	*Noradrenaline*	*Serotonin*	*Ref.*
7	8	2	28	Udenfriend *et al.* (1959)
—	—	3–10	12–57	Foy & Parratt (1960)
—	—	—	30–78	Vettorazzi (1974)
—	22	1·4	—	Riggin *et al.* (1976)
—	643	103	75	Stachelberger *et al.* (1977)
95	—	—	26	Kenyhercz & Kissinger (1978)

B. *Citrus*

Several phenolic amines including tyramine, octopamine and synephrine occur in citrus fruits (Stewart & Wheaton, 1969; Wheaton & Stewart, 1970) though these are absent from grapefruit (Wheaton & Stewart, 1970). Synephrine is used pharmacologically as a stimulant, decongestant and in the treatment of hypotension in oral form, with a minimum dose of 100 mg of D,L-synephrine tartrate. This amount, equivalent to about 17 mg of L-synephrine, would occur in 150 ml of tangerine juice in which the concentration is about 100 μg/ml (Stewart & Wheaton, 1964). Tyrosine is the precursor of the phenolic amines in citrus (Wheaton & Stewart, 1969) (Fig. 4).

Feruloylputrescine (*N*-(4-aminobutyl)-4-hydroxy-3-methoxycinnamamide) was found in grapefruit and oranges but it was not detected in tangerines or lemons. In grapefruit the content ranged from 22 to 34 μg/ml of juice (Wheaton & Stewart, 1965*a*,*b*). Feruloylputrescine and related compounds have hypertensive activity (Panaschchenko & Ryabinin, 1961).

C. *Apples*

Apple fruits, cultivars Cox's Orange Pippin, Ontario and Jonathan, contain isoamylamine and hexylamine. These amines were present in both the pre- and post-climacteric phases. Ethylamine was also found during the climacteric respiratory increase when the tissues become brown. Fruit browned by freezing did not contain ethylamine, although isoamylamine and hexylamine were still evident (Hilkenbaümer *et al.*, 1960). The presence of ethylamine and hexylamine in Cox's Orange Pippin and Golden Delicious was also demonstrated by Hartmann (1967*a*,*b*) but only traces of isoamylamine could be found. *n*-Butylamine was found

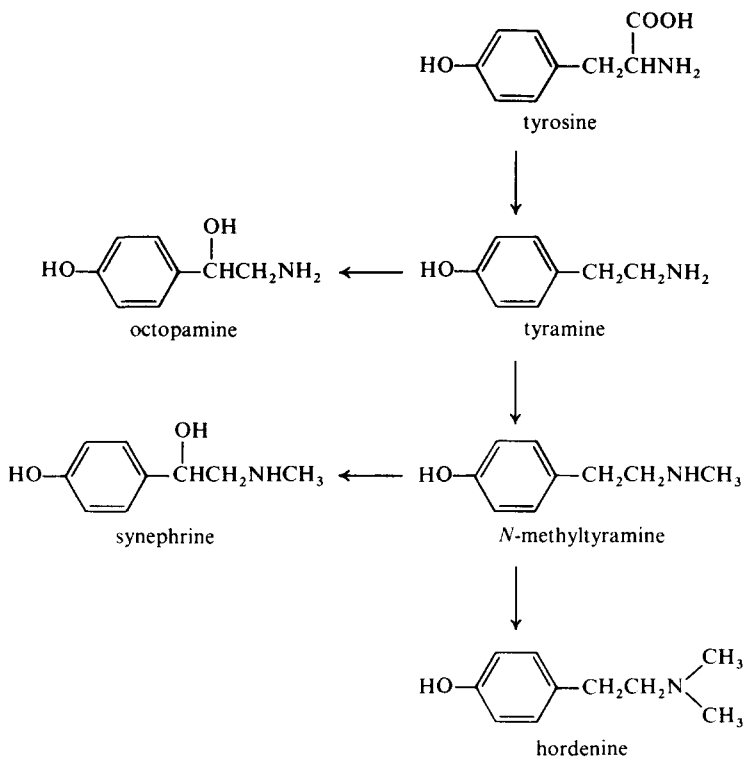
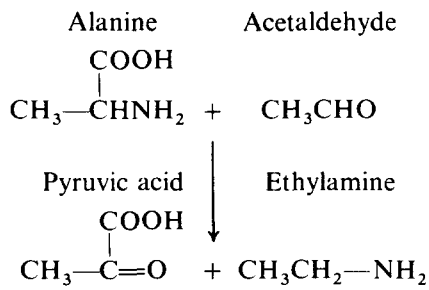


Fig. 4. Formation of hordenine in the roots of barley seedlings and of phenolic amines in *Citrus*.

in five varieties but isobutylamine could not be detected. Hartmann (1967*b*) showed that these amines may be formed *in vivo* by amination of the corresponding aldehydes. Acetaldehyde, propionaldehyde, n-butyraldehyde, isobutyraldehyde, n-valeraldehyde, isovaleraldehyde and n-hexanal gave the corresponding amines, and amine formation by the decarboxylation of the corresponding amino acids could not be demonstrated. Acetaldehyde, n-butyraldehyde and n-hexanal have already been demonstrated in apple fruits (Meigh, 1956; Drawert *et al.*, 1965). Highest



amine concentrations were found under conditions which favoured aldehyde formation in the apples. Younger apples were more active than older apples in aminating the aldehydes, but it was not possible to detect transaminase activity in tissue extracts (Hartmann, 1967*b*). Many of the simple amines are now known to be formed in other higher plants by aldehyde amination, with L-alanine as the most efficient donor (Hartmann *et al.*, 1972).

D. *Barley seedlings, hops and beer*

In barley seedlings, the methylated amines hordenine and gramine occur in the roots and shoots respectively. Hordenine may be found in malt and malt extract (McFarlane, 1966). The hordenine concentration of beer is in excess of 2 ppm (Drews *et al.*, 1957). The biosynthesis of hordenine from tyrosine in barley

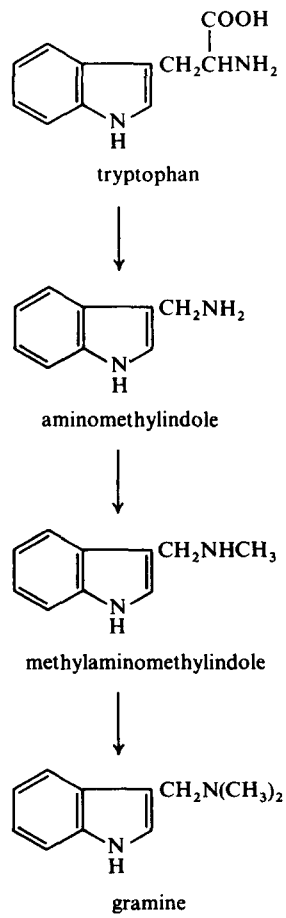


Fig. 5. Formation of gramine in barley seedlings.

(Rabitzch, 1959; Schultz *et al.*, 1975) is shown in Fig. 4. Hordenine is a diuretic, and its effect as a remedy for diarrhoea and dysentery (Ghosal & Banerjee, 1969) may be ascribed to its antibacterial properties (McCleary *et al.*, 1960; Subba Rao, 1970). Hordenine is not found in dormant seeds of barley (Mann *et al.*, 1963) but it does occur in dormant millet seeds (Sato *et al.*, 1970). Tyramine occurs at up to 11 ppm in beer (Sen, 1969) (see Table 5). Gramine is formed in barley seedling shoots from tryptophan with the loss of a methylene group in the side chain which is effected by an unknown mechanism (Von Gross *et al.*, 1967, 1974; Schneider & Wightman, 1974) (Fig. 5). Unlike other indoleamines, gramine shows little pharmacological effect in higher animals (Ho *et al.*, 1970).

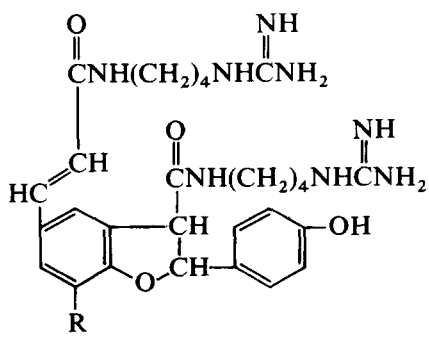
Amongst other amines in beer, dimethylamine, which is important as a potential precursor of the carcinogen dimethylnitrosamine, occurs at a concentration of up to 780 μg/litre (Drews *et al.*, 1957; Koike *et al.*, 1972). The dimethylamine is probably derived from gramine and hordenine during the kilning process of malt production.

Other amines found in beer by Drews *et al.* (1957) include methylamine (5 μg/litre), ethylamine (10 μg/litre), butylamine (< 5 μg/litre) and amylamine (10 μg/litre). Choline ($HOCH_2CH_2N(CH_3)_3OH$) occurred at up to 250 mg/litre (Drews *et al.*, 1957). In another study, ethylamine (to 300 μg/litre), isobutylamine (to 100 μg/litre), isoamylamine, di-isobutylamine and tripropylamine, and trimethylamine (to 60 μg/litre) were detected (Koike *et al.*, 1972). Addition to beer of about six times the normal amounts of ethylamine, isobutylamine, isoamylamine, dimethylamine and trimethylamine produced no significant effect on flavour.

Ethylamine and dimethylamine appeared to be the dominant volatile amines of beer. All of the volatile amines found in beer could be detected in the roots of barley seedlings (Slaughter & Uvgard, 1971). The pyrrolidine found in beer may arise by decarboxylation of proline (Slaughter, 1970). *p*-Hydroxybenzylamine was found in water extracts of barley seed and malt (Slaughter & Uvgard, 1972).

In barley seedlings agmatine is found as a conjugate with coumaric acid. Dimers of coumarylagmatine, known as the hordatines, also occur, and these are powerful

Hordatines { A: R = —H; B: R = —OCH_3



inhibitors of fungal spore germination, being fully inhibitory at 5 ppm (Stoessl & Unwin, 1970; Smith & Best, 1978). It is not known if these compounds are present in malt, but if they are, they may affect the growth of the yeast in the fermentation process.

Histamine is present in fresh hops (30–40 μg/g) and hop extract (120–160 μg/g). Unhopped wort contains no histamine but histamine is found in hopped wort (Vogel *et al.*, 1962). The histamine in beer (up to 300 μg/litre) (Chen & Gheluwe, 1979) is probably derived from the hops (Vogel *et al.*, 1962). The hop plant (*Humulus lupulus*) is a close relative of the nettle (*Urtica* spp.) which is notorious for the histamine content of its stinging hairs (Di Vialli *et al.*, 1973).

E. *Seeds*

The main constituents of the wax of green coffee beans were arachidoyl-(C-20), behenoyl-(C-22) and lignoceroyl-(C-24) serotonin in the ratio of 12:12:1 (Folstar *et al.*, 1979). The beverage quality of coffee beans could not be correlated with changes in putrescine, spermidine or spermine (Amorim *et al.*, 1977) and it seems unlikely that polyamines contribute to the odour of soybeans (Wang *et al.*, 1975).

Polyamines occur in high concentrations in the embryos of wheat seeds, and they increase on storage from 600 μg/g (6 months old) to 1100 μg/g (4 years old). No difference was found in the polyamine content of viable and non-viable wheat embryos (Anguillesi *et al.*, 1974). The content of spermidine and spermine of wheat seed increased rapidly after fertilisation. In the embryo of the mature seed the spermidine content reached 1 mg/g (Moruzzi & Caldarera, 1964; Bagni *et al.*, 1967). Putrescine, cadaverine, spermidine and spermine also occurred in the embryos of barley, rice, oats, corn and sorghum, the greatest total amount of the di- and polyamines being found in barley (Moruzzi & Caldarera, 1964).

Wheat bran contains a conjugate termed niacytin from which nicotinic acid, phenolic esters and sugars have been isolated, together with an amine tentatively identified as 2-aminophenol in which the amino group was unsubstituted (Kodicek & Wilson, 1960; Mason & Kodicek, 1970).

Bagni (1968) estimated spermidine and spermine in the seeds of a large number of higher plants. In the genus *Vicia* (six species) the spermine content was consistently less than that of spermidine, while for *Phaseolus* (eight species) this ratio was reversed. *Phaseolus vulgaris* (runner bean) seeds contained 310 μg/g of spermine. Serotonin which occurs in high concentrations (550 μg/g) in walnut embryos (Bergmann *et al.*, 1970) appears to be utilised as a storage substance (Grosse, 1977). Other seeds known to contain amines are listed in Tables 9 and 10.

F. *Chocolate and migraine*

In some patients, headache appears to be induced by certain foods, especially by chocolate and less frequently by cheese and other milk products, alcohol and fish. Investigations into the factors in these foods inducing these attacks have produced

conflicting results. However, it was observed that some of the foods causing headache were amongst those causing hypertension in patients taking MAO inhibitors. Hanington (1967) showed that 100 mg of tyramine induced migraine in a group of people with a dietary history of migraine in whom a control dose of lactose had no effect. However, although chocolate, which is a very common trigger of dietary migraine, does not appear to contain tyramine, it was eventually shown to contain phenethylamine. Sandler *et al.* (1974) therefore fed 3 mg of phenethylamine to a group of migraine patients. Out of 30 who suffered no effect with lactose, 18 developed headache with phenethylamine. On amine ingestion, headache developed about 12 h later. Similarly, migraine occurs about 12 h after ingestion of chocolate. Activity of MAO type B, capable of oxidising phenethylamine, was reduced in migraine sufferers, though paradoxically both dietary and non-dietary migraine patients showed the same reduction. Medina & Diamond (1978) suggest that chocolate and wine do not cause headaches, but increase susceptibility to them.

Phenethylamine in chocolate is derived from the roasted cocoa bean and its concentration in the final product varies considerably. The concentration in raw beans, low-, medium- and high-roast beans from Ghana is <2, 9, 10 and 12 μg/g respectively (Saxby, 1979), which is consistent with the formation of this amine by decarboxylation of phenylalanine during roasting. Milk chocolate and unsweetened chocolate have been found to contain 0·7 and 14 μg/g respectively (Schweitzer *et al.*, 1975). Phenethylamine was also detected in plain chocolate at up to 27 μg/g by Chaytor *et al.* (1975). However, it could not be found in a variety of other chocolate products including unsweetened, sweetened, dairy milk, dark or white chocolate or drinking chocolate by Koehler & Eitenmiller (1978) or Ingles *et al.* (1978).

The high concentrations of phenethylamine in cheese (Table 3) and sausage (Table 6), foods which are less effective than chocolate for the induction of migraine, cast some doubt on the phenethylamine hypothesis. Even so chocolate has been implicated in a single but well documented (Krikler & Lewis, 1965) hypertensive attack in a patient taking MAO inhibitors (Pargyline). In this case the attack occurred 1 h after eating 60 g of milk chocolate. It was suggested that amines may arise during the fermentation process which lasts for up to 12 days prior to drying and shipment.

G. *Tea*

γ-Glutamyl-methylamide (GMA) and -ethylamide (theanine) found in tea are formed by condensation of methylamine or ethylamine with glutamic acid by analogy with the formation of glutamine. These amides are formed in the roots and exported to the leaves (Suzuki, 1973). The ethylamine is derived from L-alanine by decarboxylation (Takeo, 1974) (Fig. 6). Tea is probably the main source of the ethylamine found in human urine (Asatoor, 1966). Theanine accounts for more than 50% of the total amino acid content and 1% of the dry weight of the leaves (Wickremasinghe, 1978).

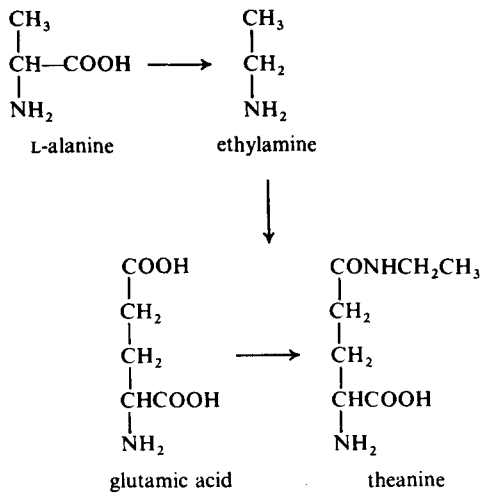


Fig. 6. Formation of theanine in tea.

N-Methylglutamine occurs at lower concentrations than theanine (Konishi & Takahashi, 1966). Methylamine and ethylamine have been demonstrated in tea leaves (Serenkov, 1962) and other amines found in tea leaves are reviewed by Maga (1978). The formation of the amides may be due to the non-specificity of glutamine synthetase, since theanine is also formed by acetone powders of pea seed and pigeon liver. The availability of ethylamine rather than the occurrence of a specific enzyme may therefore determine theanine formation in tea (Sasaoka *et al.*, 1964; Konishi *et al.*, 1969).

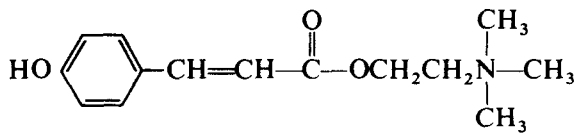
Conjugates similar to theanine have been found in edible mushrooms. A phenylhydrazine/glutamic acid conjugate (β-*N*-[γ-L(+)glutamyl] 4-hydroxymethylphenylhydrazine) may attain 0·3% of the dry weight of certain *Agaricus* species (Levenberg, 1961, 1964). N^5-(4-Hydroxyphenyl)-L-glutamine has also been isolated from *Agaricus hortensis* (Jadot *et al.*, 1960).

Excretion of 3,4-dimethoxyphenethylamine on ingestion of tea (Stabenau *et al.*, 1970) could not be confirmed in subsequent work (Narasimhachari *et al.*, 1972).

H. *Rapeseed meal and egg taint*

In higher animals, trimethylamine is normally oxidised in the liver to trimethylamine-*N*-oxide prior to excretion. However, trimethylamine may accumulate in certain circumstances. Chickens which have been fed with rape seed meal may lay eggs which are tainted with trimethylamine. Free trimethylamine is not found in the rape seed meal but is present as a precursor known as sinapine (the choline ester of sinapic acid).

It also appears that a component of this meal prevents oxidation of exogenously formed trimethylamine, which then accumulates in the yolk of the egg (up to 8 μg per



Sinapine

egg). Only certain breeds of hen laying brown eggs are affected and the trait may be genetically inherited. The best approach to control may be to produce varieties of rape which have low concentrations of sinapine (Hobson-Frohock *et al.*, 1975).

I. *Favism*

Favism is a haemolytic disease which affects certain individuals after eating raw or cooked broad beans. Symptoms include fatigue, fever and renal failure in severe cases. It is particularly frequent in the inhabitants of the countries surrounding the Mediterranean Sea. For instance, in Rhodes there are about 40 cases per 1000 population. It is associated with a deficiency of glucose-6-phosphate dehydrogenase activity and of reduced glutathione, determined by a genetic factor which is inherited on the X-chromosome. Favism is more common in children under 10 years of age,

Divicine (reduced)

and it occurs mainly in the male. On present evidence the substances responsible for favism are the pyrimidines divicine and isouramil, which are found in the beans as the glycosides vicin and convicin respectively. It appears that these pyrimidines react with reduced glutathione. Research is limited by the fact that favism cannot be induced in animals (Mager *et al.*, 1980).

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Food Chemistry 6 (1980–81) 201–211

NON-PROTEIN AMINO ACIDS OF PLANTS

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ABSTRACT

Protein forms the major nitrogenous constituent of plants, but other essential nitrogen-containing materials present in all plants include the nucleic acids, chlorophyll and indolylacetic acid. Some plant species produce secondary nitrogenous compounds such as alkaloids, cyanogenetic glucosides and non-protein amino acids. This paper discusses the group of more than 200 non-protein amino acids now characterised as plant products. Emphasis is placed on the variety of chemical structures encountered, upon the occurrence and biosynthesis of representative examples, and upon the toxic nature of certain compounds in relation to their behaviour as metabolic analogues or antagonists.

1. INTRODUCTION

Plants take up nitrogen, mainly in the form of nitrate, through their root systems and, after reduction to ammonia, introduce this nitrogen into organic compounds. Most of the nitrogen is found essentially in protein molecules containing 20 different amino acids linked by peptide bonds. Very roughly four-fifths of a plant's total nitrogen is present in protein distributed between its various organs. There are, however, wide variations in this ratio when the compositions of different plant species are compared, and also between the protein levels in the different organs of a single species. Roots and fleshy storage organs (tubers, rhizomes and bulbs) generally contain smaller proportions of their total nitrogen in the form of protein than do leaves.

Although protein is quantitatively the most important nitrogenous constituent of plants and, in the form of enzymes, forms a vital component of the metabolic apparatus of all cells, other types of nitrogen compounds recognised as essential to the activity of plants are derived from the amino acids that represent the early

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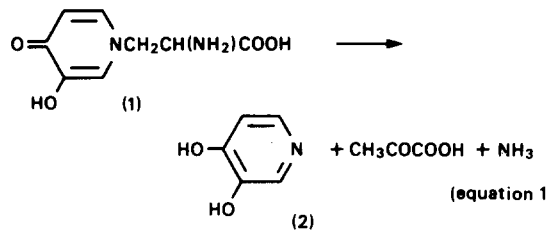
products of assimilation of inorganic nitrogen. These additional products include the pyrimidine and purine constituents of the nucleic acids, the chlorophyll pigments and auxin (indolylacetic acid). Like protein, these products are synthesised by all plant species. There are, in contrast, a range of nitrogenous compounds whose distribution is not universal within members of the plant kingdom, and whose *raison d'être* is not fully clear. These compounds, which include the alkaloids, the cyanogenetic glucosides and the non-protein amino acids, form part of the complex of secondary products of the plant kingdom. It is impossible to generalise about the distribution of these compounds, or about the concentrations in which they occur. Whilst many of the secondary products appear to be restricted to a limited range of plant species, often a group exhibiting close botanical relationships, a few occur in almost all species examined: concentrations may vary between species from amounts barely detectable by the most sensitive analytical procedures to accumulations representing several percent of the plant's dry matter. The alkaloids form the most numerous group of these secondary products, but the non-protein amino acids now include about 250 compounds derived from the plant world; other non-protein amino acids are produced by micro-organisms, frequently as components of antibiotics. This account considers some general principles relating to the existence of non-protein amino acids in higher plants; it does not attempt to provide a comprehensive description of a still expanding group of compounds.

2. AMINO ACIDS AND NUTRITION

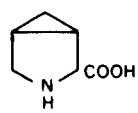
Animals and man depend upon an adequate supply of protein in their diet—or, more strictly, upon the ingestion of minimum daily quantities of certain of the amino acid constituents of protein (the essential amino acids). Ingested protein is enzymically hydrolysed to the constituent amino acids as an initial metabolic step in animals and man. Although considerable exchange of nitrogen between different carbon skeletons can occur, and some amino acids can be synthesised *de novo*, this facility does not extend to the essential amino acids because the biosynthetic enzymes required to elaborate their carbon skeletons are lacking in man. Therefore, in order to synthesise his own bodily protein, man depends upon the acquisition from food of preformed molecules of eight such essential amino acids. The fact that nitrogen, as amino groups, can be exchanged between carbon skeletons is consistent with a pool of nitrogen that can be mobilised, at least in limited directions, for biosynthesis in animals and man. The extent to which ingested secondary products can contribute to this pool is uncertain, because few critical experiments have been performed. However, many non-protein amino acids undergo transamination reactions, and some form substrates for amino acid oxidases; therefore enzymic mechanisms exist for utilising the nitrogen present in some compounds for other purposes. Certain non-protein amino acids undergo more specific metabolic



changes, e.g. mimosine (1) is split into dihydroxypyridine (2), pyruvate and ammonia in the rumen of cattle (eqn. 1) (Hegarty *et al.*, 1976), but information of this type is very limited. Although metabolism of some compounds then can contribute to pools of metabolites, and thereby possibly provide a 'sparing action', there is no evidence for any non-protein amino acid being an indispensable dietary requirement of man or animals.

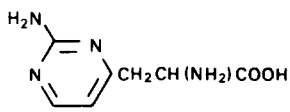
3. OCCURRENCE AND DISTRIBUTION

Like the secondary products, individual non-protein amino acids normally do not occur universally in the members of the plant kingdom. The pattern of distribution varies widely for different compounds. Some are known only as constituents of the species from which they were isolated and characterised; *cis*-3,4-methanoproline (3) from *Aesculus parviflora* is such a compound (Fowden *et al.*, 1969). In sharp



(3)

contrast, γ-aminobutyric acid has been detected in extracts of almost all plants examined. The distribution pattern of most other compounds falls between these extremes. Many compounds appear confined to a small number of plant species, usually a group within a sub-genus or genus, or a section of a family. There are many examples of studies of the distribution of non-protein amino acids at this level: examples include studies of lathyrine (4) and other compounds within the genus *Lathyrus* (Bell, 1962), of β-pyrazol-1-ylalanine (5) in sections of the family Cucurbitaceae (Dunnill & Fowden, 1965) and of canavanine (6) within the Papilionoideae (Turner & Harborne, 1967).



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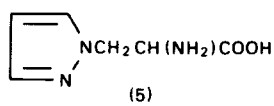

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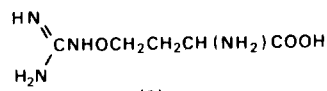
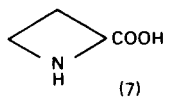


(6)

Other compounds occur in more than a single family. A group of C_6 and C_7 amino acids containing a cyclopropane residue are present in some, but apparently not all, species of the botanically related families Sapindaceae, Hippocastanaceae and Aceraceae (Fowden *et al.*, 1970). Azetidine-2-carboxylic acid (7) was considered initially as an imino acid unique to species within the Liliaceae and Amaryllidaceae (Fowden & Steward, 1957), but was characterised later as a constituent of species in quite unrelated groups of plants, e.g. in some legumes (Sung & Fowden, 1969), in a red marine alga (Impellizzera *et al.*, 1977) and, in trace amounts, in sugar beet (Chenopodiaceae) (Fowden, 1972). The distribution of γ-methyleneglutamic acid (8), γ-methyleneglutamine (9), γ-methylglutamic acid (10), and γ-hydroxy-γ-methylglutamic acid (11) also appears sporadic across the plant families; initially isolated from peanut (a dicotyledonous legume) (Done & Fowden, 1952), γ-methyleneglutamic acid and γ-methyleneglutamine were described subsequently in tulip (a monocotyledon, Liliaceae) (Zacharius *et al.*, 1954) and in a number of fern species.



(7)

$HOOCC(=CH_2)CH_2CH(NH_2)COOH$ (8)

$H_2NOCC(=CH_2)CH_2CH(NH_2)COOH$ (9)

$HOOCCH(CH_3)CH_2CH(NH_2)COOH$ (10)

$HOOCC(OH)(CH_3)CH_2CH(NH_2)COOH$ (11)

It is often possible to trace the biogenetic evolvement of a group of structurally related compounds by examining their distribution within the members of a genus. A compound representing the end-product of a biosynthetic pathway almost always exhibits a more restricted distribution than a compound forming an earlier intermediate in the pathway. This situation follows from evolutionary processes within a related group of plants leading to the addition, or deletion, of genes specifying particular enzymic proteins: biosynthetic pathways terminate at different points dependent upon the complement of the necessary enzymes within particular species. A nice example of this situation is encountered within the genus *Lathyrus*, and therefore an extended discussion of the phenomenon is included in this symposium (see Bell, 1980–81).

The concentrations of individual compounds in plants vary between wide limits. A few compounds accumulate to high levels when expressed on a dry weight basis, and concentrations as high as 10% have been recorded, e.g. for 3,4-dihydroxyphenylalanine in seeds of *Mucuna* species (Bell & Janzen, 1971). Other compounds occurring at several percent of the dry matter include canavanine (in certain legume seeds) (Turner & Harborne, 1967), mimosine in seeds of *Leucaena* species, especially *L. leucocephala* (Brewbaker & Hylin, 1965), and azetidine-2-carboxylic acid in the rhizome of *Polygonatum multiflorum* (Solomon's seal) (Fowden, 1959). The presence of a non-protein amino acid usually is detected initially by some form of chromatographic survey of plant extracts—two-dimensional paper chromatography, thin-layer chromatography or by use of an

amino acid autoanalyser. All such methods necessarily have a concentration limit below which a particular compound is unlikely to be detected, and strictly one can refer only to a substance as being undetected, not absent. During fractionation of amino acids present in extracts of large bulks of plant material, the presence of compounds not recognised by initial chromatographic screens may become evident, azetidine-2-carboxylic acid was recognised in this way as a constituent of sugar beet following industrial processing of beet extracts (Fowden, 1972).

4. STRUCTURES AND BIOSYNTHESIS

Most of the non-protein amino acids from plants contain an α-amino group, and therefore are represented by the general structure $RCH(NH_2)COOH$ common to the protein amino acids. Based on the structural characteristics of R, the compounds can be classified as alkyl, hydroxy, acidic, basic, sulphur-containing, aryl or heterocyclic amino acids. R then typically shows the same variety of structural features present in the 20 protein amino acids, but additional structures are recognised in the non-protein amino acids; these include ethylenic and acetylenic linkages, cyclopropyl residues, many types of heterocyclic ring systems (furan, pyran, pyrazole, pyrimidine, pyridine, isoxazoline, etc.), and imino acids based on four-atom (azetidine) and six-atom (piperidine) heterocycles. Several non-protein amino acids are simple homologues (higher or lower) of protein amino acids. Others contain a single small group substituted into a protein amino acid, e.g. the γ-substituted glutamic acids mentioned previously. Sometimes a grouping within a protein amino acid is replaced in a non-protein derivative by a similar sized atom or group of atoms so giving a compound isosteric with the protein constituent. Canavanine is an isostere of arginine, having an O atom in place of the CH_2 group adjacent to the guanidino group. Other examples of isosteric pairs include glutamine (12) and albizziine (13), where the C-4 methylene group of glutamine is replaced by NH in albizziine; and lysine (14) and S-aminoethylcysteine (15), where

$H_2NOCCH_2CH_2CH(NH_2)COOH$ (12)

$H_2NOCNHCH_2CH(NH_2)COOH$ (13)

$H_2NCH_2CH_2CH_2CH_2CH(NH_2)COOH$ (14)

$H_2NCH_2CH_2SCH_2CH(NH_2)COOH$ (15)

C-4 methylene in the centre of the carbon skeleton of lysine is replaced by a S atom in the isostere. The similarity of molecular features encountered between homologous amino acids, and between the parent amino acid and products derived by simple substitution is often associated with related metabolic activity, the derived amino acid acting as an analogue (or antagonist or antimetabolite) of the structurally related protein amino acid.

Biosynthesis of an amino acid normally is approached in two ways: we wish to know the origin of the carbon skeleton unique to the particular compound, and also at what stage the amino group is introduced during the elaboration of the final molecule. For several of the protein amino acids, amination is the last step. Inorganic nitrogen, as ammonia, is assimilated into the amino group of glutamic acid; then the α-NH_2 is transferred by a series of transamination reactions to receptor α-oxo acids. Several non-protein amino acids participate in transamination reactions with considerable facility, and it is reasonable to assume that they may derive their α-amino groups by transamination from glutamic acid (or perhaps alanine or aspartic acid) as the last step in biogenesis.

Although certain non-protein amino acids undergo transamination readily, much evidence suggests that as a group the compounds generally are characterised by a metabolism sluggish in comparison with that of the protein amino acids. The experimental study of biosynthetic pathways is then difficult: few examples exist of non-protein amino acids whose synthesis in plants has been documented at the enzymic level and, where information on biosynthetic pathways exists, it has been obtained mainly by radioisotopic labelling techniques. In many of the latter studies, the experimental design has anticipated that similarities will exist between the pathways leading to particular groups of non-protein amino acids and to structurally similar protein constituents.

The dicarboxylic and diamino acids form a metabolically interrelated group with the imino acids; ureido, guanidino, and acetyl derivatives of the diamino acids may also be related in a common scheme (Fig. 1). The relationships established for the protein amino acids (glutamic acid (16), proline (18), and arginine (20), with ornithine (19), as an intermediate: $n = 2$ for all compounds) are shown in outline.

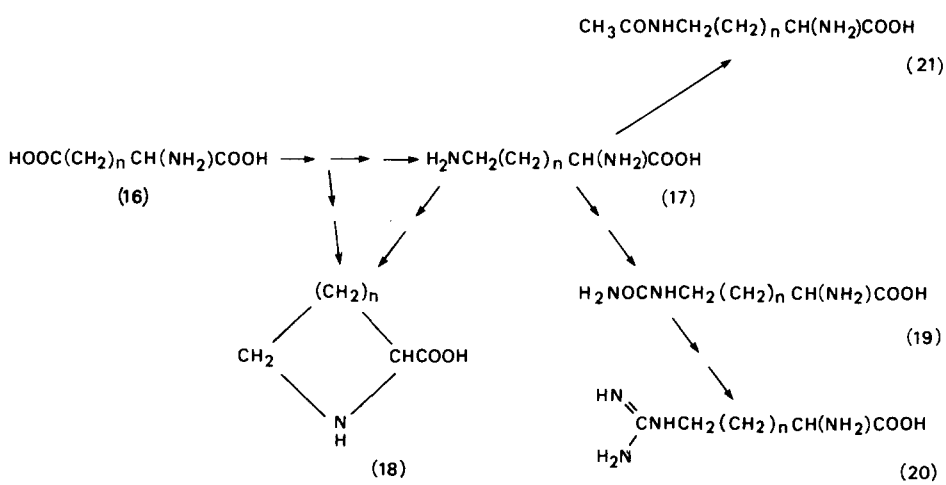
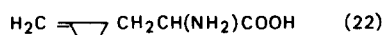


Fig. 1.

Although studies have been undertaken, it is not established that diaminobutyric acid (17, $n = 1$) and azetidine-2-carboxylic acid (18, $n = 1$) can be derived from aspartic acid (16, $n = 1$): isotope labelling experiments indicate that methionine is the primary precursor of azetidine-2-carboxylic acid (Leete, 1964). α-Aminoadipic acid (16, $n = 3$) is present at low concentration in some higher plants; although lysine is formed from this acid in fungi, higher plants utilise an alternative pathway for lysine biosynthesis not based on α-aminoadipate. Lysine is converted rapidly to the related imino acid (pipecolic acid, 18, $n = 3$) in certain test plants (Fowden, 1960), and is also a precursor of homoarginine in some species of *Vicia* (Bell & Tirimanna, 1965). α,β-Diaminopropionic acid (17, $n = 0$) is a constituent of some legume seeds, and is often associated with the presence of albizziine (19, $n = 0$, the ureido derivative) and the β-*N*-acetyl derivative (20, $n = 0$). The β-*N*-oxalyl derivative, which behaves as a neurolathyrogen, is discussed by Bell (1980–81). There is no evidence for the existence in plants of α-aminomalonic acid, aziridine-2-carboxylic acid or β-guanidino-α-aminopropionic acid (all $n = 0$).

The key steps involved in the formation of another group of non-protein amino acids may be based on the type of chain-lengthening process encountered in the production of leucine from valine. The genera *Blighia* (Sapindaceae) and *Aesculus* (Hippocastanaceae) elaborate a group of C_7 amino acids showing branching of the carbon skeleton at C-4 (Fowden *et al.*, 1970): the group contains compounds possessing cyclopropyl residues (β-(methylene cyclopropyl)alanine, 22, hypoglycin A), homoisoleucine and its unsaturated form, 2-amino-4-methylhex-4-enoic acid (23). ^{14}C-Labelling studies with developing fruits of *A. californica* have established



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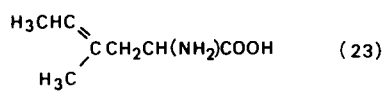

(23)

that isoleucine is a precursor of the C_7 structure, and that the intermediate steps involve the loss of the C-1 atom of isoleucine in a manner similar to the elimination of the C-1 of valine during its conversion to isoleucine (Fowden & Mazelis, 1971). Similar labelled precursor techniques have indicated that γ-methylglutamic acid is formed from leucine in *Gleditsia triacanthos* (Peterson & Fowden, 1972). The key transformation then involves the oxidation of one of the chain terminal methyl groups of leucine to carboxyl; although the *Gleditsia* seedlings also contained γ-methyleneglutamic acid, this unsaturated amino acid did not acquire any detectable ^{14}C-label from leucine.

A group of aromatic amino acids based on phenylalanine and tyrosine, but possessing a *m*-carboxyl constituent occur in some members of the families Resedaceae, Iridaceae and Cucurbitaceae. The biosynthetic pathway producing these modified amino acids consists of a series of steps closely analogous to those

involved in the formation of phenylalanine and tyrosine. The pathway commences from shikimate, but an alternative rearrangement mechanism leads to the retention of the shikimate carboxyl-C (as the *m*-carboxyl substituent on the phenyl ring) rather than its elimination as carbon dioxide as occurs in the normal route to phenylalanine and tyrosine (Larsen *et al.*, 1972).

It is evident that, for a group numbering more than 200 compounds, we have little precise information concerning biosynthetic pathways, and even less about enzymic mechanisms involved. There are then many challenging problems, but some may prove intractable by reason of the limited natural distribution of species and the apparent low intrinsic rates of synthesis of the compounds.

5. ANALOGUE ROLES

Some non-protein amino acids behave as analogues of structurally related protein constituents: in some cases this is reflected as an antimetabolic acitivity interfering with the normal mechanisms of amino acid biosynthesis or catabolism, or with amino acid uptake across cell membranes or incorporation into cellular protein. Any one of these antagonistic mechanisms might be classed broadly as antinutritional, and there are several well-known examples of non-protein amino acids causing toxic symptoms in either man or animals. The lathyrogenic amino acids present in seeds of some *Lathyrus* and *Vicia* species is considered in detail by Bell (1980–81). Other examples include mimosine, a principal amino acid component of forage (seed and foliar material) of *Leucaena leucocephala*, which causes loss of hair in horses and cattle, and of the fleece of sheep eating large quantities of plant material (Fowden *et al.*, 1967); the selenium-containing amino compounds, especially *Se*-methylselenocysteine and its γ-glutamyl peptide, accumulated in high concentration by certain vetches (including *Astragalus bisulcatus*) and responsible for losses of cattle and sheep grazing pastures growing on selenium-rich soils (Rosenfeld & Beath, 1964); and hypoglycins A and B, which are present in the arils (the edible portion of the fruit) of *Blighia sapida* when unripe and which produce headache, convulsion or even death (Jamaica vomiting sickness) in people, especially children, who unwarily eat them at the unripe stage (Fowden *et al.*, 1967). The mechanisms surrounding such toxicities are often not well understood at a biochemical level. There is some evidence suggesting that the seleno amino acids may act by antagonising aspects of the metabolism of cysteine and methionine; similarly, mimosine may interfere with the utilisation of tyrosine by animals. But such direct antagonisms are unlikely by themselves to account for the full range of toxic symptoms encountered. Again, whilst studies with plant enzymes indicate that hypoglycin A may act as an anomalous substrate for leucyl-tRNA synthetase, and thereby possibly limit the activation of leucine, a more important antagonistic mechanism seemingly involves oxidative deamination and decarboxylation of

hypoglycin A to give α-(methylenecyclopropyl)acetic acid, which behaves as an inhibitor of the fatty acid β-oxidation process (Fowden *et al.*, 1979).

The examples just described probably represent the most striking toxicities attributed to non-protein amino acids. There are, however, a considerable number of other compounds that display amino acid analogue behaviour, and which with certainty act directly upon basic processes concerned with amino acid metabolism or protein synthesis. For instance, glutamine donates its amide-NH_2 group to receptor compounds of various types in reactions catalysed by amido-transferase enzymes. Albizziine, a glutamine isostere, acts as a strong inhibitor of many of these enzymic reactions. In addition to acting as a competitor of an enzyme's substrate, as in this example, an analogue may mimic the behaviour of an end-product of a reaction by acting as a feed-back inhibitor of early key enzymes in the biosynthetic pathway or by repressing the formation of such enzymes: it is now recognised that only the first of these alternatives operates as a regulatory mechanism in plant systems (Miflin & Lea, 1977). In illustration, we may note that lysine exerts a strong influence over the rate of its own biosynthesis by inhibiting the first and third enzymes concerned in a seven-step synthetic pathway, and that *S*-aminoethylcysteine can substitute for lysine in this regulatory role (Mazelis *et al.*, 1977; Bright *et al.*, 1978). Analogue behaviour at the level of amino acid uptake by cells, i.e. at the permease enzyme level, has been demonstrated most clearly with bacterial systems. Uptake of proline by *Escherichia coli* cells is reduced markedly in the presence of imino acid analogues, such as azetidine-2-carboxylic acid or 3,4-dehydroproline: the kinetics of uptake are those expected presuming mutual competition between the normal substrate and the analogue for sites on the proline permease (Rowland & Tristram, 1975).

There is a wealth of clear evidence indicating that amino acid analogues can act as alternative substrates for specific aminoacyl-tRNA synthetases and, in some instances, can be incorporated subsequently in newly synthesised protein molecules. Azetidine-2-carboxylic acid (A2C) again provides a good example of this behaviour. At enzyme saturating concentrations, the analogue can be activated by prolyl-tRNA synthetase at rates equivalent to almost half that measured for proline (Norris & Fowden, 1972), and when present at concentrations considerably in excess of proline, azetidine-2-carboxylate residues are introduced into protein molecules at many of the positions normally occupied by proline. Protein molecules formed under these conditions will be abnormal, show diminished biological (enzymic) activity, and thereby be responsible for the ensuing growth inhibitions or other adverse effects. In a similar manner, canavanine can act as an alternative substrate for arginyl-tRNA synthetase (Fowden & Frankton, 1968), 2-amino-4-methylhex-4-enoic acid for phenylalanyl-tRNA synthetase (Fowden *et al.*, 1970) and certain of the γ-substituted glutamic acids for glutamyl-tRNA synthetase (Lea & Fowden, 1972). The ability of an analogue to act as a substrate for the corresponding aminoacyl-tRNA synthetase normally does not extend to its action with the enzyme present in species producing the analogue. Logically, this is not unexpected since such specificity of enzyme

action provides a mechanism whereby the producer species protects itself against an otherwise lethal constituent. Sharp discrimination at the level of substrate selectivity by prolyl-tRNA synthetase is seen when the enzymes from *Polygonatum multiflorum* (an A2C producer species) and *Phaseolus aureus* (a non-producer) are compared: the enzyme from the former species fails completely to activate A2C, whilst that from the non-producer species activates the analogue at about 40% of the rate determined for proline. Certain other aminoacyl-tRNA synthetases show similar differences in substrate specificity dependent upon the source of the enzyme, and discrimination at the level of amino acid activation may be a common mechanism whereby plants producing analogues resist possible antimetabolic effects.

Modification of the inhibitory action of analogues on amino acid permeases or on biosynthetic enzymes form alternative ways by which cells or organisms can acquire resistance to analogues. For instance, mutant strains of *E. coli* capable of growing normally in the presence of proline analogues possess an altered proline permease showing little or no affinity for the analogue when compared with wild-type strains (Tristram & Neale, 1968). It is also possible to select for plant cell lines, using tissue cultures, in which the normal activity of analogues as end-product inhibitors of biosynthetic enzymes has been lost or much reduced. Under these circumstances, the cells continue to produce the end-product which then accumulates at higher concentrations than in normal cells (Widholm, 1976). Therefore, it may be possible to use analogues to select lines of crop plants producing and storing larger amounts of nutritionally important amino acids, such as methionine and lysine, i.e. in ways advancing the quality of food for both man and animals.

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Food Chemistry 6 (1980–81) 213–222

THE STRUCTURE AND BIOSYNTHESIS OF LATHYROGENS AND RELATED COMPOUNDS

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ABSTRACT

Lathyrus *species induce toxic syndromes, but the clinical term 'lathyrism' has been used more widely than merely for symptoms produced by ingestion of these species. It is strongly emphasised that 'toxic' and 'non-nutritious' are adjectives relevant only to the particular organism ingesting that material. Compounds such as oxalyldiaminopropionic acid,* L-*homoarginine, 4-hydroxyhomoarginine, lathyrine, tetrahydrolathyrine,* L-*2,4-diaminobutyric acid and its oxalyl derivatives, and 3-aminopropionitrile are described, and their toxicity or possible toxicity to animals (including man) discussed.*

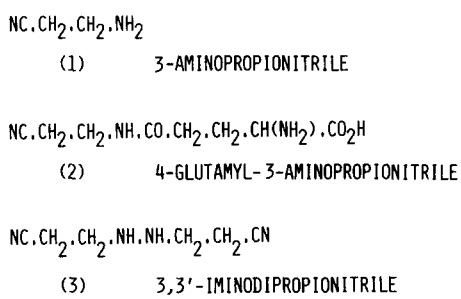
1. INTRODUCTION

Classical lathyrism is a neurological disease which affects men, cattle and horses. In man it is characterised by an irreversible paralysis of the legs and in extreme cases death. At the present time this disease is largely confined to the sub-continent of India and is found amongst peoples whose diets include a high proportion of foods made from the seeds of the legume *Lathyrus sativus* or less frequently from those of *L. cicera* and *L. clymenum*. Particularly severe outbreaks of this disease have occurred when other sources of food have been in short supply.

Strictly speaking a lathyrogen is a compound capable of inducing lathyrism but the term has been extended to other compounds isolated from *Lathyrus* species which induce toxic syndromes other than those of classical lathyrism. One such artificially induced syndrome which is characterised by skeletal changes in experimental animals is caused by 3-aminopropionitrile (1) which occurs as its 4-glutamyl derivative (2) in the seeds of *L. odoratus* (the sweet pea). The term

Food Chemistry 0308-8146/81/0006-0213/$02·50

lathyrogen has also been applied to synthetic compounds producing effects similar to those of the 3-aminopropionitrile, for example 3,3′-iminodipropionitrile (3). To add even further to the confusion, the term lathyrism itself has been applied not only to the naturally occurring neurological disease but also to the artificially induced effects. In an attempt to resolve this ambiguity of nomenclature, the naturally occurring disease is now often referred to as neurolathyrism and the artificially



(1) 3-AMINOPROPIONITRILE

(2) 4-GLUTAMYL-3-AMINOPROPIONITRILE

(3) 3,3′-IMINODIPROPIONITRILE

induced syndrome affecting the skeleton and blood vessel walls as osteolathyrism or experimental lathyrism. This terminology reflects an over-simplification of the situation, however, as the various species of *Lathyrus* synthesise more than two compounds which are physiologically active in other organisms and these include at least four which are toxic to mammals. In the present review I shall try to summarise our present knowledge of these and other 'non-nutritional' nitrogenous compounds of *Lathyrus*. I must emphasise and re-emphasise, however, that the term 'non-nutritious', like the term 'toxic', only has meaning if the organism to which the compound is 'non-nutritious' or 'toxic' is specified.

2. OXALYL DERIVATIVES OF 2,3-DIAMINOPROPIONIC ACID

While seeking the cause of classical neurolathyrism in man, two groups of workers in India simultaneously isolated the neurotoxin 2-amino-3-oxalylaminopropionic acid (oxalyldiaminopropionic acid, ODAP (4)) from seeds of *Lathyrus sativus* (Rao *et al.*, 1964; Murti *et al.*, 1964). The same compound was subsequently shown to be a major component of all three *Lathyrus* species which are implicated as causes of classical human neurolathyrism (Bell, 1964). In each of these, and in all other species in which it has been detected, ODAP is accompanied by lower concentrations of the isomeric 2-oxalylamino-3-aminopropionic acid (5). The two isomers are readily separated and identified by paper high voltage electrophoresis at pH 1·9 (Bell & O'Donovan, 1966).

In aqueous solution the two compounds form an equilibrium mixture and it is probable that non-enzymatic chemical rearrangement is responsible for the presence of the minor component in the plants. The biosynthesis of the major

component has been shown to involve the enzymatic transfer of an oxalyl group to the 3-amino group of 2,3-diaminopropionic acid (6) (Malathi *et al.*, 1970) from oxalyl co-enzyme A (Fig. 1).

While ODAP is implicated as the most probable cause of human lathyrism there is much variability in the sensitivity of different animals to the compound and its site of action still remains uncertain. The greater susceptibility of young and acidotic

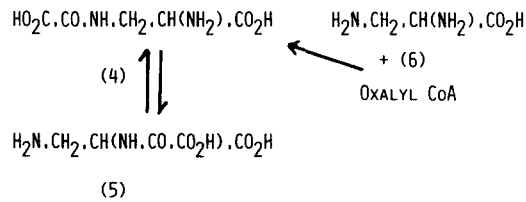


Fig. 1.

animals to the toxin were thought at one time to be due to the greater permeability of the blood/brain barrier of these animals. Recent research has shown, however, that the concentrations of ODAP in the central nervous system of acidotic and normal monkeys following intravenous or intraperitoneal injection are very similar (Rao, 1977). The oral or intraperitoneal administration of ODAP to adult mice has also been shown to result in the ODAP entering the brain, causing characteristic intoxication (Mehta *et al.*, 1979).

The concentration of ODAP in seeds of different varieties of *L. sativus* shows considerable variability, values ranging from 0·12 to 2·25% of dry seed weight having been reported (Roy & Narasinga Rao, 1968). This variability offers the hope that toxin-low varieties of this protein-rich legume will in future years provide a safer source of food and fodder in many parts of India.

Although *Lathyrus* species are the only ODAP-containing plants which regularly constitute part of the human diet it is worth noting that this compound has also been detected in the seeds of legume species of other genera including *Acacia* and *Crotalaria* (Qureshi *et al.*, 1977). One such species is *Acacia albida*, a plant of economic importance in the arid regions of the world. Although ODAP inhibits feeding in the desert locust (Navon & Bernays, 1978) there is no evidence that the seeds of toxin-low strains of *L. sativus* are more susceptible to attack by the larvae of the seed beetle *Callosobruchus chinensis* than are the seeds of high toxin varieties (Roy & Bhat, 1975).

ODAP has recently been synthesised in good yield by reacting, 2,3-diaminopropionic acid with potassium methyl oxalate (Rao, 1975), and 2-oxalylamino-3-aminopropionic acid by reacting oxalylchloride with 3-benzyloxycarbonylamino-2-aminopropionic acid and then removing the 3-amino protecting group by hydrogenolysis (Fig. 2) (Wu *et al.*, 1976). The chromatographic

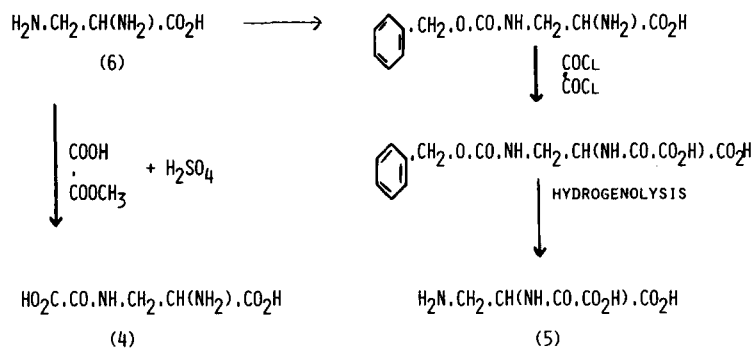


Fig. 2.

separation of mixed isomers, either from plant extracts or in synthetic reaction mixtures, can be achieved on ion-exchange resins at pH 2·9 (Harrison *et al.*, 1977).

3. L-HOMOARGININE

All *Lathyrus* species which synthesise ODAP also synthesise and accumulate L-homoarginine (7) in their seeds (Bell, 1964). The incorporation of ^{14}C-labelled lysine into L-homoarginine in seedlings of *L. venosus* (Sarmiento *et al.*, in prep.) suggests that L-homoarginine is synthesised in a manner analogous to the synthesis of arginine from ornithine. The same enzymes may indeed be responsible for the synthesis of both guanidino compounds but this remains to be confirmed. In the laboratory, L-homoarginine may be readily prepared by reacting *O*-methylisourea with L-lysine (Stevens & Bush, 1950).

In some organisms such as the bacterium *Staphylococcus aureus* and the yeast *Candida albicans* L-homoarginine has proved to be toxic (Rao *et al.*, 1963). In the rat liver, however, homoarginine is broken down enzymically to the essential amino acid lysine (8) and urea (9), homoarginine supplementation consequently enables

$H_2N.C(:NH).NH.CH_2.CH_2.CH_2.CH_2.CH(NH_2).CO_2H$

(7) HOMOARGININE

$H_2N.CH_2.CH_2.CH_2.CH_2.CH(NH_2).CO_2H$

(8) LYSINE

$H_2N.CO.NH_2$

(9) UREA

these animals to thrive on a lysine-deficient diet (Stevens & Bush, 1950). These findings illustrate clearly how meaningless it is to term a compound 'non-nutritional' without specifying the organism(s) in which it is non-nutritional.

4. 4-HYDROXYHOMOARGININE, LATHYRINE AND TETRAHYDROLATHYRINE

A survey of free amino acids in the seeds of some 60 species of *Lathyrus* (Bell, 1964) showed that 40 synthesised and accumulated homoarginine (7) and of these 16 also contained 4-hydroxyhomoarginine (10) and lathyrine (11).

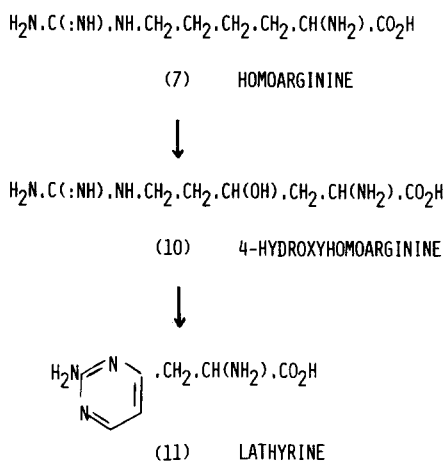
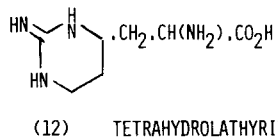


Fig. 3.

Structural similarities suggested that homoarginine might be the precursor of these two compounds and it has been shown that label from ^{14}C homoarginine is incorporated into 4-hydroxyhomoarginine and lathyrine in seedlings of *L. tingitanus* (Bell & Przybylska, 1965) (Fig. 3). Tetrahydrolathyrine (12), a possible intermediate in the synthesis of lathyrine, has recently been isolated from a species (*Lonchocarpus costaricensis*) of another legume genus (Fellows *et al.*, 1979). It is also of interest that orotic acid and uracil can act as precursors of lathyrine in *L. tingitanus* (Brown & Al-Baldawi, 1977).



(12) TETRAHYDROLATHYRINE

5. L-2,4-DIAMINOBUTYRIC ACID

While L-homoarginine is the principal amino acid found free in the seeds of most *Lathyrus* species, in some it is replaced by L-2,4-diaminobutyric acid (13) (Ressler *et al.*, 1961; Bell, 1962). This lower homologue of ornithine is highly toxic to rats inducing convulsions and death when administered in their diet. The cause of death has been identified as ammonia toxicity resulting from the inhibition of ornithine transcarbamylase by 2,4-diaminobutyric acid (Fig. 4) (O'Neal *et al.*, 1968).

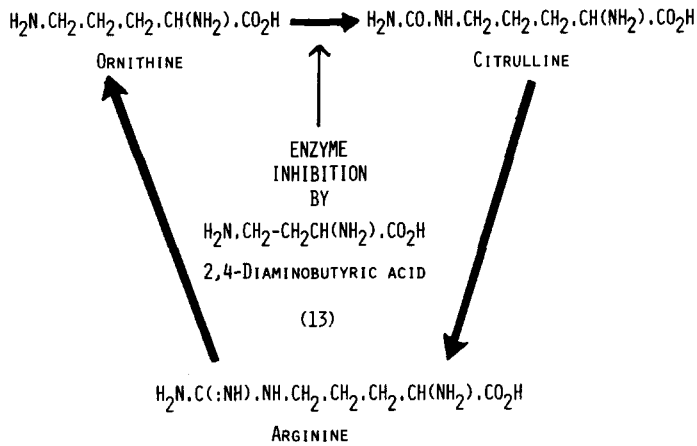


Fig. 4.

6. OXALYL DERIVATIVES OF L-2,4-DIAMINOBUTYRIC ACID

2,4-Diaminobutyric acid is frequently accompanied (Bell & O'Donovan, 1966) by major concentrations of 2-amino-4-oxalylaminobutyric acid (14) and lower concentrations of 2-oxalylamino-4-aminobutyric acid (15). Of these two compounds the major component has been shown to be toxic in vertebrates (Rao & Sarma, 1966) and to act as a feeding inhibitor in insects (Navon & Bernays, 1978). 2,4-Diaminobutyric acid itself is not restricted to species of *Lathyrus*, however; indeed it was first reported in *Polygonatum multiflorum* (Fowden & Bryant, 1958).

$HO_2C.CO.NH.CH_2.CH_2.CH(NH_2).CO_2H$

(14) 2-AMINO-4-OXALYLAMINOBUTYRIC ACID

$H_2N.CH_2.CH_2.CH_2.CH(NH.CO.CO_2H).CO_2H$

(15) 2-OXALYLAMINO-4-AMINOBUTYRIC ACID

The oxalyl derivatives also occur in the seeds of at least 11 species of *Acacia* (Evans *et al.*, 1977) and 14 species of *Crotalaria* (Pilbeam & Bell, 1979).

In many species of *Acacia* an oxalylamino acid tentatively identified as N^3-oxalylalbizziine has been identified (Evans & Bell, in prep.). The synthesis and toxicity of this compound are being investigated at the present time.

7. 3-AMINOPROPIONITRILE

As previously mentioned, the seeds of *L. odoratus* contain the γ-glutamyl derivative of 3-aminopropionitrile (2). The free nitrile and the glutamyl derivative have both proved toxic in experimental animals, inhibiting the enzymes responsible for the synthesis of the cross-linkages in collagen (Levene, 1962) and elastin (O'Dell *et al.*, 1966). The effects of these compounds in experimental animals are generally referred to as osteolathyrism but as far as I am aware there are no reports of osteolathyrism occurring naturally, nor are the seeds of *L. odoratus* used as food or fodder anywhere in the world.

3-Aminopropionitrile not only causes osteolathyrism in vertebrates, but is toxic to a wide range of other organisms including the larvae of the seed beetle *Callosobruchus maculatus* (Janzen *et al.*, 1977). This toxicity to potential insect predators may well provide the plants which accumulate the glutamyl derivative of the nitrile with a selective advantage. The possibility that 3-aminopropionitrile may also play a role in plant/plant interrelationships, providing plants which accumulate it with the ability to compete successfully with other plant species growing in the same habitat, is suggested by the observation that this compound, which is synthesised by *Lathyrus odoratus*, is extremely toxic to the seedling of *L. aphaca*, a species of the same genus which does not synthesise it (Wilson & Bell, 1978). It has been found, moreover, that the root exudates of *L. odoratus* seedlings are rich in free 3-aminopropionitrile (Wilson & Bell, in prep.). This finding is of particular interest suggesting as it does a positive secretion mechanism involving the hydrolysis of the stored γ-glutamyl compound. In contrast to the seedlings of *L. aphaca* the growth of those of *L. odoratus* appears to be stimulated by externally supplied 3-aminopropionitrile which increases gibberellin production (Wilson & Bell, in prep.). It may then be that this lathyrogen fulfils the dual role of stimulating seedling growth in *L. odoratus* while at the same time discouraging competition from other plant species in the same habitat.

8. OTHER 'NON-NUTRITIONAL' FACTORS

Two other non-protein amino acids which have been isolated from the seeds of *Lathyrus odoratus* are 4-hydroxynorvaline (16) (Fowden, 1966) and 4-methylglutamic acid (17) (Przybylska & Strong, 1968). As yet little is known of the role or

effects of these two compounds in other organisms. The biosynthesis of 4-hydroxynorvaline has not been investigated. Incorporation of label from ^{14}C-labelled leucine (Peterson & Fowden, 1972) suggests that 4-methylglutamic acid is formed by the oxidation of one of the methyl groups of leucine to a carboxyl group. The accumulation of *O*-oxalylhomoserine (18) in the pods of *L. sativus* has also been reported (Przybylska & Pawelkiewicz, 1965).

$CH_3.CH(OH).CH_2.CH(NH_2).CO_2H$

(16) 4-HYDROXYNORVALINE

$HO_2C.CH(CH_3).CH_2.CH(NH_2).CO_2H$

(17) 4-METHYLGLUTAMIC ACID

$HO_2C.CO.O.CH_2.CH_2.CH(NH_2).CO_2H$

(18) 4-OXALYLHOMOSERINE

9. CONCLUSIONS

Species of the economically important legume genus *Lathyrus* synthesise a great variety of low molecular weight nitrogen compounds (Table 1) which are not primary metabolites in these or other organisms. Some of these compounds are toxic to man and his domestic animals and an understanding of their chemistry, distribution and biological effects is of importance in medicine and agriculture.

The toxicity of these compounds to man and/or his domestic animals is probably fortuitous, however. It is most probable that the pressures which have led to the natural selection of toxin-synthesising species have been applied not by man or cows, but rather by other phytophagous animals such as insect larvae, locusts and small rodents, or possibly by competing plant species. ODAP, which we have considered as a human toxin, is also, for example, toxic to the nymphs of the grasshoppers *Locusta migratoria* and *Anacridium melanorhodon* (Evans & Bell, 1979) and to the seedlings of *Lathyrus* species other than those which synthesise it (Wilson & Bell, 1978). L-Homoarginine, which is not toxic in experimental mammals, is nevertheless toxic to micro-organisms and inhibits seedling development in some plant species.

Lathyrogen-containing species may therefore have evolved because these compounds have provided a selective advantage to the plants which synthesise them, affording protection against potential predators or discouraging the growth of potential competitors.

TABLE 1
EFFECTS OF LATHYROGENS AND RELATED COMPOUNDS IN VARIOUS ORGANISMS

Compound	*Occurrence*	*Physiological activity*
2-Amino-3-oxalylamino-propionic acid	*Lathyrus*, *Acacia* and *Crotalaria* seeds	Neurotoxic in mammals and birds, toxic in insects and inhibits feeding in locusts
1-Oxalylamino-3-amino-propionic acid	*Lathyrus*, *Acacia* and *Crotalaria* seeds	Not toxic in chicks
2-Amino-4-oxalylamino-butyric acid	*Lathyrus*, *Acacia* and *Crotalaria* seeds	Neurotoxic in chicks, inhibits feeding in locusts
4-Amino-2-oxalylamino-butyric acid	*Lathyrus*, *Acacia* and *Crotalaria* seeds	—
2,4-Diaminobutyric acid	*Polygonatum*, *Lathyrus* species	Neurotoxic in mammals, toxic in insects
3-Aminopropionitrile	*Lathyrus* root exudate	Inhibits collagen and elastin synthesis in mammals and seedling growth, toxic in insects
4-Glutamyl-3-amino-propionitrile	*Lathyrus* seeds	Inhibits collagen and elastin synthesis
Homoarginine	*Lathyrus*, *Lotus* seeds, *Acacia* leaves	Toxic in micro-organisms
4-Hydroxyhomoarginine	*Lathyrus* seeds	Inhibits seedling growth
Lathyrine	*Lathyrus* seeds	—
4-Oxalylhomoserine	*Lathyrus* leaves	—
4-Hydroxynorvaline	*Lathyrus* seeds	—
4-Methylglutamic acid	*Lathyrus* seeds	—

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Food Chemistry 6 (1980–81) 223–234

GLUCOSINOLATES AND RELATED COMPOUNDS

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ABSTRACT

The characteristic flavour of cruciferous foods and the spicy character of some other plants are connected, though not necessarily synonymous, with their glucosinolates content. The understanding of the biological role of the glucosinolates has only just begun. In this paper, their occurrence, biosynthesis, catabolism and biological significance are discussed in detail.

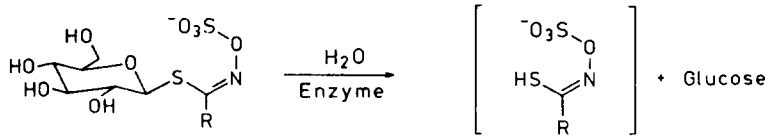
1. BACKGROUND, DEFINITIONS AND SCOPE

The inclusion of a contribution dealing primarily with glucosinolates in a symposium devoted to 'Some Non-nutritional Nitrogenous Constituents of Foods' may, at first glance, seem somewhat out of place insofar as this class of natural compounds traditionally is thought of as one composed of molecules characterised primarily by their sulphur contents. However, structural inspection of the glucosinolates (1) immediately reveals that they are perfectly respectable 'nitrogenous constituents', albeit with an unusual and intriguing molecular architecture. Viewed in a biogenetic perspective, the nitrogenous features of the glucosinolates, with, as we shall see, their parental roots in the amino acid pool, become even more conspicuous. Add hereto that typical loci of the non-nutritional glucosinolates are certain plant families rich in species of vast importance as food and fodder, and it will, I hope, become apparent that their inclusion in the present context is in fact both justified and potentially important.

The pungency we cherish in cruciferous food products such as cabbage, radish, horseradish, mustard, cress and watercress is invariably associated with their contents of glucosinolates. The same holds true of the spicy character of capers and

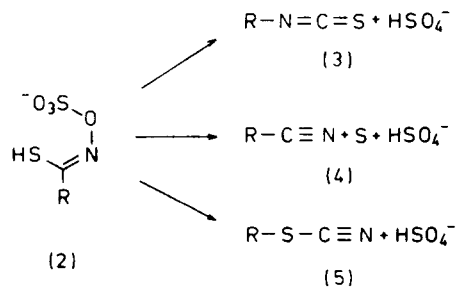
Food Chemistry 0308-8146/81/0006-0223/$02·50

several other products from subtropical and tropical plants of widely varying botanical families, although one should not, of course, be led to believe that biting taste and pungency in plant products is necessarily synonymous with their contents of glucosinolates. Today we know of about 90 individual glucosinolates (1) occurring in nature and varying solely in the character of the sidechain R. Characteristic for all of them is their natural association with enzyme systems, myrosinases, catalysing their hydrolysis to the unstable aglucones (2), the further



(1) (2)

fate of which depends, in each case, on the character of the sidechain R and the conditions prevailing during the enzymic process. Various pathways from the aglucones (2) to stable end-products are known and frequently simultaneously involved. The most important are: (i) intramolecular, non-enzymic rearrangement to isothiocyanates (mustard oils) (3); (ii) fragmentation to nitriles (4) (with liberation of elementary sulphur); and (iii) rearrangement, in rare cases, to thiocyanates (5). The factors controlling the formation of and ratio between (3), (4), and (5) are important, but still incompletely understood.



(2)

Once established, the glucosinolate structure (1) soon invited speculations as to its biogenetic derivation and it is a pleasing fact that we today possess a detailed, though not entirely complete picture of the pathways leading from amino acids to the finished products. Far less precise, however, is our understanding of the catabolic fate of these and their enzymically produced degradation products, and we are only beginning to comprehend the significance and biological role of the glucosinolates and the many products derived from them under natural conditions. Several reviews exist, covering, comprehensively (Kjær, 1960; Ettlinger & Kjær, 1968), or more selectively (Kjær, 1974, 1976; Kjær & Olesen Larsen, 1973, 1976,

1977, 1980), the various aspects of the topic. Here we shall pursue a more limited goal: briefly to discuss some selected aspects of the occurrence, biosynthesis, enzymic degradation and biological function of the glucosinolates, particularly relevant for the occasion.

2. OCCURRENCE

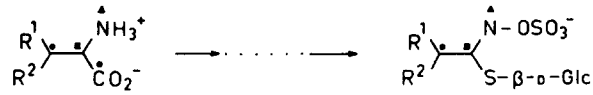
As far as we know the natural occurrence of glucosinolates is restricted to dicotyledonous angiosperms yet with, it seems, a discontinuous distribution within this great botanical division. Hence, their occurrence may well be indicative of phylogenetic affinities between the various loci. In this connection it should be noted that for systematic purposes a 'negative character', such as, for example, the non-occurrence of glucosinolates in a given taxon, is as important and potentially useful as a positive finding, though infinitely more difficult to establish. An ever-increasing enhancement in sensitivity of the analytical tools and methods employed routinely in serial analyses may render a compound pattern recorded today far more complex than one produced a few years ago from the same material. Inevitably, this process will continue and our present picture will soon be superseded by one still richer in detail. Even so, it remains that we do possess a rather precise conception as to where in the system glucosinolates congregate. Thus, within large and important families such as Brassicaceae and Capparaceae, glucosinolates seem to be consistently present. Similarly, we know of no taxon from the smaller families Moringaceae, Pentadiplandraceae, Resedaceae, and Tovariaceae devoid of glucosinolates. Perhaps the order Capparales *sensu* Dahlgren (1975), including all of these with the families Limnanthaceae, Tropaeoloceae, Bretschneideraceae, Salvadoraceae, Gyrostemonaceae, and Bataceae, correctly delineates the 'pool' of natural glucosinolates although the occurrence of these was admittedly used as an ancillary character in the construction of the order. A few sporadic occurrences outside Capparales, thus defined, are known (e.g. in Caricaceae and Euphorbiaceae) but these may, of course, conveniently be attributed to evolutionary convergence.

In the sequel we shall focus on glucosinolates in taxa of potential interest as food for man and domestic animals. Such plant materials are virtually limited to Brassicaceae and, to a smaller extent, Capparaceae, both large families comprising about 3000 and 600 species, respectively. Only a handful of these have acquired notable practical significance but, when so, they are to be counted among the really important crops. We need only remind ourselves of the enormous global production of oil seed crops of various species of *Brassica* (rape, turnips, mustard) or of the production of vegetables such as cabbage, radish, horseradish, cress, and watercress, to appreciate the interest associated with a detailed understanding of the plethora of constituents, including the glucosinolates, occurring in this production. By way of illustration, the global *Brassica* seed production is stated to exceed 10^7

tons annually and the consumption of *raw* radish in Japan alone has been estimated at 3×10^5 tons per year (Kjær *et al.*, 1978). Numerous problems, associated with cultivation, storage, taste, toxicology, etc., are intimately related to the *in vivo* synthesis and subsequent catabolic fate of the glucosinolates. Hence, we shall enquire into certain aspects of these processes.

3. BIOSYNTHESIS

The available evidence, notably specific labelling experiments, points to one general pathway for the *in vivo* synthesis of all glucosinolates, proceeding from α-amino acids, through several intermediates, to the finished products (6) with the labelling pattern shown. Two conclusions immediately emerge: (i) C-1 of the amino acid is



(6)

lost during the conversion, and (ii) all intermediates must contain nitrogen. Considerable efforts have been exerted to clarify the individual steps in the above conversion; before discussing these, however, the chemical character of the sidechains should be considered.

Within the collection of natural glucosinolates one group stands out by having sidechains identical with those of the protein amino acids (Table 1). Additional to this, a subgroup can be distinguished encompassing structural variants, yet with an unchanged carbon skeleton, such as hydroxylated, methoxylated, acyloxylated, glycosyloxylated and sulphonated derivatives. As far as we know, most or all of

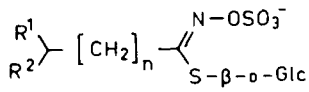
TABLE 1
GLUCOSINOLATES (6) WITH PROTEIN AMINO ACID SIDECHAINS

Formula (6)		*Corresponding protein amino acid*
R^1	R^2	
H	H	Alanine
Me	Me	Valine
$[Me]_2CH$	H	Leucine
Et	Me[a]	Isoleucine
C_6H_5	H	Phenylalanine
p-HO.C_6H_4	H	Tyrosine
3-Indolyl	H	Tryptophan
Me.S.CH_2	H	Methionine[b]

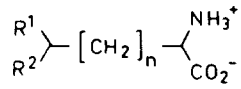
[a] (*S*)-configuration.
[b] From Grob & Matile (1980).

these modifications take place somewhere along the path rather than at the starting amino acid level. Altogether, however, the eight protein amino acids account for only about one-fourth of the known glucosinolates. It should be noted also that less than half of the known protein amino acids can be recognised in the known glucosinolate sidechains, although it seems quite likely that additions will appear.

Another conspicuous group within the class of structurally identified glucosinolates is composed of a series of homologues (7, $n \geq 1$), presented in Table 2.



(7)



(8)

Again, within this series, modifications such as terminally unsaturated sidechains (known for $R^1 = CH_2{:}CH$, $R^2 = H$; $n = 0$–4), sulphoxide analogues of the MeS-substitutes (known for $n = 2$–10), the corresponding sulphones (known for $n = 2, 3$), a single dehydro- and ring-methoxylated derivative, and a number of variously hydroxylated species, have been encountered. Most likely these structural modifications also are brought about along the biosynthetic paths. Together this group accounts for about 50 identified glucosinolates (Kjær, 1960, 1974, 1976; Ettlinger & Kjær, 1968; Kjær & Olesen Larsen, 1973, 1976, 1977, 1980).

With regard to the biosynthetic derivation of the corresponding amino acids (8) compelling experimental evidence, essentially based on specific labelling, supports the homologisation process depicted in Fig. 1; characteristically, it involves a C-2 fragment (acetate) with subsequent loss of a C-1 fragment (CO_2); this elongation principle is similar to that operating during the *in vivo* conversions of, for example,

TABLE 2
GLUCOSINOLATES (7) DERIVED FROM HOMOLOGUES OF PROTEIN AMINO ACIDS

Formula (7)			*Protein amino acid converted into homologue(s)*
R^1	R^2	n	
H	H	1, 3, 4[a], 5[a]	Alanine
OH[b]	H	2	Serine
Me	Me	3[a]	Leucine (valine)
Et	Me	1[c], 2[a,d]	Isoleucine
Me.S	H	2–10[f]	Methionine
C_6H_5	H	1–3[g]	Phenylalanine
MeO_2C[e]	H	2	Glutamic acid

[a] From Kjær *et al.* (1978).
[b] Only known as the benzoate.
[c] (*S*)-configuration.
[d] Of unestablished configuration.
[e] Only known as the methyl ester.
[f] Some homologues only known as sulphoxides.
[g] For $n = 2$ and 3, *cf.* Grob & Matile (1980).

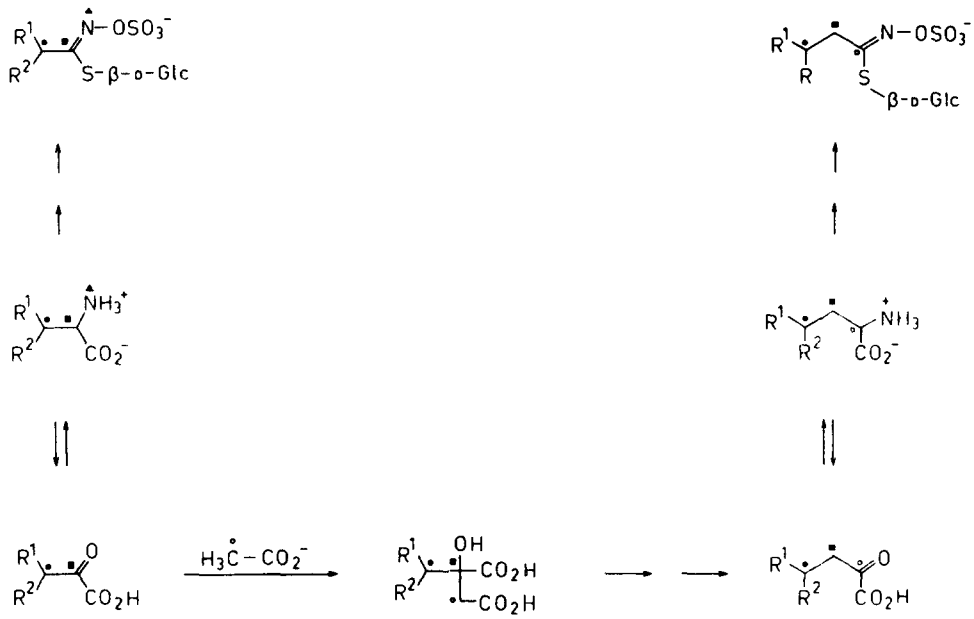


Fig. 1.

valine into leucine, and aspartic acid into glutamic acid, but draws particular attention because of its remarkable efficiency and highly repetitive character, with the Me.S.(O).$[CH_2]_{11}$ sidechain, derivable from methionine, representing the acme thus far.

Only a few of the known glucosinolates cannot easily be traced to a likely amino acid progenitor. They comprise sidechains such as Pr.CO.$[CH_2]_{3-4}$ and Et.CO.$[CH_2]_4$ (Kjær, 1960; Ettlinger & Kjær, 1968), Me.$[CH_2]_2[CH(OH)]_4[CH_2]_3$ (Gaind *et al.*, 1975), and (*p*)-HO.$C_6H_4C(Me)_2.CH_2$ (El Migirab *et al.*, 1977); here, our ignorance is clearly an invitation to further studies.

In view of the active operation of the chain-elongating process one might perhaps expect to encounter protein amino acid homologues (8) in taxa containing the corresponding glucosinolates; that is indeed the case. Reported occurrences of 2-amino-5-methylthiopentanoic acid (homomethionine) in horseradish and 2-amino-4-phenylbutyric acid (homophenylalanine) in watercress are in fact such examples, although it appears that under normal 'flow conditions' accumulation of such homologues tends to be low.

We now turn to a discussion of the pathway leading from the amino acids, whether protein type or elongated, to the glucosinolates. For details and references the various review articles should be consulted (Kjær, 1960, 1974, 1976; Ettlinger & Kjær, 1968; Kjær & Olesen Larsen, 1973, 1976, 1977, 1980). The generally accepted pathway, established by numerous feeding experiments with specifically labelled

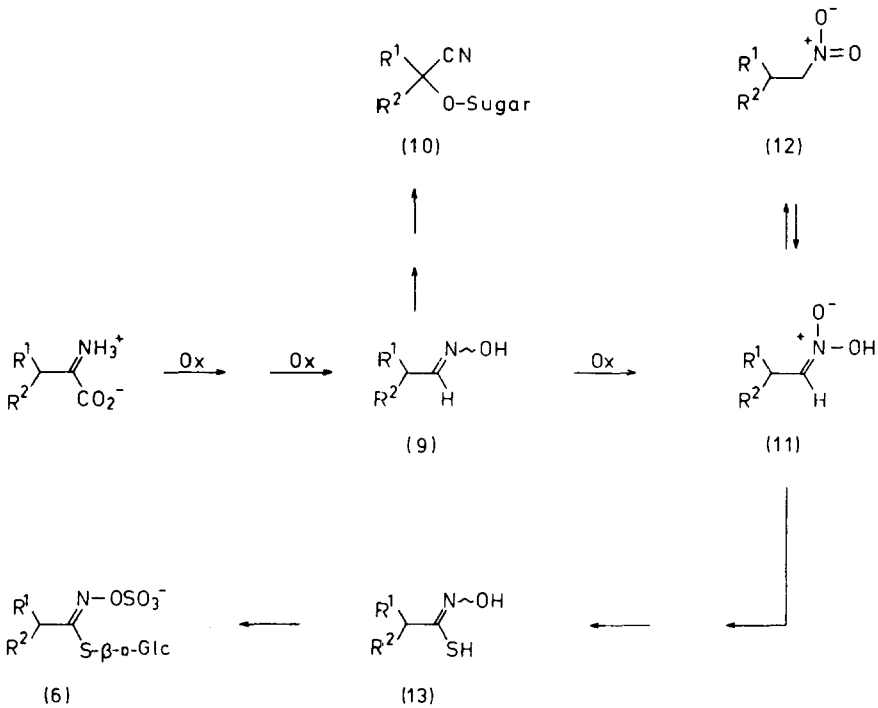


Fig. 2.

compounds, is presented in Fig. 2. Obviously, *N*-oxidation is a salient feature in the overall transformation which we shall, for practical reasons, discuss in two stages: (i) the steps leading to the aldoximes (9), and (ii) the further conversion of the latter into glucosinolates (6).

Today, aldoximes are accepted as key intermediates in the biosynthesis of two major classes of plant products: the cyanogenic glycosides (10) and the glucosinolates (6) (Kjær, 1960, 1974, 1976; Ettlinger & Kjær, 1968; Kjær & Olesen Larsen, 1973, 1976, 1977, 1980). As we have seen, the latter have a fairly restricted distribution; the cyanogenic glycosides (10), on the other hand, are encountered in a far wider range of the plant kingdom including monocotyledons and certain classes of lower plants. As far as we know, aldoximes are produced by only one path (Mahadevan, 1973); if correct, this synthetic route hence is widespread and, perhaps, of great antiquity in a phylogenetic context. It is remarkable, however, that the known cyanogenic glycosides (10) all derive from aldoximes which, in their turn, arise from certain ordinary protein amino acids, but not from their homologised counterparts. Convincing evidence has recently been adduced in favour of the first step on the road from L-tyrosine to dhurrin (10, R^1 = (*p*)-HO.C_6H_4; R^2 = H; sugar = glucose; (*S*)-configuration) in sorghum seedlings being the oxidation of

tyrosine to *N*-hydroxytyrosine (Møller & Conn, 1979). The conversion into (*p*)-hydroxyphenylacetaldoxime (9, R_1 = (*p*)-HO . C_6H_4; R_2 = H) probably proceeds via further oxidation to the α-nitroso acid, followed or accompanied by decarboxylation. Adoption and generalisation of this sequence to include all taxa synthesising glucosinolates, however, rests solely on analogy. In this context it should be noted that the occurrence of cyanogenic glycosides and glucosinolates within the same taxon seems close to mutually exclusive: the spacious, biosynthetic avenue carrying amino acids oxidatively through to aldoximes at this point apparently branches into two unconnected alleys: a broader one, leading to the cyanogenic glycosides, and a more narrow one, terminating in the glucosinolates; we shall briefly explore the latter.

The conversion of an aldoxime (9) into a glucosinolate (6) is formally an oxidation. *N*-Oxidation, affording (11) (i.e. the aci-tautomer of a primary nitro compound (12)), followed by reaction with a sulphur-containing entity, introducing the thiolfunction, to give the thiohydroximic acid (13), enzymatic *S*-glucosylation of the latter, and, finally, *O*-sulphonation, represents the accepted overall pathway to the glucosinolates (6). However, important details are still outstanding. Thus, we do not know with certainty how sulphur is introduced although cysteine is suspect of playing the carrier's role; if so, a subsequent, C–S lyase-induced severance of the $S–C_3$ linkage is probably implied. As to the last steps, the enzymic glucose transfer from UDP-glucose and the similarly catalysed *O*-sulphonation from PAPS, we are on much firmer ground (Kjær, 1960, 1974, 1976; Ettlinger & Kjær, 1968; Kjær & Olesen Larsen, 1973, 1976, 1977, 1980).

Even when the general *in vivo* pathway to glucosinolates thus has been revealed, our knowledge as to the timing and detailed character of the numerous sidechain modifications (hydroxylation, elimination, *S*-oxidation, etc.) is regrettably limited. Similarly, the factors controlling the production of the individual glucosinolates and their mutual conversions are virtually unknown. A better understanding of the regulatory mechanisms would be of pre-eminent practical importance in connection with, for example, plant-breeding programmes aimed at the production of cultivars with low or, in some cases, high contents of glucosinolates. The enormous recent refinement of isolation and separation methods, combined with a dramatic increase in the speed and accuracy with which serial analyses can be performed, makes it riskless to prophesy that we shall soon possess a much better understanding of the subtle regulatory mechanisms operating along the biosynthetic roads to glucosinolates.

4. CATABOLISM

About 150 years ago, two crystalline compounds, later designated 'sinigrin' and 'sinalbin', were isolated by French chemists from black and white mustard seeds,

respectively. Further, the mustard pungency was shown to result from interaction between the crystalline isolates and an auxiliary extract from the seeds (Kjær, 1960; Ettlinger & Kjær, 1968). These observations, forming a corner stone in the development of modern natural product chemistry, amount to an early and remarkably precise recording of what is known to us as an enzyme-catalysed hydrolysis.

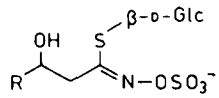
Today, several enzymes from plant sources, collectively termed 'myrosinases', are known to catalyse the hydrolysis of the thioglucosidic linkage in glucosinolates. They differ in their cofactor requirements, some needing vitamin C, others not, rather than in their specificities (Björkman, 1976). The enzymes are localised in special cells, separated from the glucosides, which in themselves seem to be at least partly compartmentalised (Jørgensen *et al.*, 1977). Thus, pungency becomes conditional on rupture of the cell structure.

X-ray structure determination has been used to establish the (*Z*)-configuration of allylglucosinolate, a stereochemistry that has since been tacitly adopted for all other glucosinolates. Hence, the aglucones resulting from hydrolytic detachment of glucose, whether catalysed by the plant's own enzyme(s) or extraneously added myrosinase, have been assigned the structure (2); this species is properly constituted for a spontaneous, intramolecular Beckmann-type rearrangement to occur, producing an isothiocyanate (3) with concomitant release of sulphate, and this, indeed, represents the major path of degradation under conditions normally prevailing in crushed or injured plant tissue. However, an alternative mode of decomposition exists: simple fragmentation to nitrile and elementary sulphur. It has been convincingly demonstrated that low pH values favour the nitrile formation although cations other than protons (e.g. Fe^{++}) can promote the same reaction. In practice, mixtures with isothiocyanates as the predominant constituents, nitriles as the minor constituents, are frequently obtained. For practical reasons, the large group of steam-volatile isothiocyanates (and nitriles) has attracted the lion's share of interest, being easier to isolate, separate and characterise than the smaller, non-volatile group, which call for solvent extraction and frequently rather tedious separation and purification operations. It is well to remember that both types are equally important in producing a complete picture of a given taxon. It must also be appreciated that, no matter how sophisticated, the analysis of the enzymically produced constituents will, at best, afford only an approximate picture of the genuine glucosinolate contents. Hence, recent efforts have been directed towards a direct determination of the glucosidic progenitors.

Formerly, the structural identification of the enzymic hydrolysis products, and hence of the glucosinolates, often presented true chemical challenges. Today, however, the access to powerful chromatographic techniques, combined with efficient spectroscopic methodology (MS, n.m.r.), has made the recognition of known, and the discovery of new, glucosinolates almost a matter of routine. This development is to be welcomed, however, as a prerequisite for deepening our

understanding of the regulated biosynthetic pathways and hence of the parameters we may wish to influence.

Mostly, the naturally derived isothiocyanates are sufficiently stable to permit their isolation as such. Exceptions exist, however. Thus, in aqueous systems, *p*-hydroxybenzylglucosinolate does not give *p*-hydroxybenzylisothiocyanate on enzymic hydrolysis, but rather *p*-hydroxybenzyl alcohol, the solvolysis product. Similarly, 3-indolylmethylglucosinolate yields stable end products, such as 3,3'-bisindolylmethane, which again can be accounted for in terms of solvolysis of the initially formed isothiocyanate (Kjær, 1960, 1974, 1976; Ettlinger & Kjær, 1968; Kjær & Olesen Larsen, 1973, 1976, 1977, 1980). Glucosinolates with a β-hydroxylated sidechain (14) on enzymic hydrolysis afford isothiocyanates that spontaneously cyclise to 5-substituted oxazolidine-2-thiones (15), some of which, notably the (*S*)-5-vinyl derivate (16) (goitrin), occur in important food plants (cabbage, rape a.o.) and draw attention because of their goitrogenic effect.



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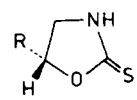
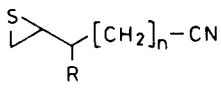


(15)

(16) R = CH_2 : CH

Our understanding of the processes accompanying the enzymic glucosinolate hydrolysis are, however, far from complete. Thus, the formation of thiocyanates (5), rather than isothiocyanates, is characteristic of certain glucosinolates when cleaved in certain taxa; for example, autolysis of seeds of *Thlaspi arvense* results in the production of both allyl isothiocyanate and allyl thiocyanate, whereas benzylglucosinolate, when treated with disintegrated seeds or fresh parts of *Lepidium ruderale*, or seed extracts of ordinary cress (*L. sativum*), affords benzyl thiocyanate, additional to benzyl cyanide and benzyl isothiocyanate. Similarly, plant extracts of *Eruca sativa* mediate the conversion of 4-methylthiobutylglucosinolate, a natural constituent of the taxon, into 4-methylthiobutyl thiocyanate; *seed* extracts, however, direct the fission to give the corresponding isothiocyanate. We know that the aglucones, and not the parent glucosinolates, are the common progenitors of the alternative reaction products, and we know that in the allyl case rearrangement to the thiocyanate is accompanied by an interchange of the allyl group termini. A satisfactory rationalisation of these observations is still outstanding, but various possibilities have been discussed in a recent review (Benn, 1977).

Perhaps even more puzzling is the repeatedly observed formation of cyanoepithioalkanes (17) from glucosinolates possessing terminally unsaturated sidechains (18), again governed by enzymes. The operation of an 'epithio specifier protein' has been invoked, acting as a sort of enzyme cofactor, in itself unable to bring about the thioglucoside hydrolysis but instrumental in directing the product composition away from nitrile and isothiocyanate and towards cyanoepithioalkane.



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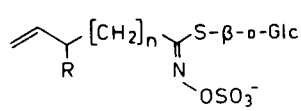

(18) R = H, or OH, n = 0-2

This remarkable and virtually unprecedented principle of product regulation deserves continued interest. Various mechanistic interpretations of the sulphur-transfer reaction are presented in a recent review (Benn, 1977).

5. BIOLOGICAL SIGNIFICANCE

The genuine glucosinolates are colourless, inconspicuous ions with a poorly understood biological function. It seems convincingly established though that allylglucosinolate, but not the isothiocyanate deriving from it, can stimulate feeding in the phytophageous, crucifer-adapted insect species *Pieris brassica*. In most other cases, however, doubt prevails as to an observed biological response being attributable to the parental glucosinolates or rather to their often volatile catabolic products.

Such doubt is excluded, however, when it comes to the flavour and odour of glucosinolate-containing food products. Here, isothiocyanates, nitriles, and perhaps other catabolic products as well, contribute significantly to the palatability and flavour of such materials. Equally obvious, such products are in many cases involved not only in attracting insects, adapted to glucosinolate-containing taxa, but also in inducing them to egg-laying and feeding. Much remains, however, to be clarified in the insect–plant relationships within this area, e.g. the extent of the recorded food deterrent role played by glucosinolates. Much practical and economic interest is obviously associated with such problems.

The toxicological properties of the glucosinolates and, notably, their catabolic products, deserve, we believe, much more interest. We know that isothiocyanates, in general, are vesicant and mutagenic, nitriles toxic, and thiocyanate ion and oxazolidinethiones goitrogenic, but we do not seem to possess a satisfactory and reliable picture of the hazards, if any, associated with an often life-long ingestion of diets rich in raw, glucosinolate- and enzyme-containing food materials such as cabbage, mustard and radish.

6. CONCLUSION

The glucosinolates constitute a large collection of naturally occurring ions with a strikingly uniform chemical structure and an intriguing natural distribution. Their biosynthetic derivation and catabolism are reasonably well understood. This is not true, however, of the regulatory mechanisms, controlling the actual level of the

individual glucosinolate, or the detailed biological function. We should also like to know more about toxicity.

May I close by quoting an old friend and colleague, stating (Benn, 1977): 'the importance of the glucosinolates resides in their disappearance'. I should like to join issue with him on this point, if for no other reason than a very selfish one: as a chemist, life without glucosinolates, their challenges and intricacies, would become unbearably dull—missing flavour.

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PROTEIN INHIBITORS OF ENZYMES

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ABSTRACT

The range of proteins from plants which form reversible stoichiometric protein–protein complexes with enzymes, thus inhibiting their catalytic functions, is surveyed. Details of the biochemistry of the proteinase- and α-amylase inhibitors found in the important food crops such as the cereals, legumes and potatoes are given. Recent experiments on the nutritional significance, possible therapeutic use, and value of the inhibitors as laboratory tools are also discussed. The possible physiological role of the inhibitors in protecting plants against microbial and insect pests is considered briefly.

1. INTRODUCTION

Proteins with the peculiar property of forming stoichiometric protein–protein complexes with various enzymes resulting in the competitive inhibition of their catalytic functions are known to be extremely widespread in the plant kingdom. The existence of such inhibitory proteins was initially discovered by Weinland in 1903 who used the term 'antienzymes' to explain the resistance of certain nematodes to digestion by the enzymes of the alimentary canal. The occurrence of similar chemical compounds in plant tissues was suspected for a number of years and finally confirmed in the 1940s when Kunitz isolated and purified a heat-labile protein from soybeans which inhibited trypsin and Kneen and Sandsted found an α-amylase inhibitor in cereal grains.

The best known of these protein inhibitors are those affecting the activities of the proteinase enzymes. Table 1 shows the wide range of different enzymes of this type which are inhibited by proteins obtained from plant tissues. It should be noted that

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TABLE 1
RANGE OF ENZYMES KNOWN TO BE INHIBITED BY PROTEINS FROM PLANTS

Proteinases

A. *Serine proteinases*
- Chymotrypsin (EC 3.4.21.1)
- Trypsin (EC 3.4.21.4)
- Thrombin (EC 3.4.21.5)
- Plasmin (EC 3.4.21.7)
- Kallikrein (EC 3.4.21.8)
- Acrosin (EC 3.4.21.10)
- Elastase (EC 3.4.21.11)
- Nagarse, Subtilisin BPN (EC 3.4.21.14)
- *Aspergillus oryzae* alkaline proteinase (EC 3.4.21.15)

B. *Sulphydryl proteinases*
- Papain (EC 3.4.22.2)
- Ficin (EC 3.4.22.3)
- Bromelain (EC 3.4.22.5)

C. *Acidic proteinases*
- Pepsin (EC 3.4.23.1)
- Rennin (EC 3.4.23.4)
- Cathepsin D (EC 3.4.23.5)

D. *Metalloproteinases and others*
- Carboxypeptidase A (EC 3.4.12.2)
- Carboxypeptidase B (EC 3.4.12.3)
- *Bacillus subtilis* neutral proteinase (EC 3.4.24.4)
- *Bacillus nato* proteinase (EC 3.4.4.16)
- Aminopeptidase (EC 3.4.11.1) (Beitz *et al.*, 1978)

Other Enzymes
- α-Amylase (1,4-α-D-glucan glucanohydrolase) (EC 3.2.1.1)
- Acid invertase (EC 3.2.1.26) (Matsushita & Uritani, 1976)
- Endopolygalacturonase (endo-β-1,3-glucanase) (EC 3.2.1.39) (Fisher *et al.*, 1973)
- Phospho inositol kinase (EC 2.7.1.67) (Majumder & Biswas, 1973)
- Pectate lyase (EC 4.2.2.2) (Bock *et al.*, 1975)
- Endopectin lyase (EC 4.2.2.10) (Bock *et al.*, 1975)
- Catalase (EC 1.11.1.6) (Sorenson & Scandalios, 1975)
- Isocitrate lyase (EC 4.1.3.1) (Surendranathan & Nair, 1978)
- NADH-Nitrate reductase (EC 1.6.6.1) (Jolly & Tolbert, 1978')
- L-Phenylalanine ammonia lyase (EC 4.3.1.5) (Billett *et al.*, 1978)
- Lipase (EC 3.1.1.3) (Kim *et al.*, 1977)
- Guanylate cyclase (EC 4.6.1.1) (Vesely *et al.*, 1977)
- Cinnamic-4-hydroxylase (EC 1.14.13.11) (Kim *et al.*, 1977)

in some cases the proteinase inhibitors exhibit a very narrow range of specificity being capable of inhibiting only one or two closely related proteinases, whilst others of broad specificity are active against a wide range of different enzymes. Somewhat surprisingly there is as yet no evidence for the existence in plants of inhibitors of the important microbial proteinase thermolysin.

The only other group of protein inhibitors which has been intensively studied is the inhibitors of α-amylases which show a variable specificity towards the salivary and pancreatic enzymes of mammals and the α-amylase of insects, plants and microorganisms. Table 1 also lists the many other enzymes which are known to be inhibited more or less specifically by plant proteins.

The protein inhibitors have been particularly well studied in the important food crops such as the cereals, legumes and potatoes, since much of the initial work in this field came from those interested in animal nutrition who were concerned about the

possibly deleterious dietary effects exerted by the inhibitors, but they have also attracted the attention of botanists, biologists, biochemists, pharmacologists and those in the medical world. The large body of research literature which has built up in the last two decades has been extensively reviewed in recent years (Ryan, 1973; Fritz *et al.*, 1974; Tschesche, 1974; Marshall, 1975; Saunders, 1975; Buonocore *et al.*, 1977; Richardson, 1977) so within the confines of this short talk I shall only give a brief summary of the more important aspects of the biochemistry and physiology of the proteinase- and α-amylase inhibitors before concentrating in more detail on their role in human and animal nutrition, and their possible function in protecting plants against microbial and insect pests.

2. BIOCHEMISTRY

A. Proteinase inhibitors

Almost without exception the proteinase inhibitors found in plants are relatively small proteins with little or no additional carbohydrate moieties. At the lower end of the size range the smallest inhibitors for which the complete amino acid sequences are known are the carboxypeptidase inhibitor from potatoes (MW 4300) (Hass *et al.*, 1975), the bromelain inhibitor from pineapple (MW 5600) (Reddy *et al.*, 1975) and the trypsin inhibitor from aubergine (MW 5700) (Richardson, 1979). The majority of the other proteinase inhibitors whose primary structures are known contain between 70 and 90 amino acids corresponding to a molecular weight of 8000–10,000 (Fritz *et al.*, 1974; Richardson, 1974; Richardson & Cossins, 1975; Wilson & Laskowski, 1975; Joubert *et al.*, 1979). Many of the apparently larger ($>$ 10,000 MW) inhibitors are actually polymeric (usually di- or tetrameric) proteins whose protomers have minimum molecular weights under 10,000.

Another notable feature of the proteinase inhibitors from plants is the frequency with which they exhibit high levels of microheterogeneity. For example, at least 10 isoinhibitors of chymotryptic inhibitor I have been isolated from potato tubers of the variety 'Ulster Prince' (Richardson *et al.*, 1976) and families of isoinhibitors are known for the pineapple bromelain inhibitor (Reddy *et al.*, 1975) and the soybean inhibitors (Hwang *et al.*, 1977).

Inspection of the primary sequences of several of the proteinase inhibitors reveals a striking degree of structural homology between them. Figure 1 illustrates this homology by making comparisons at the intergeneric (*Phaseolus/Glycine*), interspecific (*Phaseolus vulgaris/Ph. lunatus*) and intraspecific (the isoinhibitors of *Ph. lunatus*) levels. This figure also demonstrates the fact that several of the plant proteinase inhibitors contain repetitive sequences or regions of internal homology, which are thought to have possibly resulted from the extension of shorter polypeptide chains by a process of gene duplication. Another feature of these separate but homologous regions within the same molecule is that they very often

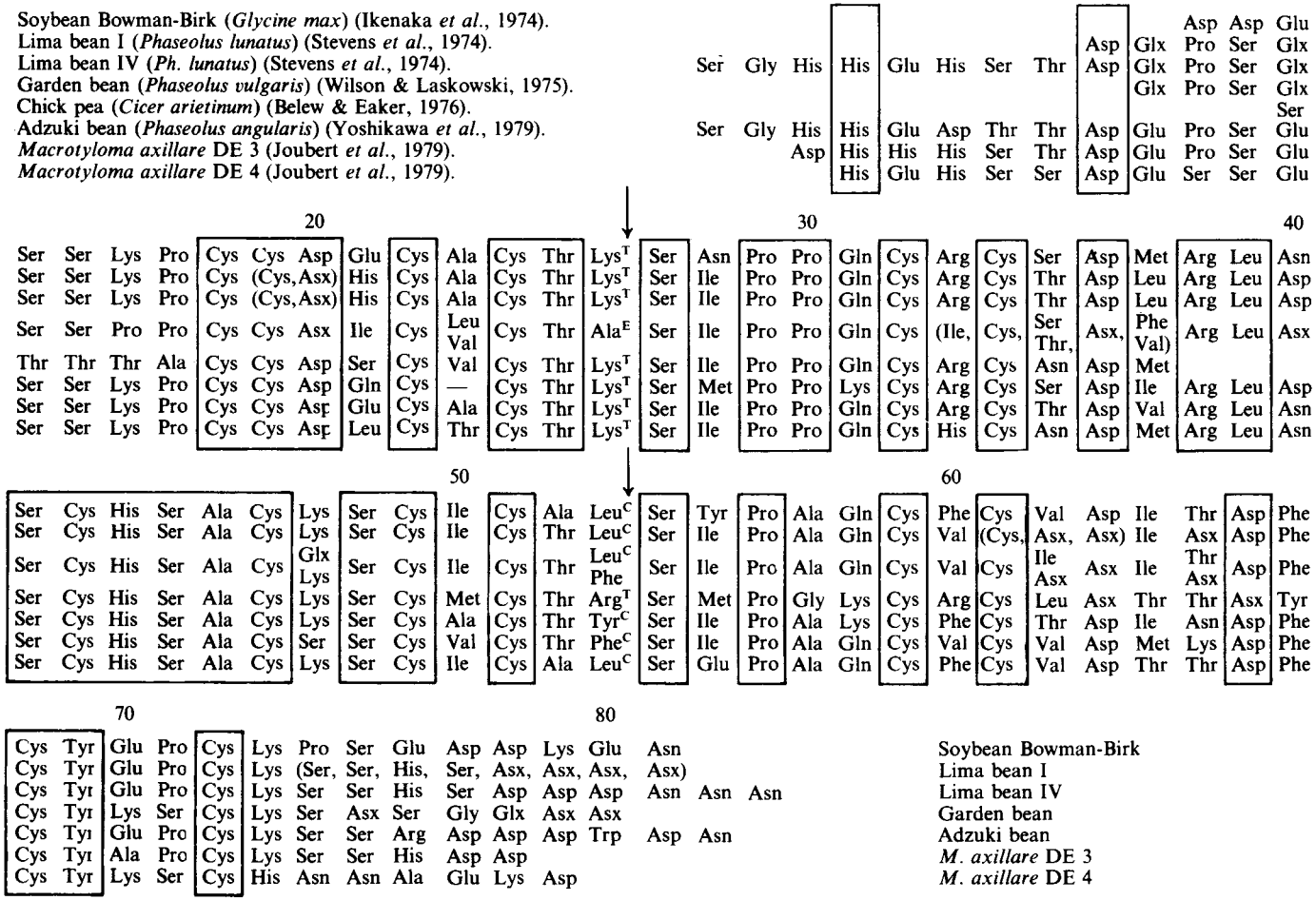

Fig. 1. Comparison of the amino acid sequences of the 'double-headed' proteinase inhibitors from various members of the Leguminosae. Segments of identical (homologous) sequences in the proteins are enclosed in boxes. The reactive (inhibitory) sites of each protein are indicated by the arrows; T, site reacting with trypsin; C, chymotrypsin; E, elastase. The sequences are arranged to facilitate comparison of the regions of internal homology (repetitive sequences) around the reactive sites (e.g. residues 14–36 and 41–63).

contain the reactive (or inhibitory) sites which interact with the proteinases affected. Thus it can be seen in Fig. 1 that several of the inhibitors are 'double-headed' proteins capable of inhibiting both trypsin and chymotrypsin at separate peptide bond sites. On the other hand, some inhibitors have a single peptide bond which serves as the reactive site for a single inhibited proteinase or acts against several enzymes. Such examples are referred to as 'single-headed' inhibitors.

The sequence of the 'double-headed' garden bean inhibitor shown in Fig. 1 deserves some comment. It clearly differs from the other related sequences shown by having replacement of amino acids at both reactive sites. The usual Lys residue at position 26 is replaced by an Ala residue. The site thus loses its ability to bind trypsin, and binds instead the related proteinase elastase. Similarly the replacement of the usual Leu (Position 53) by an Arg at the second reactive site makes it inactive towards chymotrypsin and converts it to a trypsin binding site. These mutational changes, which presumably occurred over an evolutionary period of millions of years, have recently been imitated in the laboratory in a matter of days by Laskowski *et al.* (see Fritz *et al.*, 1974) who showed that the Arg^{63}–Ile^{64} reactive peptide bond in the soybean (Kunitz) tryspin inhibitor could be experimentally modified in several ways by chemical and enzymatic means without the inhibitor losing its activity. For example the Arg^{63} could be replaced by Lys^{63} in which case the altered inhibitor continues to behave like the native form, or alternatively by Trp^{63} in which case the protein becomes a good inhibitor of chymotrypsin. They also demonstrated that certain changes of the amino acid (Ile) in position 64 were permissible without destruction of the inhibitor capacity (Kowalski & Laskowski, 1976). Other modifications worthy of note are the cleavage of certain 'double-headed' inhibitors by chemical and enzymatic means into two smaller 'single-headed' active fragments (Odani & Ikenaka, 1973) and the reduction in size of certain native 'single-headed' inhibitors without loss of inhibitory activity (Iwasaki *et al.*, 1974, 1975).

The reactive sites are usually identified by carrying out a limited hydrolysis (or proteolysis) of the inhibitor with catalytic amounts of the enzyme at low pH (2·0–4·0) and then separating the cleaved (modified) fragments after any necessary reduction and S-alkylation. Alternatively, removal of the newly formed carboxy-terminal residue (e.g. Met, Phe, Tyr or Leu in chymotryptic inhibitors, Lys or Arg in trypsin inhibitors) by the action of either carboxypeptidease A or B usually abolishes the inhibitory activity of the protein (Fig. 2).

Table 2 shows the different reactive sites which have been identified by these methods in the plant inhibitors. Generally these sites conform to the tentative rules drawn up by Osawa & Laskowski (1966) and Laskowski & Sealock (1971) with the most obvious conclusion that the reactive site peptide bond is present as a 'cyclic substrate' in a ring closed by a disulphide bridge (or disulphide loop).

Evidence obtained from X-ray crystallographic studies suggests that the proteinases and their inhibitors have complementary structures and associate in their complexes as rigid molecules. In the case of inhibition of the serine proteinases

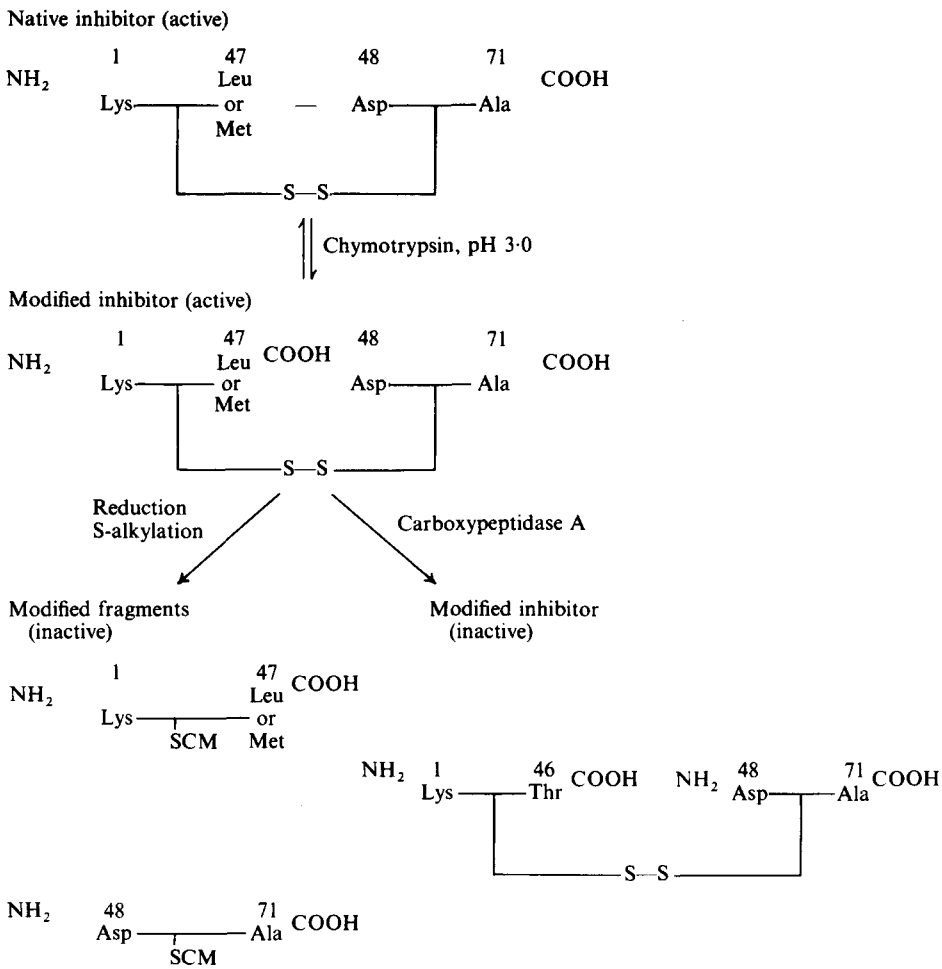

Fig. 2. Chemical events occurring during conversion of native potato chymotryptic inhibitor I to modified form by catalytic amounts of chymotrypsin at pH 3·0. (After Richardson *et al.*, 1977.)

(trypsin and chymotrypsin) it seems that the structure at the active site in the enzyme:inhibitor complex is that of a tetrahedral adduct in which the carbonyl carbon of the active site amino acid (Lys or Arg) is involved in a covalent linkage with the O^{γ} oxygen of the catalytic serine in the enzyme molecule (Fritz *et al.*, 1974; Janin & Chothia, 1976). The high resolution (2·6 Å) three-dimensional map prepared for the soybean (Kunitz) inhibitor: trypsin complex from X-ray data shows that only 12 out of the 181 amino acids in the inhibitor actually make contact with the enzyme (Sweet *et al.*, 1974). These 12 of course include the reactive peptide bond Arg^{63}–Ile^{64}. Similar studies have been carried out on the small potato inhibitor of

TABLE 2
THE REACTIVE (INHIBITORY) SITES OF PROTEINASE INHIBITORS FROM PLANTS

Enzyme inhibited	*Reactive site residues*	*Species/inhibitor*	*Ref.*
Trypsin	Arg–Ala	Wheat, rye	Richardson (1977)[a]
	Arg–Ile	Soybean (Kunitz)	Richardson (1977)[a]
	Arg–Leu	Maize	Richardson (1977)[a]
	Arg–Ser	Garden bean	Richardson (1977)[a]
		Vicia angustifolia var. *segetalis*	Abe *et al.* (1978)
	Arg–Asn	Aubergine	Richardson (1979)
	Lys–Ser	Soybean (Bowman-Birk)	Richardson (1977)[a]
		Chickpea	Richardson (1977)[a]
		Lima bean	Richardson (1977)[a]
		Potato (IIa)	Richardson (1977)[a]
		Phaseolus vulgaris var. *nanus*	Richardson (1977)[a]
		Adzuki bean	Yoshikawa & Ogura (1978)
		Ph. coccineus	Hory & Weder (1976)
		Macrotyloma axillare (DE 3 and DE 4)	Joubert *et al.* (1979)
Chymotrypsin	Leu–Ser	Lima bean (Var. I)	Richardson (1977)[a]
		Soybean (Bowman-Birk)	Richardson (1977)[a]
		Ph. coccineus	Hory & Weder (1976)
		Ph. vulgaris. var. *nanus*	Richardson (1977)[a]
	Leu–Val	Potato (Inhibitor pI 7·3)	Valueva *et al.* (1977)
	Phe–Ser	Lima bean (Var. IV)	Richardson (1977)[a]
		Macrotyloma axillare (DE 3)	Joubert *et al.* (1979)
	Tyr–Ser	Adzuki bean	Yoshikawa & Ogura (1978)
	Lys–Ser	Potato IIa/IIb	Richardson (1977)[a]
	Arg–Ile	Soybean (Kunitz)	Richardson (1977)[a]
	Leu–Asp	Potato (Inhibitor I)	Richardson (1977)[a]; Richardson *et al.* (1977)
	Met–Asp	Potato (Inhibitor I)	Richardson *et al.* (1977)
Elastase	Ala–Ser	Garden bean	Richardson (1977)[a]
		Soybean (Inhibitor CII)	Odani & Ikenaka (1977)
Nagarse	Lys–Ser	Potato IIa/IIb	Richardson (1977)[a]

[a] See references therein.

the carboxypeptidases A and B. As might be expected, in this case the carboxyl terminus of the inhibitor including the residues Tyr^{37} and Gly^{39} was shown to be the region of contact when the inhibitor fitted like a cap over the shallow depression at the active sites of the enzymes (Hass *et al.*, 1976; Ako *et al.*, 1976).

B. α-Amylase inhibitors

The plant protein inhibitors of α-amylase have molecular weights in the range 12,000–60,000 but as in the case of the proteinase inhibitors many of the larger α-amylase inhibitors dissociate in the presence of urea, guanidine HCl or sodium dodecyl sulphate into sub-units with molecular weights close to 12,000. Another similarity with the proteinase inhibitors is the very high degree of heterogeneity

shown by the α-amylase inhibitors. For example, the inhibitory proteins from the wheat kernel which make up some 65% of the whole albumin fraction have been divided into three main families on the basis of their different molecular weights, but within each family there are several active isoinhibitors that differ slightly in their electrophoretic mobilities (Buonocore *et al.*, 1977). The inhibitors from the cereals appear to be simple proteins like the proteinase inhibitors with at most a single reducing sugar per mole, but the α-amylase inhibitors isolated from various beans (Marshall & Lauda, 1975; Pick & Wöber, 1978) are glycoproteins with as much as 15% of carbohydrate in their structures.

Little is known about the structure of the plant inhibitors of α-amylase. As yet we only have details of the amino acid sequences of short fragments of two of the wheat isoinhibitors (Redman, 1976; Petrucci *et al.*, 1978) and the complete sequence of the 0·30 α-amylase inhibitor from the wheat variety 'Flanders' (Richardson & Kashlan, 1980). As a consequence, little is also known about the structural features of the α-amylase inhibitors which are essential for their action. Most of the inhibitors are inactivated by reduction with mercaptoethanol and subsequent carboxymethylation, but the degree of inactivation brought about by treatment of the proteins with various proteases or cyanogen bromide varies depending on the inhibitor treated and the source of the α-amylase used (Buonocore *et al.*, 1977). Silano *et al.* (1977) and Buonocore *et al.* (1980) have described a theoretical model to account for the observed interactions of the various wheat isoinhibitors with α-amylase.

C. General

A feature common to many but not all of the proteinase and α-amylase inhibitors is their surprising resistance to denaturation by heat, a property which has frequently been exploited during their purification in the laboratory. For example, several isolation procedures include a preliminary step in which the plant extract or homogenised suspension is heated at 80–100 °C for approximately 10 min during which period various contaminating proteins become precipitated and can be removed by filtration or centrifugation. The inhibitors themselves are left essentially unaltered by this process. This peculiar resistance to heat has been attributed to a tightly coiled conformation imposed by the large number of disulphide bonds found in many of these inhibitors, but this is unlikely to be the explanation for this property in the chymotryptic inhibitor I from potatoes which has only one disulphide bond in a polypeptide chain of 71 amino acid residues (Richardson & Cossins, 1975). The varying susceptibility of the inhibitors to heat, such as that experienced during cooking procedures is obviously an important factor in any consideration of the possible role of these proteins in nutrition and will be discussed in a later section.

The biochemical properties of the inhibitors are such that the technique of affinity chromatography has been a particularly useful tool in their purification (Mosolov *et al.*, 1979). One of the attractions of this method is that crude or only partially purified extracts of plant material at near neutral pH values can be applied to

columns containing the enzyme (proteinase or α-amylase) bound to an insoluble resin or polymerised dextran (such as Sepharose). As the extract passes through the column the inhibitors form stable complexes with the immobilised enzyme and are retained on the column during subsequent washing which removes most of the other impurities. The inhibitors can then be released from their complexes by lowering the pH and increasing the ionic concentration of the eluting buffer. This rapid and highly specific method is potentially very useful for purifying the inhibitors of α-amylase and other enzymes (Sorenson & Scandalios, 1975), but when dealing with the proteinase inhibitors suffers from the drawback that partial proteolysis of the reactive site of the inhibitor may occur, leading to the formation of modified forms of the inhibitor (see Fig. 2). On the other hand, there is little doubt that the immobilised proteinase inhibitors covalently attached to inert supports will continue to be widely used in affinity chromatography as a method for the purification of specific proteases (Reeck *et al.*, 1971; Otsuka & Price, 1974; Ager & Hass, 1977) and for the removal of contaminating protease activity from other enzyme preparations (Amneus *et al.*, 1976; Bartling & Barker, 1976). Similarly the α-amylase inhibitors have been used for the purification of several α-amylases by affinity chromatography (Buonocore *et al.*, 1975).

3. NUTRITIONAL SIGNIFICANCE

The exact nutritional significance of the plant enzyme inhibitors in the diets of animals and man is difficult to assess. The first implication of these proteins as the cause of certain nutritional disorders came when growth inhibition was observed in animals fed with plant products known to contain high levels of proteinase inhibitors and/or α-amylase inhibitors. Later feeding experiments in which rats and chickens were supplied with raw soybeans or with supplements of the partially purified trypsin inhibitors resulted in marked pancreatic hypertrophy and excessive enzyme secretion. It was also noticed that the inhibitors appeared to cause a metabolic disturbance in the utilisation of the sulphur amino acids methionine and cysteine. Some of the suggested explanations of these disorders are illustrated in Fig. 3.

Much of the discussion on the significance of the plant proteinase inhibitors in human nutrition has been based on experiments in which bovine, ovine or porcine proteinases were used instead of human proteinases because of their ready commercial availability. The relevance of such data is not always immediately apparent; indeed some workers have reported that certain proteinase inhibitors had little if any effect on human proteinases (Feeney *et al.*, 1969) and therefore that the plant inhibitors were of little relevance in human nutrition. However, recent work has shown that the purified inhibitors as well as crude extracts of raw soybeans do completely inhibit *in vitro* human trypsin and chymotrypsin activity (Krogdahl &

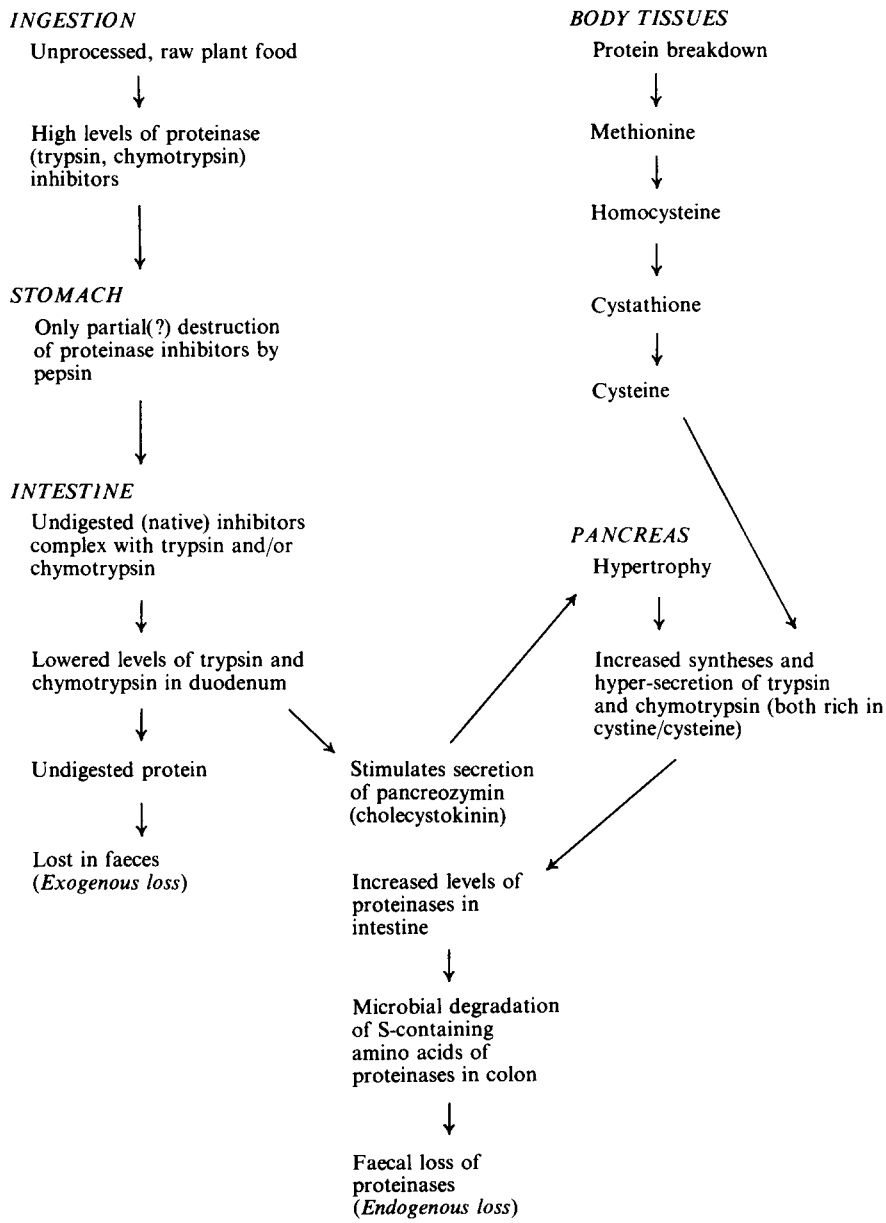

Fig. 3. Scheme to explain the possible deleterious effects of proteinase inhibitors on the nutritive value of proteins.

Holm, 1979). Similar studies on a range of other proteinase inhibitors from a variety of plant sources would clearly be very valuable.

There is also a need for detailed studies on the ability of the plant inhibitors to survive in an active form during passage through the stomach. The likelihood of a particular inhibitor causing adverse physiological effects in the intestine depends to a considerable extent on its stability under acid (pH 2–3) conditions, and its resistance to digestion by pepsin. Many inhibitors are known to be very stable under these conditions but a few are quickly destroyed (see references listed in Richardson, 1977). Recent studies with the soybean Bowman-Birk trypsin inhibitor indicate that most of this inhibitor is degraded during its passage through the stomach and intestine of chicks, and that there is negligible absorption of the native inhibitor, with most of the degradation products being excreted in the faeces (Madar *et al.*, 1979).

It is known that various treatments of plant foods (such as the germination of leguminous seeds, fermentation and controlled heating) can lead to an improvement in their nutritive value (Liener & Kakade, 1969), which is often presumed to result from a reduction in the level of the inhibitors. It should be remembered, however, that these processes do not always necessarily lead to a complete destruction of the inhibitors. On the contrary, Palmer *et al.* (1973) have pointed out that the concentration of trypsin inhibitors in one species, the kidney bean, actually increases during germination whilst at the same time there is a substantial improvement in their nutritive value. Moreover, the proteinase inhibitors from different sources vary considerably in their thermal stability, and the extent to which they are destroyed by heat *in vivo* is a function of several variables such as particle size and moisture content. For example, the inhibitors in chick peas retained all of their activity after being heated at 80 °C for 5 min and were only 50 % inactivated by boiling at 100 °C for 5 min or roasting at 130 °C for 1 min (Belew *et al.*, 1975). Similarly, the trypsin inhibitors in Faba beans (*Vicia faba* var. *minor*) still retained 20 % of their original activity after heating in a boiling water bath for 60 min (Bhatty, 1975). On the other hand, it has been shown that the various proteinase inhibitors in potatoes, many of which are very heat stable in the purified state and cause severe growth depression when fed to chicks, are rapidly denatured in the intact tuber during cooking and then provide a valuable source of cysteine-S (Pearce *et al.*, 1979).

In some cases the excessive amount of heating required to destroy the inhibitors can lead to a decline in the overall biological value of the food. The same is true for some other methods of processing. For example, treatment of raw soybean meal with 1 % formaldehyde inactivated 99 % of the trypsin-inhibitory and 97 % of the chymotrypsin-inhibitory activities, reduced pancreatic hypertrophy and restored the enzymic levels in the pancreas and intestine of chicks to that of a heated soybean diet, but these positive effects of formaldehyde were counteracted by its adverse effect on protein utilisation leading to a reduction in body weight gain (Nitsan & Bruckental, 1977).

The inhibitors are still present on the surface of insolubilised soy proteins (Kakade *et al.*, 1974) and although they are further inactivated during the process of converting the material into fibre it has been necessary to have a semi-automated method of determining the residual levels of trypsin inhibitor as a production control parameter in the manufacture of textured soyproteins (Egberg *et al.*, 1975).

There is little doubt that suitable pretreatment of otherwise toxic plant products can greatly improve their nutritional quality. Specially prepared soybean infant formulas did not give rise to pancreatic hypertrophy or hyperplasia in the rat and were equal to controls in their calorific and protein utilisation values (Churella *et al.*, 1976). Similarly, Rackis *et al.* (1979) have recently shown that various processed forms of soy flour used as a sole source of dietary protein in long-term feeding trials did not enlarge the pancreas despite retaining nearly 50 % of their original content of proteinase inhibitors.

These findings and other results have suggested that part of the nutritional improvement produced in some plant foods by germination, heating or other processing may be due to the elimination of growth inhibitors other than the proteinase inhibitors. There is now good evidence that the soybeans and other legumes contain several other antinutritional factors such as the phytohaemaglutinins, goitrogens, cyanogenic glycosides, antivitamin factors, metal-binding constituents, lathyrogens and substances responsible for causing favism (Liener, 1974). In this connection, Abbey *et al.* (1979*a*,*b*) have recently attempted to determine the exact nutritional and physiological significance of the proteinase inhibitors from field beans (*Vicia faba*) by incorporating the partially purified inhibitors into diets devoid of other legume proteins. The inhibitors were found to depress growth and cause pancreatic hypertrophy in weanling rats but only when fed in amounts which were up to five times the normal endogenous levels found in field beans. They concluded that the proteinase inhibitors alone could not be responsible for the harmful effects of raw field bean meal, but possibly acted synergistically with other growth inhibitory factors.

The role of the plant α-amylase inhibitors in nutrition is also poorly understood. It is known that the protein α-amylase inhibitors may represent as much as 1 % of wheat flour (Deponte *et al.*, 1976) and that because of their relatively high thermostability they can survive in an active form during the baking processes involved in the manufacture of bread and breakfast cereals (Marshall, 1975; Buonocore *et al.*, 1977). Addition of these wheat inhibitors to raw starch-enriched diets of rats by Lang *et al.* (1974) led to a large decrease in starch availability, but other workers (Puls & Keup, 1973) found that the inhibitors had much less effect on the digestion of cooked starch, possibly because of a higher affinity of α-amylase for gelatinised starch as compared to raw starch. It has also been shown that the native α-amylase inhibitors isolated from wheat albumin had no effect on the growth rates of young chickens, but when the purified inhibitors were enclosed in cellulose-coated microgranules resistant to the digestive action of pepsin in the chicken gizzard they

significantly depressed the growth rate, caused pancreatic hypertrophy and increased the production of pancreatic α-amylase (Macri *et al.*, 1977). These results which point to the possible differential susceptibility of the inhibitors to peptic digestion depending on whether they are fed as an integral component of solid food or as a purified (crystalline) supplement to a diet may help to explain the apparently contradictory results of other workers. For example, another α-amylase inhibitor, phaseolamin, was originally discovered in uncooked red kidney beans as the factor responsible for impaired starch digestion (Jaffe & Lette, 1968), but subsequent work by Savaiano *et al.* (1977) has shown that the addition of this purified inhibitor to various diets did not affect the availability of starch or alter the growth rates of weanling rats.

In contrast, other workers have investigated the possible benefits of the deliberate therapeutic inclusion of α-amylase inhibitors in the diets of patients suffering from diabetes mellitus, obesity, hyperlipoproteinaemia and related diseases. Puls & Keup (1973) have shown that post-prandial hyperglycaemia and hyperinsulinaemia in human volunteers, rats and dogs resulting from loading with raw starch could be progressively reduced by the addition of amylase inhibitor preparations to the food intake. The desirability of such oral administration of the inhibitors over a continuous period has, however, been questioned in view of their known pathological effects on the pancreas (Buonocore *et al.*, 1977).

It has been reported (Strumeyer, 1972) that α-amylase inhibitors occur in the gliadin (proteins soluble in 70% ethanol) fraction of wheat kernel, leading to the speculation that they might be responsible for the sensitivity to wheat flour observed in patients suffering from coeliac disease (gluten-induced enteropathy). One of the symptoms in such patients is an impaired ability to metabolise starch resulting from a lowered production of α-amylase by the pancreas, but there is by no means general agreement that this is the primary lesion in coeliac disease. Moreover, a large number of pure gliadin fractions have since been tested for antiamylase activity by Pace *et al.* (1978) who were unable to find any inhibition. These workers also showed that α-amylase inhibitors normally occurring in the albumin fraction of wheat can be extracted from flour or commercial gluten with solvents (70% ethanol, dilute acid) that were considered typical for wheat gliadins. Also, the wheat albumin α-amylase inhibitors which are very active towards mammalian enzymes caused no reaction in coeliac disease when tested by Auricchio *et al.* (1974).

4. PHYSIOLOGICAL ROLE IN THE PLANT

The relatively high levels of the enzyme inhibitors in many plants clearly pose an interesting question as to their physiological purpose. One suggestion is that the proteinase inhibitors which occur particularly in storage tissues such as seeds and tubers might function as depot or safe storage forms of protein which are immune to

digestion until required during germination or sprouting. Using specifically labelled antibodies, Shumway *et al.* (1976) have shown that the chymotryptic inhibitor I forms a significant component of the vacuolar storage protein bodies in many members of the Solanaceae. There is also evidence that the proteinase inhibitors of several legume seeds are associated with the same storage organelles (Pusztai *et al.*, 1977; Miege *et al.*, 1976). The level of the inhibitor found in potato tubers declines during sprouting and there is a corresponding accumulation of related proteins in the young growing sprouts. This continues until the mature plants begin to set tubers, at which time the inhibitor disappears from the vegetative tissues and starts to build up in the new tubers (Ryan, 1973). Similarly, the amount of inhibitor in old callus tissues decreases rapidly when the cultures are transferred to a new medium, suggesting that there is a degradation and utilisation of the inhibitor protein for the establishment of new callus growth (Wong *et al.*, 1975). The picture of the inhibitors having a storage role in seeds, however, is confused by the fact that although the levels of the inhibitors do decline (Richardson, 1977) or disappear completely (Ikeda & Kusano, 1978) during germination in some species, in others there is an actual increase in inhibitor content (Richardson, 1977; Palmer *et al.*, 1973).

Another possible function for the proteinase- and α-amylase inhibitors in dormant seeds and tubers might be to control the breakdown of the stored reserves of protein and starch. Certainly quite a number of plant proteinases are inhibited by the inhibitors found in the same tissue (Richardson, 1977; Xavier & Ainouz, 1977) but some are not (Richardson, 1977; Santarius & Belitz, 1978). In this connection, Baumgartner & Chrispeels (1976) came to the conclusion that the specific inhibitor of the major endopeptidase in mung bean cotyledons had little or no role in regulating normal protein turnover, but was more likely to protect the cytoplasm from the effects of an accidental rupture of the proteinase containing protein bodies in this species. In any event it is unlikely that enzyme/inhibitor interactions are the only controlling factor in the regulation of proteolysis since we know that during germination some of the important proteases are formed *de novo* and some control is likely to be exerted by the proteases and their substrates being suitably separated in different cellular compartments. The same is probably true also for the control of starch breakdown in wheat where although there is an inverse relationship between the levels of α-amylase and α-amylase inhibitor activity throughout the period of seed maturation, at no stage of kernel development did the extracted inhibitors actually inhibit the enzyme (Pace *et al.*, 1978). Similarly, the amylase inhibitor in beans had no effect on the endogenous enzyme (Marshall & Lauda, 1975; Pick & Wöber, 1978). On the other hand, there is now some experimental evidence to suggest that the inhibitors of invertase and phospho inositol kinase found in various plant tissues may have a direct role in the regulation of their endogenous enzymes (Matsushita & Uritani, 1976; Majumder & Biswas, 1973).

There seems to be increasing agreement that the most likely major role of the proteinase and α-amylase inhibitors in plants is one of protection against the attacks

of animals, insects and microorganisms. Perhaps the most obvious example of this is in the endozoic dispersal of seeds. Birds and other animals which eat fruits or whole plants are known to subsequently excrete some seeds in a viable condition (e.g. tomatoes growing in sewage works). The presence of proteinase- and α-amylase enzyme inhibitors in relatively high concentrations in so many seeds must increase the chances of their passing through the animals alimentary canal without being damaged by the digestive system.

Several of the plant enzyme inhibitors are very effective in blocking the action of the proteases and amylases found in the guts of the larval forms of insects such as *Tribolium* and *Tenebrio* which are pests of stored grain and seeds (see references in Richardson, 1977). The presence of the inhibitors can be expected to make the plants and seeds less palatable, perhaps lethal to the insects and thus confer some selective advantage to the plant. The hypothesis is supported by the work of Silano *et al.* (1975) who found that most of the insects that attack wheat grain and flour seem to have very high amylase activities and amylases that are inhibited by the wheat inhibitors whereas insect species that do not normally feed on wheat had relatively low amylase activities and amylases that were resistant to inhibition by the wheat proteins. Other recent work has shown that the trypsin inhibitor in cowpea seeds (*Vigna unguiculata*) which is toxic to the larvae of the bruchid beetle *Callosobruchus maculatus* was present in about twice the concentration in seeds of a variety resistant to the attacks of the pest, compared to the highest level found in susceptible varieties (Baker, 1978). Moreover, addition of the inhibitor up to the level of the resistant variety resulted in a susceptible variety becoming resistant. Similar feeding experiments have demonstrated the toxicity of soybean trypsin inhibitor to larvae of the corn-borer *Ostrinia nubilalis* (Steffens *et al.*, 1978). There also appears to be a correlation between the levels of proteinase inhibitors in different varieties of barley and the severity of damage by grass-hoppers (Weiel & Hapner, 1976).

Perhaps the most persuasive argument for the inhibitors having a protective role was the demonstration by Green & Ryan (1972) that leaves of solanaceous plants infested with adult Colorado beetles or their larvae accumulated much greater amounts of proteinase inhibitor than uninfected control plants. This response to insect damage could be simulated mechanically by wounding or crushing the leaves with tools. Other results suggested that a chemical signal or hormone, now known as PIIF (proteinase inhibitor inducing factor), was released at the site of wounding and was capable of being translocated out of that damaged tissue to the rest of the plant where it induced accumulation of inhibitor. Ryan (1973) has described this system as a primitive immune response contributing to the overall defence of the plant and suggested that the discovery of mutant varieties of plants with enhanced or reduced levels of proteinase inhibitors might be very valuable in studies on this form of biological pest control.

A related discovery of great interest was the finding by Peng & Black (1976) that the proteinase inhibitor levels in tomato plants increased following infection with

the pathogenic fungus *Phytophthora infestans*, but this host response only occurred in varieties of tomato that were resistant to the pathogen and not in susceptible varieties. It is not known whether this increased synthesis of proteinase inhibitor by the tomato host plant is merely part of a general wound reaction or a specific response to the presence of a proteinase from the fungal pathogen. However, recent work has shown that the proteases released from the phytopathogenic fungus *Fusarium solani* are inhibited by a number of plant proteinase inhibitors (Mosolov *et al.*, 1976) and highly specific proteins occur in kidney beans that inhibit a protease of *Colletotrichum lindemuthianum* (Mosolov *et al.*, 1979) and also the cell wall degrading enzyme endopolygalacturonase (Fisher *et al.*, 1973), which is believed to be of key importance in the pathogenesis caused by this fungus.

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Food Chemistry 6 (1980–81) 255–261

THE PRODUCTION OF LYSINOALANINE AND RELATED SUBSTANCES DURING PROCESSING OF PROTEINS

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ABSTRACT

Processing foods may cause unintended changes in their constituent proteins. Bovine milk protein studies have assisted in understanding the formation of lysinoalanine, which has toxic properties, and some phosphoproteins.

Protein lysyl residues may enter the Maillard reaction with degradation products of carbohydrates, but losses of lysine can occur in the absence of free carbohydrate. Other alanines have also been detected. The study of bovine milk casein complex, via its four components, has yielded additional understanding of lysinoalanine formation, and has indicated that it corresponded to the lysine loss, but not to the serine loss.

The studies report the effect of gentle heat and alkali at neutral pH, but greater heat at the same pH has yielded lysinoalanine. Cyanate, formed from milk urea, may lead to homocitrulline formation.

The conversion of native proteins into processed food forms usually involves subjecting the proteins to physical conditions markedly different from those of their natural environment. A process may, for example, include one or more heat treatments at pH values significantly removed from that of neutrality for the purpose of modifying the structures of the proteins sufficiently to endow them with desirable technological properties. However, it is being recognised increasingly that such treatment may promote many modifying reactions so that its use may cause changes to be produced in the chemical, physical and nutritional properties of food proteins in addition to those intended. It is important therefore to consider in some detail the chemistry of these reactions and of their products. The occurrence in a wide range of heat-treated proteins of one such product, lysinoalanine, has recently been reviewed (Gross, 1977; Friedman, 1977) although a detailed description of the

Food Chemistry 0308-8146/81/0006-0255/$02·50

reactions leading to its formation has necessarily been limited by a lack of knowledge of the primary structures of the proteins themselves. The state of knowledge of the chemistry of bovine milk proteins has, however, provided an opportunity for the study of the formation of lysinoalanine in proteins and phosphoproteins of known primary structures and it is with some of the findings from this study that this communication is mainly concerned.

It has long been accepted that lysyl residues of proteins are susceptible to reaction with degradation products of carbohydrates during storage and processing by way of the Maillard reaction and that significant amounts of lysine may be lost in this way. More recently it has been shown by Bohak (1964) that with certain proteins the presence of free carbohydrate was not necessary for losses of lysine to occur during treatment with alkali. Thus the lysine content of ribonuclease was reduced by such treatment while that of pepsin was not affected. The product of this reaction was stable to the conditions normally employed for acid hydrolysis of proteins to their constituent amino acids and it was characterised as N^6-(DL-2-amino-2-carboxyethyl)-L-lysine or lysinoalanine. It was deduced that its formation was due to nucleophilic addition of a lysyl residue to a dehydroalanyl residue which had itself been generated in the alkaline conditions by a β-elimination reaction from a cystyl residue. It was further suggested that this reaction was favoured by close proximity of the lysyl and cystyl residues but not necessarily excluded if the distance between them was apparently great. Lysinoalanine was also obtained from the phosphoprotein, phosvitin, which contains no cystine and from the protein–polysaccharide complex of cartilage in a quantity in excess of that which could be accounted for by its cystine content (Whiting, 1971). It is apparent, as is illustrated in Fig. 1, that the intermediate dehydroalanyl residue necessary for the formation of lysinoalanine may be produced by a phosphoseryl, glycosylated seryl or cystyl residue.

Lysinoalanine is not the only product which may result from addition of protein components to dehydroalanyl residues in alkaline conditions. Lanthionine which has been detected in alkali-treated wool has been reported to be formed by addition of cysteine and the same authors reported that ammonia, which was probably

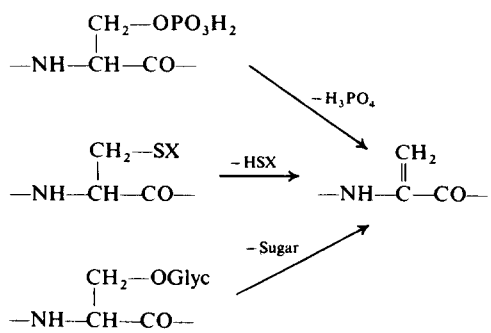

Fig. 1.

derived from amide-containing amino acid residues, reacted to yield β-aminoalanine although in low yield only (Asquith *et al.*, 1969). Under alkaline conditions arginyl residues are known to be converted to ornithinyl residues and the presence of ornithinoalanine observed originally in alkali-treated wool and more recently in similarly treated sunflower protein has been ascribed to reaction of ornithinyl and dehydroalanyl residues (Provansal *et al.*, 1975). These reactions are summarised in Fig. 2. In addition it should be remembered that β-elimination from substituted threonyl residues may give rise to β-methyldehydroalanyl residues which in turn may be expected to yield a series of methylated derivatives by reactions analogous to those contained in Fig. 2.

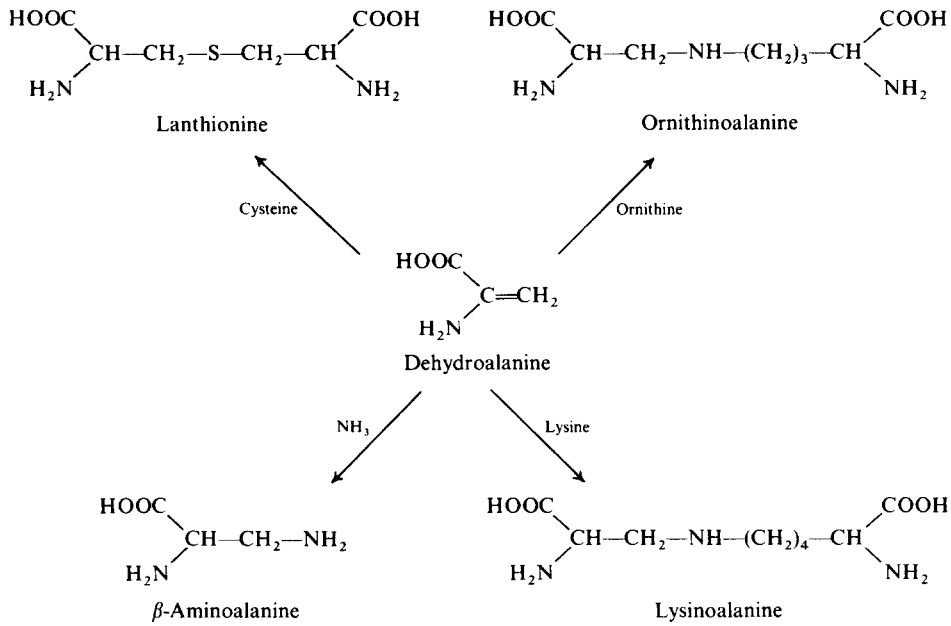

Fig. 2.

Lysinoalanine has attracted attention following the discovery, in experiments with rats, of its toxic properties (Woodard & Short, 1973). It is noteworthy that its presence has been detected in a wide variety of foods, some of which had not been subjected to treatment with alkali but to heat only as, for example, occurs in the preparation of evaporated milk (Sternberg *et al.*, 1975). Thus, while increase in temperature and in pH may both be regarded as promoting the formation of lysinoalanine and analogous products, the application of either by itself may be sufficient to ensure the production of detectable amounts of these compounds.

Further information on the formation of lysinoalanine has been obtained from studies on the behaviour of individual members of the casein complex of bovine milk

when subjected to heat in alkaline solution (Lorient, 1979; Manson & Carolan, 1980). The results have some significance from a nutritional viewpoint also since bovine casein is presently the source of about one-quarter of the protein requirement of the population of the United Kingdom and all of this is now processed to some degree before consumption.

The casein complex of bovine milk contains four components each of whose primary structure has been fully elucidated. They are α_{s1}-casein (Mercier *et al.*, 1971; Grosclaude *et al.*, 1973), α_{s2}-casein (Brignon *et al.*, 1977), β-casein (Ribadeau Dumas *et al.*, 1972), and κ-casein (Mercier *et al.*, 1973). They each have distinctive amino acid compositions and all contain phosphate in the form of monoesters of orthophosphoric acid with seryl residues. The number of phosphoseryl residues present per mole of α_{s1}, α_{s2}, β and κ-casein is 8, 10–13, 5 and 1 respectively, and a form of α_{s1}-casein containing 9 phosphoseryl residues and designated α_{s0}-casein is also known (Manson *et al.*, 1977). Only one extended sequence of amino acid residues, —Ser(P)—Ile/Leu—Ser(P)—Ser(P)—Ser(P)—Glu—Glu—, is common to α_{s1} and β-casein although part of this, —Ser(P)—Ser(P)—Ser(P)—Glu—Glu—, occurs twice in α_{s2}-casein. Of the four caseins, only α_{s2} and κ-casein contain cysteine and κ-casein, since it contains variable amounts of carbohydrate linked O-glycosidically with seryl residues, may also be considered to be a glycoprotein. The individual caseins contain among them all the structural groups which can give rise to dehydroalanyl residues (Fig. 1).

When α_{s0} and α_{s1}-caseins were treated with 0·2 N sodium hydroxide at 40 °C for 4 h all of their constituent phosphate was liberated. A comparison of the amino acid compositions of the treated and untreated proteins which is summarised in Table 1 indicated that only lysine, serine and lysinoalanine values had been affected by this treatment. In both proteins the amount of lysinoalanine formed corresponded closely to the amount of lysine lost but differed markedly from the amount of serine lost. Clearly not all of the lost serine had been converted to lysinoalanine. In addition, the yield of lysinoalanine from α_{s0}-casein was significantly greater than that from α_{s1}-casein. Structural studies (Manson *et al.*, 1977) have shown that α_{s0}-casein differs from α_{s1}-casein only in respect of one additional phosphoseryl residue which is located immediately adjacent to a lysyl residue. These results have been

TABLE 1

NET CHANGES IN AMINO ACID COMPOSITION OF α_{s0} AND α_{s1}-CASEIN AFTER TREATMENT WITH 0·2 N NaOH AT 40 °C FOR 4 h EXPRESSED IN MOLES AMINO ACID PER MOLE PROTEIN

	α_{s0}-*Casein*		α_{s1}-*Casein*	
	Gain	*Loss*	*Gain*	*Loss*
Lysinoalanine	2·7		2·0	
Lysine		2·7		1·9
Serine		4·1		3·3

interpreted as showing that the sequence —Ser(P)—Lys— was converted much more efficiently to lysinoalanine than other phosphoseryl residues in α_{s0} and α_{s1}-casein (Manson & Carolan, 1980) and support the contention advanced by Bohak (1964) that proximity of lysyl and dehydroalanyl residues in a polypeptide chain promotes lysinoalanine formation.

When the behaviour of α_{s1}-casein was compared with that of β-casein under conditions similar to those described above it was again apparent that the amount of lysinoalanine present at a given time was balanced almost exactly by the lysine lost but that the destruction of serine was always greater than was apparently required (Table 2). Thus the destruction of all 8 phosphoseryl residues of α_{s1}-casein yielded 5

TABLE 2

NET CHANGES IN AMINO ACID COMPOSITION OF α_{s1} AND β-CASEINS WITH TIME, ON TREATMENT WITH 0·2 N NaOH AT 40 °C. THE RESULTS ARE EXPRESSED IN MOLES AMINO ACID PER MOLE PROTEIN

Time (h)	*Gain*	*Loss*	
	Lysinoalanine	*Lysine*	*Serine*
α_{s1}-Casein			
2	0·9	0·9	1·7
6	2·6	2·8	4·7
10	4·3	4·1	6·3
26	4·9	4·7	7·8
48	4·7	4·7	8·1
β-Casein			
2	0·3	0·3	0·9
6	1·4	1·6	3·0
10	1·9	1·9	3·9
26	2·3	2·1	5·1
48	2·1	2·2	5·3

equivalents of lysinoalanine while the 5 phosphoseryl residues of β-casein produced approximately 2 equivalents of lysinoalanine. In each protein the destruction of the equivalent of 3 phosphoseryl residues has apparently resulted in the production of no lysinoalanine. Further study of this reaction has been made using the phosphopeptide which constitutes the first 25 residues of β-casein (Manson & Annan, 1971). This contains 4 of the 5 phosphoseryl residues of β-casein but no lysyl residue. When it was subjected to alkaline treatment in the presence of α-*N*-acetyllysine the amount of lysinoalanine obtained when all 4 phosphoseryl residues had been destroyed was the equivalent of 0·75 residue, i.e. the destruction of 3·25 phosphoseryl residues had apparently yielded no lysinoalanine. The yield of lysinoalanine was not increased when α-*N*-acetyllysine was replaced by its methylamide, by the tripeptide Thr—Lys—Tyr, by a pentapeptide Pro—Gly—Lys —Ala—Arg or by increasing the reaction time. However, small amounts of ornithinoalanine, approximating to 0·1 molar equivalent, were detected whether a

lysyl compound was present or not. These were concluded to have arisen from either the N- or C-terminal arginyl residue by the pathway outlined in Fig. 2.

On the evidence of these results it was concluded that although dehydroalanyl residues are formed readily from all phosphoseryl residues of a polypeptide chain, those present in clusters as occurs in α_{s1}, β and α_{s2}-caseins do not react significantly with lysyl residues to form lysinoalanine. The fate of the —Ser(P)—Ser(P)—Ser(P)— fragments of these three proteins is not known but some evidence exists (Manson & Carolan, 1972) which suggests that the corresponding dehydroalanyl sequence is extremely susceptible to alkali and breaks up by way of reactions whose overall rate is much faster than that which leads to lysinoalanine formation.

While these studies have been concerned with the effect of gentle heat and alkali on phosphoproteins it is noteworthy that the application of stronger heat treatments at neutral pH has been reported by Lorient (1979) to result in the formation of significant amounts of lysinoalanine. The effect of heat at neutral pH also promoted in a somewhat non-specific fashion the hydrolysis of peptide bonds and in a recent study no fewer than 64 peptides were obtained from α_{s1}-casein by this treatment (Lorient *et al.*, 1977). Not all of these, however, possessed amino acid sequences which could be related to that known for α_{s1}-casein and these were concluded to be isopeptides formed through interaction of the γ-carboxyl group of glutamyl residues with the ε-amino group of lysyl residues. Thus, heat treatment of this type as well as promoting the fragmentation of the protein by destruction of peptide bonds also induced the production of cross-linked peptides by isopeptide bond formation.

In this communication no consideration has been given to reactions which may occur during processing between proteins and other food components. Many of these have already been described (Nursten, 1980–81) but one further reaction is probably worthy of mention in conclusion. Milk contains small amounts of urea and it has been shown that cyanate formed from urea readily attacks the ε-amino group of lysyl residues located in individual caseins with the formation of homocitrulline (Manson, 1962). The conditions necessary for the conversion of urea to cyanate are present in many of the processes used in food technology so that the presence of small amounts of homocitrulline in foodstuffs containing milk protein is to be expected.

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Food Chemistry 6 (1980-81) 263–277

RECENT DEVELOPMENTS IN STUDIES OF THE MAILLARD REACTION

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ABSTRACT

The Maillard reaction is central to food chemistry and has implications on several levels, sensory, nutritional, toxicological, and technological. Its complex network of steps is outlined and the chemistry of the production of colour and flavour is dealt with from the point of view of the nature of the components involved and of the mechanisms by which they may be formed. Control of the reaction is a paramount focus of interest, but, although recent work, using newer techniques, such as HPLC, electrophoresis, ^{13}C-NMR and ESR, has illumined some facets, the complexities even of model systems are still overwhelming.

INTRODUCTION

The Maillard reaction is central to food chemistry and its implications must be considered whenever a food or food processing is under study. Maillard (Strahlmann, 1978), in first submitting the interaction of reducing sugars and amino compounds to serious study, was very conscious of its ramifications.

Systematisation of the complex network of reactions that takes place is due to Hodge (1953), whose scheme is outlined in Fig. 1. It is remarkable that more than a quarter of a century later, it is still the most apt description of the Maillard reaction.

The following four points should be noted:

1. Sugars on their own, provided they are exposed to higher temperatures, will undergo similar reactions by themselves (caramelisation), but then there can be no interaction with amino compounds and no nitrogen-containing compounds can result.

Food Chemistry 0308-8146/81/0006-0263/$02·50

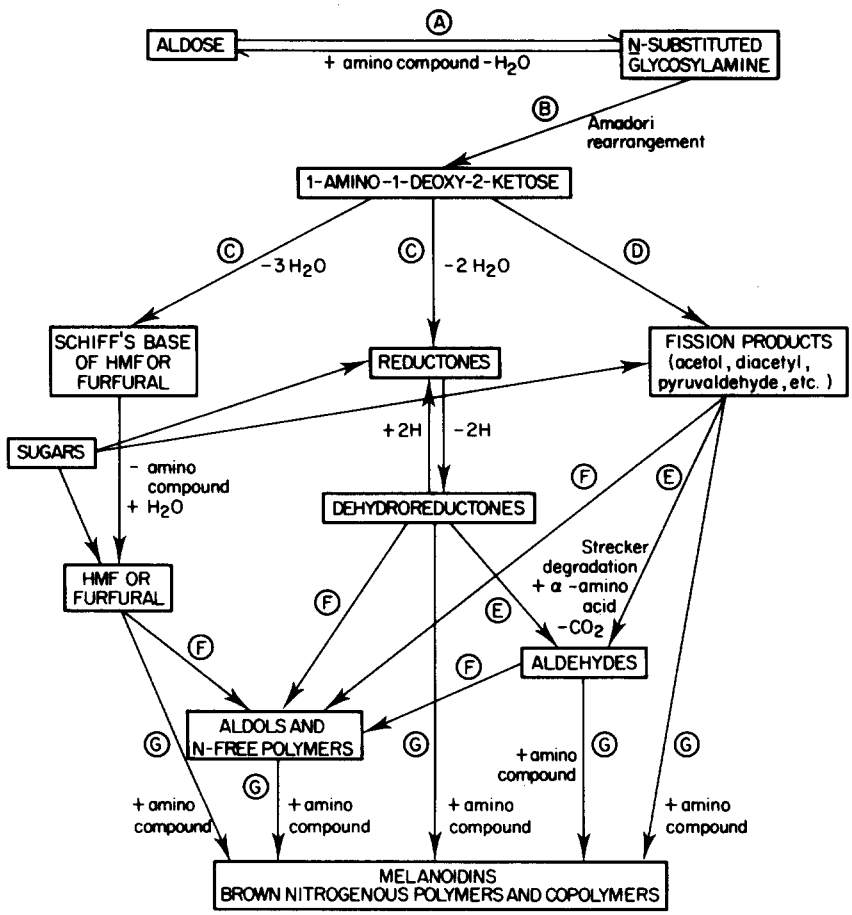


Fig. 1. Non-enzymic browning. Based on J. E. Hodge (1953).

2. The Maillard reaction (and caramelisation) is best divided into three stages. The initial one comprises glycosylamine formation (A) and subsequent rearrangement (B). The intermediate stage comprises dehydration (C), either by loss of three molecules of water to furfurals or by loss of two to reductones; fission (D), mainly by dealdolisation; and Strecker degradation (E), the interaction of amino acids and dicarbonyl compounds, which may be either dehydroreductones or dehydration or fission products. The final stage consists of the conversion of carbonyl compounds, be they furfurals, fission products, dehydroreductones, or Strecker aldehydes, into high molecular weight products, the melanoidins, with further involvement of amines where these are available.

3. Ketoses undergo similar reaction, reaction B then being termed the Heyns rearrangement.
4. Some of the reactions are reversible, notably the first step A and the dehydrogenation of reductones.

The significance of the Maillard reaction for food is at least fivefold:

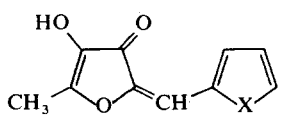
1. Production of colour. This may be desirable, as in coffee and bread crust, or undesirable, as in glucose syrup and in many intermediate moisture products.
2. Production of flavour or off-flavour. Odours and off-odours are due to volatile products, e.g. fission products and Strecker aldehydes. Substances tasting sweet or bitter may be involved, as well as effects on texture.
3. Reduction in nutritional value by involvement of ascorbic acid (a reductone) and of lysine (free or bound), an essential and often limiting amino acid. Metal-chelating properties may also be significant.
4. Toxicity through the possible formation of imidazoles and of *N*-nitroso derivatives, e.g. of Amadori compounds. The intrinsic toxicity of Maillard products and intermediates is also under study, but is made difficult by their anti-nutritional properties.
5. Antioxidant properties. These are thought to be due to the reductones formed, but chelation of heavy metals may also be involved.

Most can be said about the production of colour and of flavour and off-flavours, and the chemistry of these two topics will now be considered in more detail.

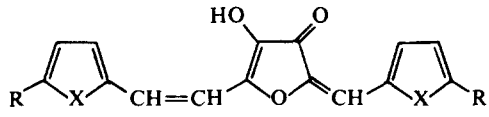
COLOUR

Considering that colour formation is the primary characteristic of the Maillard reaction, it is surprising how little is known of the structure of any chromophore present.

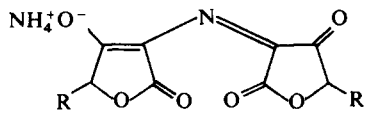
Severin and Krönig (1972) isolated the yellow compound 1, X = O, from mixtures produced by heating xylose with isopropylammonium acetate and found that 1, X = O, was also formed when xylose was replaced by arabinose or isopropylammonium acetate by amino acids, such as lysine or glycine. Ledl & Severin (1978) state that pentoses and methylammonium acetate lead to 1, X = NCH_3, yellow, in addition, and that xylose and glycine similarly give the orange compound 1, X = NCH_2COOH.



(1) R = H, CH_3 X = O, NCH_3, NCH_2COOH



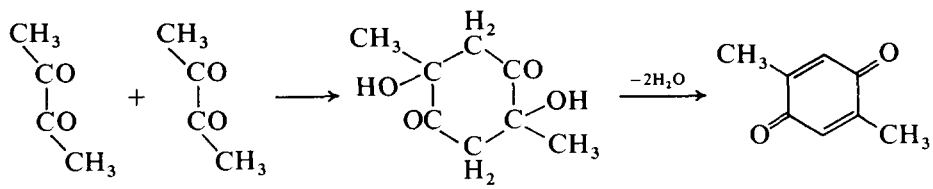
(2) R = H, CH_3; X = O, NCH_3



(3) R = —CHOH.CH_2OH

The latter authors also found that the methyl group of compounds such as 1 is sufficiently reactive to condense with carbonyls to form compounds such as 2 (R, X = H, O, deep orange; CH_3, O; or H, NCH_3).

Another possible route to a chromophore lies in the formation of quinones by double 'aldol' condensation and dehydration of 1,2-diones, e.g.



Amines readily add to quinones, oxidation then leading to coloured compounds.

The red pigment, obtained from the interaction of amino acids and dehydroascorbic acid and shown to have structure 3 by Kurata *et al.* (1973), provides yet another model chromophore for Maillard reaction products.

FLAVOUR AND OFF-FLAVOUR

Flavour aspects will be dealt with in two sections, (a) chemical nature, and (b) mechanisms of formation.

(*a*) *Chemical nature*

This part of the Maillard reaction is the least sketchily understood. Flavour tends to be dominated by odour and, to be odorous, a compound needs to be volatile, at least to some extent, even though the sensitivity of the nose is remarkable. Volatility implies relatively small molecular size and lack of polarity, both factors that tend to facilitate investigation.

Thanks mainly to combined gas chromatography–mass spectrometry, hundreds

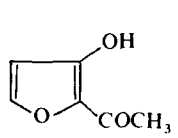
of volatile products of the Maillard reaction have been identified. They are best classified into three groups (Nursten, in press):

1. 'Simple' sugar dehydration/fragmentation products:
   - Furans
   - Pyrones
   - Cyclopentenes
   - Carbonyls
   - Acids
2. 'Simple' amino acid degradation products:
   - Aldehydes
   - Sulphur compounds
3. Volatiles produced by further interactions:
   - Pyrroles
   - Pyridines
   - Imidazoles
   - Pyrazines
   - Oxazoles
   - Thiazoles
   - Compounds ex aldol condensations

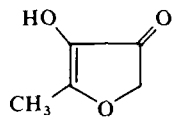
It is only the compounds in the last group, and then not all of them, which fall strictly within the purview of this symposium, but, in order to set them in proper perspective, the other two groups will first be considered briefly.

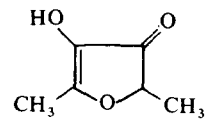
Specific compounds more significant from an odour point of view are given in Figs 2–4. Some comments on them follow below.

The compounds in Fig. 2, except the last, have odours described predominantly as caramel or burnt sugar. The last has long been known to be the character-impact compound of butter. These compounds play a role where one might expect, i.e. in

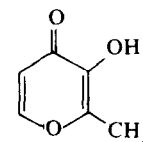


isomaltol

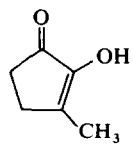




furaneol



maltol



methylcyclopentenolone

$CH_3COCOCH_3$
diacetyl

Fig. 2. Important 'simple' sugar dehydration/fragmentation volatiles.

the flavour of caramel, malt and bread, but also elsewhere, e.g. furaneol is an important constituent of essences from pineapples and strawberries and its lower homologue of beef broth.

As regards volatiles formed from amino acids (Fig. 3), the aldehydes occur widely in heated foods. Isovaleraldehyde has an odour reminiscent of malt and is indeed the volatile present in greatest amount in malt extracts, contributing substantially to the

$(CH_3)_2CHCH_2CHO$	H_2S
etc.	CH_3SH

Fig. 3. Important 'simple' amino acid degradation volatiles.

aroma. Hydrogen sulphide and methyl mercaptan, derived most likely from cysteine and methionine, respectively, contribute to the off-odours which develop in a number of foods.

It is not until consideration turns to the volatiles produced by further interactions (Fig. 4) that nitrogenous volatiles are encountered. Pyrrole rings were present in two of the coloured compounds, and many relatively simple substituted pyrroles have been identified to date. 2-Acetylpyridine and its tetrahydro derivative have cracker-like odours and the presence in bread of the former has been confirmed (Folkes & Gramshaw, in press). Imidazoles are probably too polar to contribute to odour, but much attention has been focused on the 4-methyl compound from a toxicological point of view. It is present particularly in caramel made by the ammonia process and WHO has recommended a limit of 200 mg/kg.

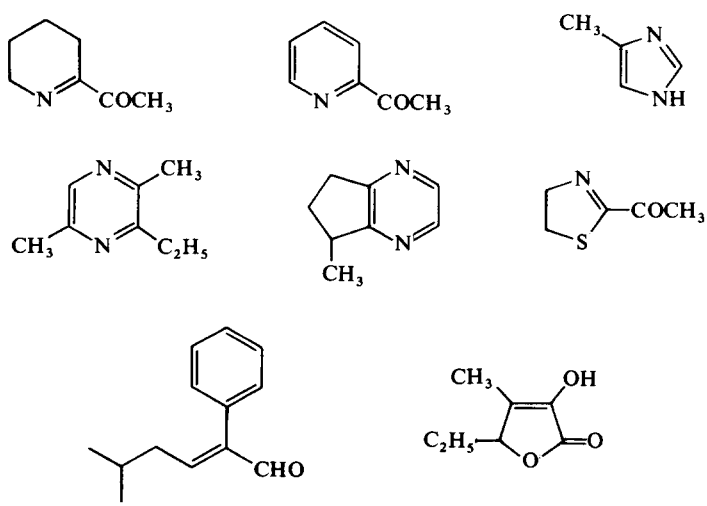


Fig. 4. Significant volatiles produced by further interactions.

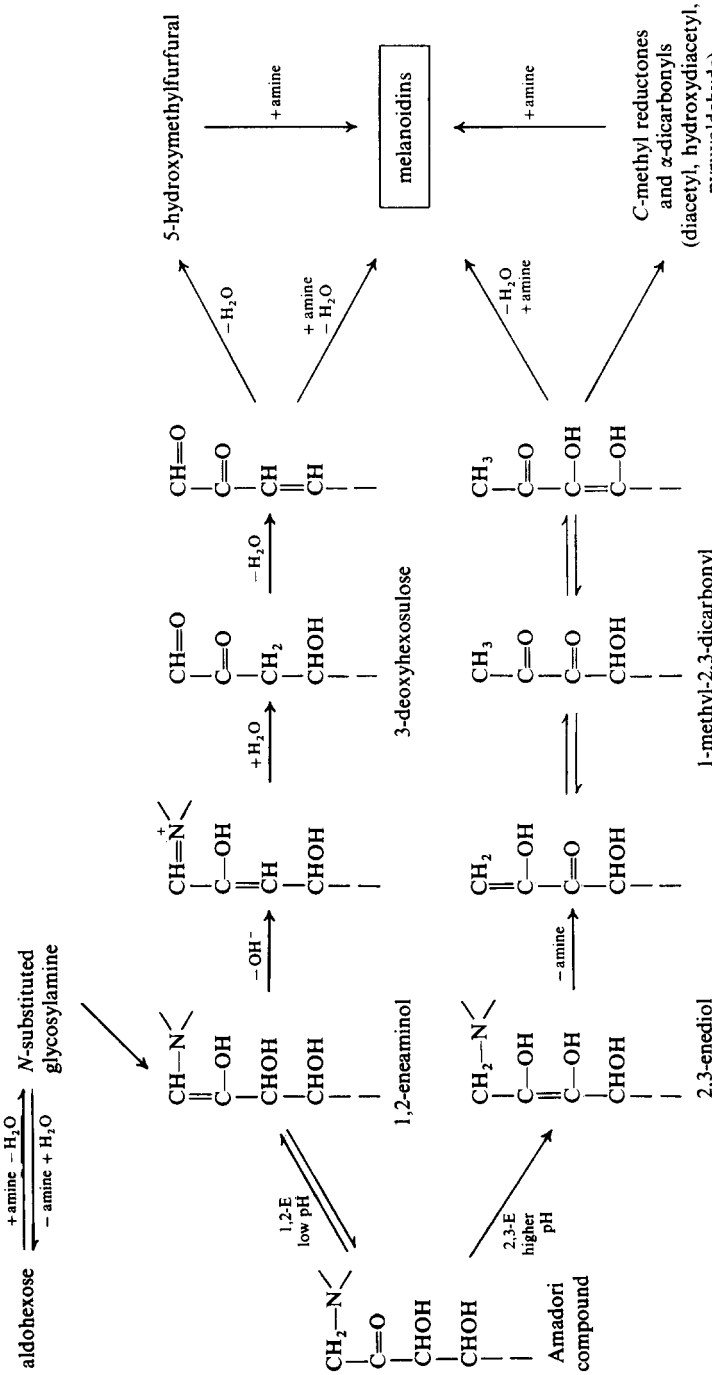


Fig. 5. Maillard reaction: two major pathways from Amadori compounds to melanoidins. Based on J. E. Hodge (1967).

Pyrazines are one of the most important discoveries in flavour chemistry, many being very powerful odorants indeed (Maga & Sizer, 1973). Alkyl substituted ones are formed readily and in considerable amounts on boiling aqueous solutions of amino acids and reducing sugars, and they are present amongst the volatiles of many foods and beverages that have been subjected to heat processing, such as roasted nuts, bread, chocolate, malt, beer and coffee. They have relatively unspecific nutty, roasted odours and more than 100 have been identified among the volatiles of foods. Two are included in Fig. 4, one because of its low threshold, the other because of its more interesting structure.

Some oxazoles and oxazolines have been found, but they do not make striking contributions to food aromas, whereas the thiazoline in Fig. 4 does play a role in bread flavour (Folkes & Gramshaw, in press).

Aldehydes can interact by aldol condensation and even 15 amino acids could thus give rise to 225 different dimeric products. Two important compounds resulting in this way, after dehydration, are included in Fig. 4. The first, from phenylalanine and leucine, is a contributor to chocolate odour (van Praag *et al.*, 1968), and the second, formed similarly from 2-oxobutyric acid, itself derived from threonine, is the character-impact compound of hydrolysed vegetable protein extracts (Sulser *et al.*, 1967).

(*b*) *Mechanisms of formation*

In order to understand the wealth of compounds generated by the Maillard reaction, it is better to have a theoretical framework, however imperfect it may yet be. The current mechanistic proposals will therefore be briefly reviewed (Figs 5–12).

Figure 5 shows some of the steps of Hodge's scheme in more detail. It should be noted:

1. That certain key steps appear to be irreversible:
   (i) *N*-substituted glycosylamine → 1,2-eneaminol;
   (ii) 1,2-eneaminol → 3-deoxyosulose;
   (iii) Amadori compound → 2,3-enediol.
2. That the relatively stable intermediate Amadori compounds can react essentially by two routes:
   1,2-E via 3-deoxyosuloses,
   2,3-E via 1-methyl-2,3-dicarbonyls,
   and that the choice is effected mainly through pH, a low pH favouring 1,2-E and vice versa.
3. Route 1,2-E can lead to furfurals, whereas route 2,3-E can lead to reductones and fragmentation. Furaneol and similar compounds appear to be formed this way. Route 2,3-E can also give rise to pyrones, as shown in Fig. 6.

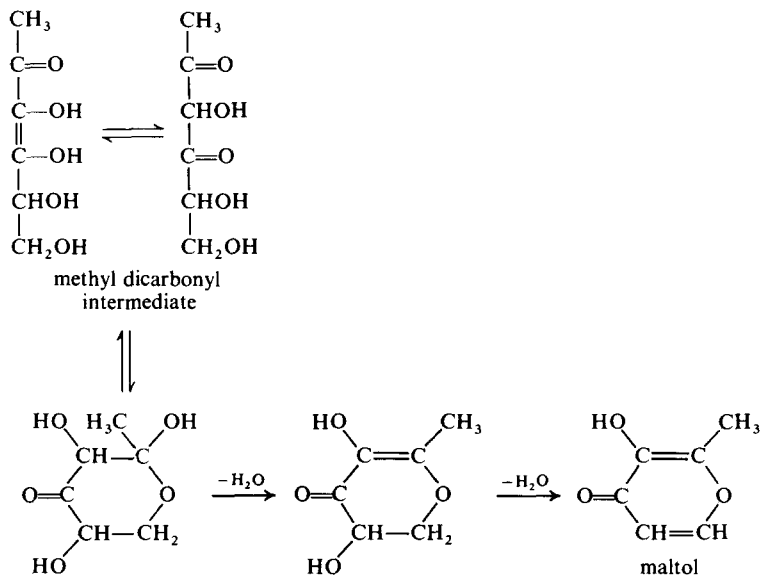


Fig. 6. Pyrone formation.

The formation of most of the volatiles derived by sugar dehydration/fragmentation has already been covered. The basic reaction of the Strecker degradation is given in Fig. 7. Note that most of the reactive dicarbonyls result from route 2,3-E, but some others are part of route 1,2-E. Most of the carbon dioxide evolved in Maillard reactions originates from the carboxyl groups of amino acids.

Turning now to the volatiles produced by further interactions, it will be seen that matters become more complex still. Pyrroles are closely related to furans and several are formed in a somewhat parallel manner, as shown in Fig. 8. The 3,4-dideoxypentosulos-3-ene may cyclise directly to furfural, but, in the presence of amino acids, it may first react at C-2 to form a Schiff's base, which then cyclises to an *N*-substituted pyrrole-2-aldehyde, a type of compound which has been isolated.

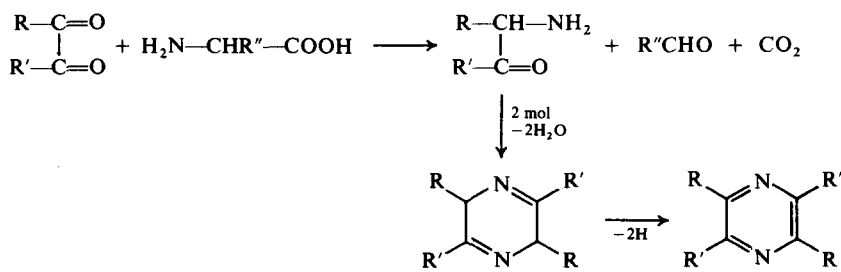


Fig. 7. Pyrazine formation.

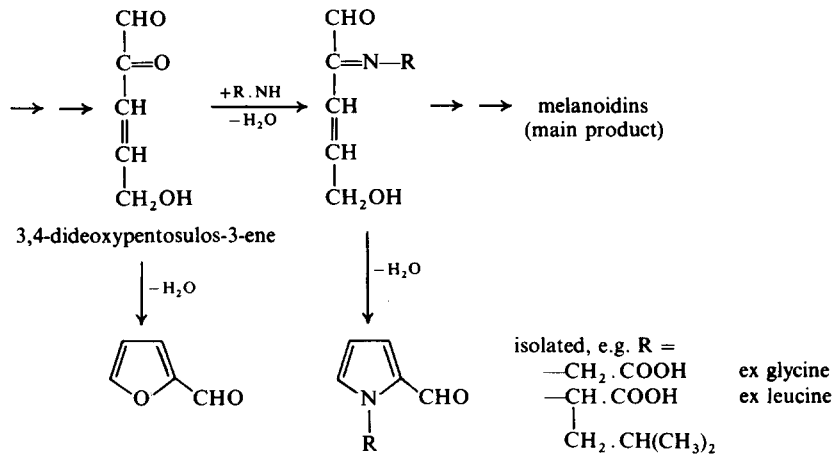


Fig. 8. Pyrrole formation (Kato & Fujimaki, 1968).

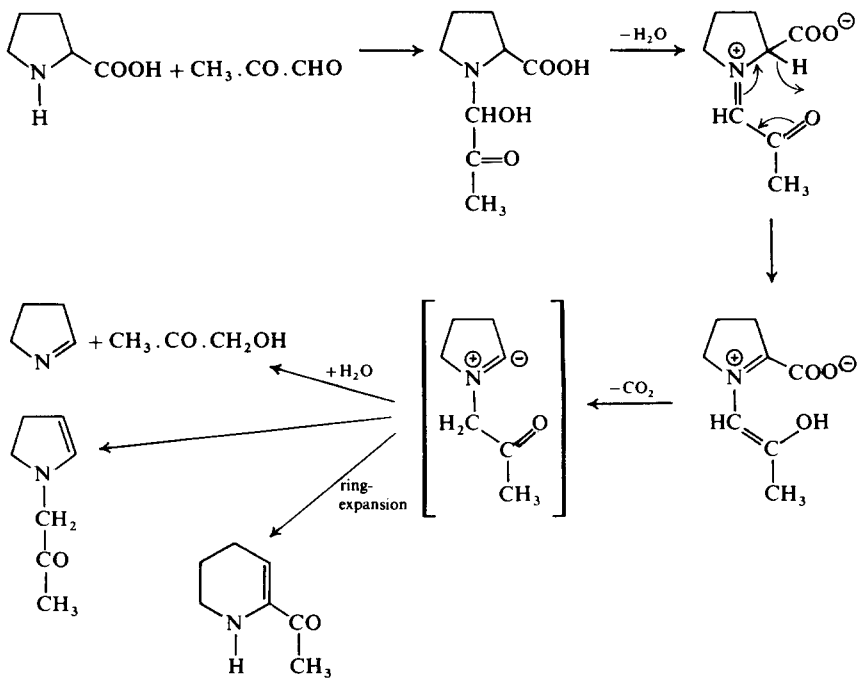


Fig. 9. Degradation of proline to 1-pyrroline, *N*-acetonyl-2-pyrroline, and 1,4,5,6-tetrahydro-2-acetopyridine. Based on Hodge *et al.* (1972).

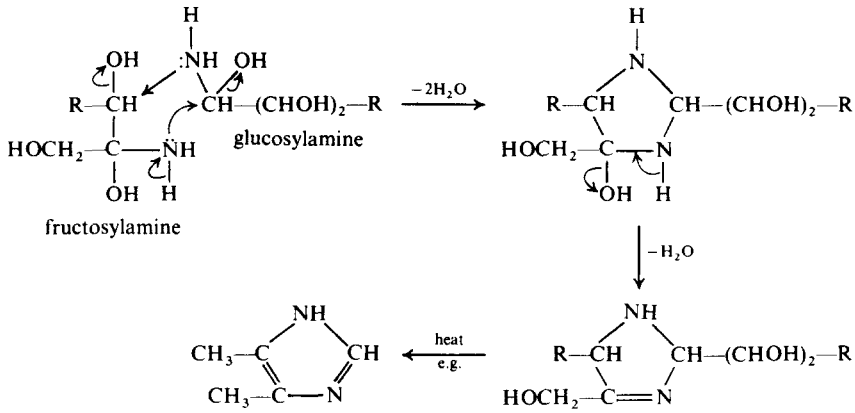


Fig. 10. Imidazole formation (Ježo, 1966).

However, proline already contains a pyrrolidine ring and this may lead to other nitrogen heterocycles, 1-pyrroline, *N*-acetonyl-2-pyrroline, and tetrahydro-2-acetopyridine, as shown in Fig. 9. Tressl (in press) recently identified more than a hundred pyrroles, pyridines, indolines, pyrrolidines, and pyrrolizines from the interaction of proline and hydroxyproline.

Imidazoles are generally thought to be formed from ammonia and sugar fragmentation products, e.g. pyruvaldehyde and formaldehyde give 4-methylimidazole. However, other mechanisms may also be operating. According to Ježo (1966), more complex imidazoles may be formed first, but decompose to simpler ones when subsequently heated strongly (Fig. 10). Yet another route, involving the non-volatile product of the Strecker degradation (see Fig. 7), has been proposed by Shibamoto & Bernhard (1978). The fact that the sidechain of 4-(2,3-dihydroxypropyl)imidazole, formed from glucose and ammonia, is optically inactive implies formation (Fig. 11) by the fragmentation route (Fujii *et al.*, 1966).

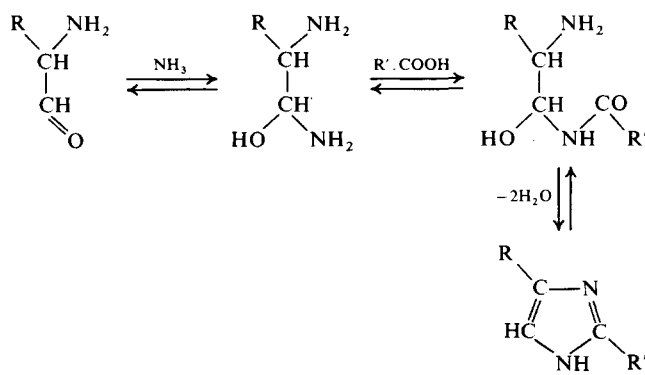


Fig. 11. Imidazole formation (Shibamoto & Bernhard, 1978).

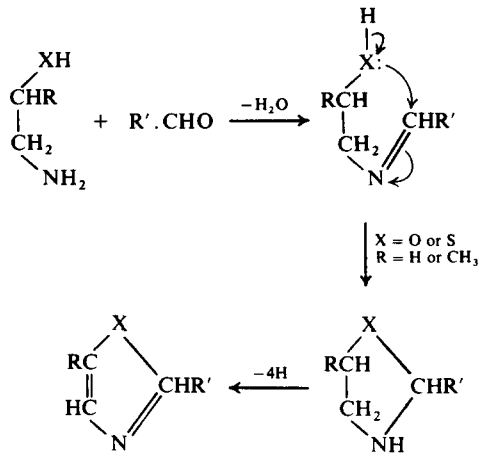


Fig. 12. Oxazole and thiazole formation (Tonsbeek *et al.*, 1971; Vitzthum & Werkhoff, 1974).

For pyrazines, it is necessary also to go back to Fig. 7, where 2 mol of the same non-volatile product were shown as interacting to form a dihydropyrazine. In reality, matters are not so straightforward and, for example, glyoxal and ammonia do not yield pyrazine only, but give rise to homologues up to the trimethyl one. Experiments by Shibamoto & Bernhard (1977) indicate that pyrazine formation from the dihydro compounds does not require oxygen and postulate formation from hydroxy derivatives by dehydration.

The formation of oxazoles and thiazoles is outlined in Fig. 12, which shows the key importance of serine, threonine, and cysteine as precursors.

CONTROL OF THE MAILLARD REACTION

Above, Hodge's framework has been filled in to some extent, but as yet all too little is known of how this complex net of interconnected reactions can be pulled about so as to yield the most desirable products.

Important factors are temperature, time, proportion and nature of reactants, and water activity. The effect of temperature on browning is marked, Q_{10} values being 3–6, but why this should be so has not been satisfactorily explained, though recently Eichner & Ciner-Doruk (in press) have reported Q_{10} values of up to 15 for the formation of Amadori compounds in tomato powder. For storage, time is of the essence, but the profile changes continually so that the optimum time will depend on the component profile sought. There are some clear effects of the nature of the reactants: pentoses react more quickly than hexoses; some products are specific to particular reactants, e.g. thiazoles from cysteine, some pyrroles from pyroline, but,

as regards proportions, there is a general tendency for model systems not to be moved away from a 1:1 ratio of sugar:amino acid. Since so many of the reactions are dehydrations and condensations, the importance of water activity is not surprising.

As soon as interactions between these factors are considered, the paucity of data becomes even more apparent. One can only surmise, for example, that the Maillard reaction can be correctly described as using amino acids to bring about reactions which would only occur otherwise at much higher temperature and/or pH. One of the important differences between amino acids is probably their effectiveness in the latter respect.

However, recent work using more modern techniques has thrown light on some facets of the Maillard reaction, and attention will now be paid to seven items:

1. Feather (in press) has shown that when a hexose is converted into hydroxymethylfurfural in strong acid in D_2O, there is no solvent incorporation into the aldehyde group or the methin group next to it, whereas incorporation does occur when starting with the corresponding Amadori compound, implying that equilibrium reactions are only involved in the latter case.
2. Feather (in press) also showed that 1-dibenzylamino-1-deoxy-D-fructuronic acid decomposes in strong acid to furfural, presumably via 1,2-enolisation, but at pH 6–8 it yields 4-hydroxy-5-methyl-3(2H)-furanone, use of ^{14}C demonstrating that the 5-methyl group is derived from C-1 of the hexuronic acid.
3. Olsson *et al.* (in press) have found the ^{13}C-NMR spectrum of high molecular weight water-soluble material from glucose and glycine to be very like that of the corresponding Amadori compound, there being no evidence of unsaturated or aromatic carbon atoms. The material is very difficult to hydrolyse, suggesting that the glucose units are linked by C–C bonds.
4. Doornbos *et al.* (in press) have used 300 MHz PMR on 6-deoxy sugars, the Amadori compounds of which cannot exist in the pyranose form, and so were able to show that the hydrogens at C-1 of both the furanose forms and of the open-chain form are separated (at pH 1), but disappear at higher pH (pH 7) because of equilibration with solvent D_2O.
5. The same authors (Doornboss *et al.*, in press) found by ^{13}C NMR that no equilibration occurred at C-3 in the reaction of rhamnose with proline, i.e. that no 2,3-enolisation took place.
6. Namiki & Hayashi (in press) found that the products of the interaction of α- or β-alanine with arabinose gave rise to ESR spectra with 17 and 23 lines, respectively. Such similar signals were attributed to the presence of the *N,N'*-dialkylpyrazine cation radicals. The radicals are detected before the Amadori compound and hence it is thought a new pathway for browning has been discovered.

7. HPLC has much potential for resolving the complex mixtures formed in browning reactions, both for analytical and preparative purposes. This is being borne out (Ledl & Severin, in press; O'Reilly, in press). Isoelectric focusing has also proved to be of help (O'Reilly, in press; Homma & Fujimaki, in press).

CONCLUSION

The Maillard reaction consists of an interrelated network of processes, rivalling in complexity those occurring in living cells. Substantial progress has been made in the identification of volatile products, but much remains to be learnt about the higher molecular weight products, including the coloured ones, the mechanisms operating, and how they may be controlled and directed. The recent application of more modern methods has provided promising leads to a fuller understanding.

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ERRATUM

We have been asked to point out that the six papers which comprised Volume 6 Number 1 of *Food Chemistry* were presented at the Symposium on Food Gels organised by the Food Chemistry Group of the Industrial Division of the Royal Society of Chemistry.

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CHANGES IN THE LEVELS OF 2-PHENYLETHYLAMINE IN CHEESE AND CHOCOLATE DURING PROCESSING AND STORAGE

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ABSTRACT

Amines from cheese and chocolate were extracted with solvent and separated from non-basic material on an ion-exchange column. The amines were reacted with trifluoroacetic anhydride after the addition of aminotetrahydronaphthalene as an internal standard. The derivatives were analysed on a SCOT column by gas chromatography. The levels of phenylethylamine increased in cocoa beans during fermentation and roasting. Some samples of cheese contained phenylethylamine, whereas others prepared aseptically did not.

INTRODUCTION

A recent publication (Koehler & Eitenmuller, 1978) on the analysis of 2-phenylethylamine in some foods, prompts us to report on some similar results obtained in these laboratories during 1975. A part of this work has already been published (Chaytor *et al.*, 1975). The present report gives the method for quantitative analysis of 2-phenylethylamine.

The relationship between the consumption of certain foods and the incidence of migraine has been known for nearly 200 years (Fothergill, 1784). Although the subject of dietary migraine continued to be of interest, it was not until recently that controlled scientific investigations were made on the subject (Smith *et al.*, 1970).

2-Phenylethylamine has been shown to initiate migraine attacks in sufferers (Sandler *et al.*, 1974). Methods for the extraction of amines from foods have been published (Chaytor & Saxby, 1975) and 2-phenylethylamine has been positively identified in plain chocolate and some cheeses (Chaytor *et al.*, 1975).

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EXPERIMENTAL

Method

Isopropanol (400 ml), dichloromethane (200 ml), aqueous 4 M potassium carbonate solution (150 ml) and finely grated sample (150 g) are added to a 1-litre separating funnel. The mixture is homogenised for 30 min with a Silverson mixer–emulsifier (laboratory model) fitted with a 1-in tubular disintegrating head and running at full speed.

The homogenate is left to settle for about 30 min. The organic solvents, containing all basic and neutral components of the sample, separate as a discrete phase above the homogenate. These are pumped through a filter tube and through a phase-separating paper into the reservoir above the ion-exchange column of Amberlyst 15.

The sample passes down the column at a rate of about 250 ml/h. The column is washed successively with three solvent mixtures: (a) 90 ml isopropanol–dichloromethane (2:1); (b) 50 ml isopropanol; and (c) 80 ml acetone–water (65:15).

All the basic material is removed from the column by elution with 250 ml 1 M hydrochloric acid (in acetone). The elution takes 1 h to complete.

The eluate is evaporated to dryness on a vacuum rotary evaporator, set initially at 30 °C until most of the organic solvent has been removed and then at 60 °C to remove the aqueous phase.

The residue is washed with ether which is decanted and discarded. Trifluoroacetic anhydride, dissolved in dry ether, is added to the residue suspended in dry ether. Contact between the residue and the anhydride is maintained for at least 30 min at room temperature. A solution of 1-amino-5,6,7,8-tetrahydronaphthalene in dry ether (500 μg in 500 μl) is added as an internal standard together with further trifluoroacetic anhydride. The solution is cautiously washed with 2·5 M potassium bicarbonate solution, rigorously dried over sodium sulphate and evaporated to about 0·2 ml. The analysis is undertaken by gas chromatography on a SCOT column (50 ft × 0·02 in i.d.) of diethylene glycol succinate operated isothermally at 140 °C. Detection is by flame-ionisation.

A calibration graph is obtained by fortification of samples of Coberine or cottage cheese with phenylethylamine and determination of the ratio of peak areas due to the amine and the added internal standard. The calibration graph for Coberine is shown in Fig. 1.

Figure 2 shows a typical chromatogram which is obtained from a chocolate extract.

Samples

Some cheeses were obtained directly from manufacturers and others were bought from local supermarkets. In the former case, the date of production was known, but cheeses bought locally were arbitrarily assumed to be two months old.

The 'aseptic cheese' was provided by the National Institute for Research in

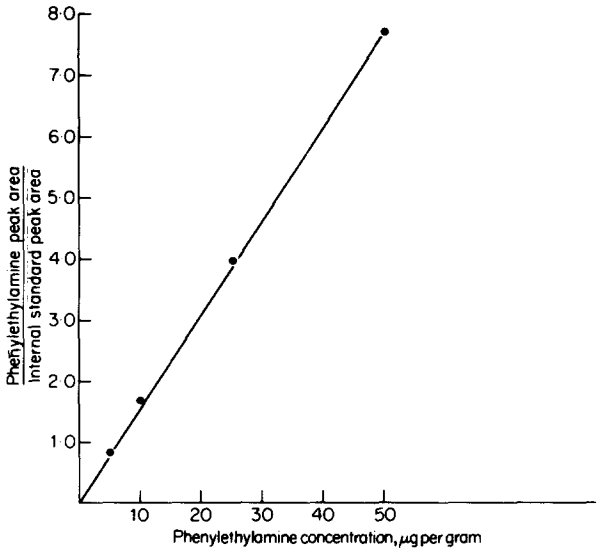


Fig. 1. Calibration curve for phenylethylamine in Coberine.

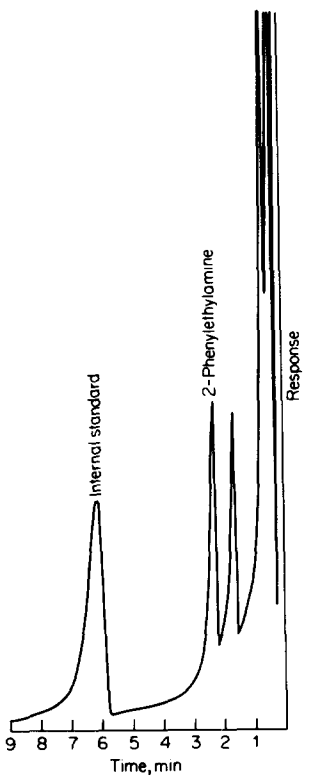


Fig. 2. Chromatogram of chocolate extract.

TABLE 1
LEVELS OF 2-PHENYLETHYLAMINE IN CHEESES DURING MATURATION

Cheese type	*Age months*	*Level ppm*	*Age months*	*Level ppm*	*Age months*	*Level ppm*	*Age months*	*Level ppm*	*Age months*	*Level ppm*
Wensleydale	2	6	5	13	7	10	8	16	9	16
Dutch Gouda	2	4	5	4	7	<4	8	<4		
English Cheshire	3	6	6	<4	11	18				
Blue Stilton	6	<4	8	<4	9	<4				
Leicester	3	<4	6	14	8	12				
Double Gloucester	4	8	9	7	9	<4				
Dutch Edam	2	10	6	14	7	14				
Mild English Cheddar	2	0	5	0						
Caerphilly	2	0	5	0						
Camembert	2	0	5	0						
St Paulin	2	0	5	0						
Dutch Jaarlsberg	2	0	5	0						
[a]English Cheddar No. 3	4	18	6	22						
English Cheddar No. 4	5	24	9	40						
Cottage cheese	2	0	9	0						
Aseptic cheese No. 1	<6	0	<8	0						
Aseptic cheese No. 2	<6	0	<8	0						
[a]English Cheddar No. 1	4	0								
[a]English Cheddar No. 2	4	0								
Danish Blue	2	0								

Cheeses stored at 4°C except where indicated.
[a] Cheeses stored at room temperature.

TABLE 2
CONCENTRATION OF 2-PHENYLETHYLAMINE IN COCOA BEANS OF DIFFERENT ORIGIN AND TREATMENT

Country of origin	*Sample*	*Concentration ppm*
Unknown	Unfermented beans	0
Unknown	Fermented, unroasted beans	<2
Unknown	Fermented, roasted beans	13
Trinidad	Unroasted shell	<2
	Unroasted beans	<2
	Roasted beans	2
Ghana	Unroasted beans	<2
	Low-roast beans	8·6
	Medium-roast beans	9·8
	High-roast beans	12
New Guinea	Unroasted beans	<2
	Roasted beans	<2
Venezuela	Unroasted beans	<2
	Roasted beans	<2
Ecuador	Unroasted beans	<2
	Roasted beans	<2

Dairying, Reading, and consisted of cheese which had been prepared under strictly aseptic conditions from a single starter.

Cocoa beans and chocolate samples were supplied by British manufacturers.

RESULTS

2-Phenylethylamine levels in cheese, cocoa beans and chocolate are given in Tables 1, 2 and 3.

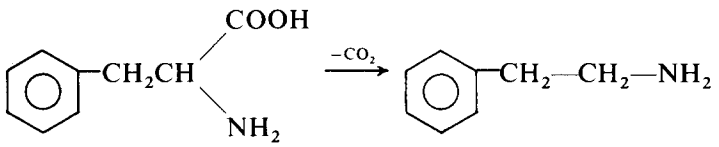
TABLE 3
CONCENTRATION OF 2-PHENYLETHYLAMINE IN CHOCOLATE CONSTITUENTS AND CHOCOLATE TYPES

Sample	*Concentration ppm*
Cocoa butter	0
Cocoa nibs	8·3
Finished chocolate	2·6
Plain chocolate A	<2
Plain chocolate B	3
Plain chocolate C	12
Plain chocolate D	2
Milk chocolate A	2·5
Milk chocolate B	5·9
Milk chocolate C	2
Milk chocolate before conching	<2
Milk chocolate after conching	<2

DISCUSSION

The levels of 2-phenylethylamine in all varieties of cheese range from 0 to 40 ppm. It is of interest to note that the highest values are considerably above those found during investigations into chocolate, in which the highest observed value was 12 ppm. There is a particular interest in the fact that chocolate is always considered to be a more severe migraine potentiator than cheese and suggests that chocolate contains a further migraine precipitant, which may not be present in cheese.

A possible precursor for 2-phenylethylamine is the amino acid phenylalanine.



Investigations (S. Berridge, pers. com.) into the amino acid composition of Cheddar cheeses during ripening show that a typical cheese contains about 1000 ppm of phenylalanine after 6 months' storage. Hence, it is clear that a cheese would contain more than sufficient phenylalanine to yield the quantities of phenylethylamine detected. However, all Cheddar cheeses exhibited gradual autolysis and phenylalanine was found in all the cheeses examined.

It would therefore appear that certain cheeses lack a specific enzyme necessary for the conversion of phenylalanine to phenylethylamine. Cheeses prepared from a single starter and produced under strictly aseptic conditions failed to produce phenylethylamine; this further emphasises the need for a specific enzyme in the formation of the amine. Table 1 also indicates that cheeses which exhibited no phenylethylamine at the beginning of the storage experiment, did not start to produce the amine on extended storage. Once again the absence of a specific enzyme is suggested. Furthermore the cheeses labelled Cheddar Nos. 1, 2 and 3 were also made from single starters, though the progress of the production was not necessarily performed under aseptic conditions. Two of these cheeses contained no phenylethylamine.

Most cheeses which do contain 2-phenylethylamine, show either a fairly steady concentration or a slight increase in concentration of the amine during storage. Several cheeses, after storage for 6–9 months at 4 °C exhibited mould growth on cut edges, whilst the three cheeses stored at room temperature became ammoniacal and fishy. The evidence suggests that 2-phenylethylamine (if present at all) is formed in the early stages of cheese manufacture and subsequent microbial activity may only enhance the concentration, if the necessary enzyme is present in the early stages.

The levels of phenylethylamine found in this study lie within the range found by Koehler & Eitenmuller (1978).

The concentration of 2-phenylethylamine in cocoa beans of different origin and

treatment is shown in Table 2. Before fermentation the beans contain no detectable amount of phenylethylamine. The process of fermentation leads to a low concentration of phenylethylamine, which increases several fold during roasting. It seems probable that the source of 2-phenylethylamine is again phenylalanine, which decarboxylates most readily under the influence of heat in the roasting stage.

Table 4, adapted from Rohan (1964) and Pinto & Chichester (1966), shows the levels of phenylalanine in cocoa beans at various stages of production. It shows that the concentration of phenylalanine rises to a maximum after fermentation and then decreases during roasting; this is consistent with the formation of 2-phenylethylamine from phenylalanine by decarboxylation. During the latter process, the concentration of phenylalanine falls by 1000 ppm, so that even if only about 1 % is due to decarboxylation, about 10 ppm of 2-phenylethylamine would be formed.

TABLE 4

CONCENTRATION OF PHENYLALANINE IN COCOA BEANS

Type	*Level in mg/100 g*
Unfermented	28
Fermented	403
Unroasted	432
Roasted	332

Tables adapted from Rohan (1964) and Pinto & Chichester (1966).

The remainder of the phenylalanine probably undergoes a Maillard reaction with reducing sugars yielding compounds which contribute to the flavour.

The results in Table 2 for Ghanaian cocoa beans show that the higher roasting yields higher levels of phenylethylamine. The table also shows that cocoa beans of different origin can differ considerably in their content of phenylethylamine.

Table 3 shows that the phenylethylamine is associated with the non-lipid portion of the cocoa bean, since none is found in cocoa butter.

The level of phenylethylamine varies from about 2 to 12 ppm in plain chocolate and 2 to 6 ppm in milk chocolate. These figures are in good agreement with those obtained by Schweitzer *et al.* (1975).

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Food Chemistry 6 (1980–81) 289–294

THE REQUIREMENT OF ESSENTIAL FATTY ACIDS FOR MAINTENANCE OF NORMAL PERIODONTAL TISSUES IN THE RAT

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ABSTRACT

Rats fed a high sugar diet deficient in essential fatty acids develop periodontal changes similar to those of periodontal disease. These changes can be prevented if the rats are injected with sufficient essential fatty acids to maintain normal growth and development of the animals. This evidence indicates that the role of essential fatty acids in maintaining normal periodontal tissue is a systemic one.

INTRODUCTION

The importance of essential fatty acids (EFAs) in the diet of animals was first demonstrated by Burr & Burr (1929) who maintained rats on a fat-free diet. Several physico–pathological changes appeared which could be prevented or reversed by including small quantities of polyunsaturated fatty acids in the animals' diet. These fatty acids became known as essential fatty acids and although a considerable number of polyunsaturated fatty acids have EFA properties, the two most important ones are linoleic and arachidonic acid. A number of reviews on essential fatty acids have appeared, e.g. Alfin-Slater & Aftergood (1968) and Sebrell & Harris (1971). The diverse symptoms ascribed to the deficiency of essential fatty acids in the rat include poor growth, scaly skin, necrosis of the tip of the tail, swelling of the feet and loss of hair. Histological changes such as swelling of mitochondria and calcification of kidney tubules and bone changes also occur (Bavetta *et al.*, 1959). The periodontal membrane of EFA deficient rats appears disorganised and is associated with alveolar bone resorption (Prout & Tring, 1971*a*). Degenerative changes have also been observed in ameloblasts and odontoblasts (Prout & Tring, 1971*b*, 1973) of EFA deficient rats. The main function of EFAs appears to be the

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maintenance of normal membrane structure since EFAs are components of phospholipids.

Changes in periodontal tissues produced by EFA deficiency in rats can be prevented by adding EFAs to the diet (Prout & Tring, 1971*a*). However, the process is complicated by the observation that periodontal changes can be produced by altering the consistency and composition of the diet while at the same time maintaining a normal nutritional balance (Baer & White, 1966). This is considered to be due to retention of food around the teeth leading to inflammation and concomitant infection.

The present study has been carried out to determine whether periodontal changes produced in rats on a diet deficient in EFAs is a direct result of systemic nutritional deficiency.

MATERIALS AND METHODS

A group of 20 Wistar rats was maintained on a high sugar (72% sucrose) synthetic diet deficient in EFAs (Seward *et al.*, 1966) fed *ad lib*. The diet contained 5% triglycerides containing saturated fatty acids of chain length 8–14 carbons. The composition of the diet was: casein 150·0 g/kg, sucrose 720·0 g/kg, triglycerides 50·0 g/kg, salt mix 40·0 g/kg and vitamin mix 40·0 g/kg, purchased from Teklad Test Diets, 2826 Latham Drive, Madison, Wisconsin 53713, USA. The rats were weaned on to the diet at the age of 14–16 days and were injected twice a week with 0·1 ml corn oil (53% linoleic acid) intraperitoneally; the dose was increased to 0·2 ml twice weekly at five weeks of age. A control group of 10 Wistar rats were fed the diet deficient in EFAs without administration of corn oil, a further control group of 20 Wistar rats were fed the deficient diet with 5% corn oil added. The rats were killed at 25–27 weeks of age, the jaws were dissected out and fixed in neutral formol saline. The tissues were decalcified with formic acid, dehydrated and embedded in Paraplast for sectioning. Serial buccolingual sections were cut at 5 μ. Every fifth section was collected and these were stained with H and E, P.A.S. or Masson Trichrome stain and examined by light microscopy. The histological observations were carried out without knowing from which group of animals the tissue had been obtained.

The animals were weighed each week during the experiment.

RESULTS

The animals maintained on the EFA deficient diet supplemented with 5% corn oil did not develop symptoms of EFA deficiency and grew normally (Bailey *et al.*, 1967; Prout & Tring, 1971*a*). Animals maintained on the EFA deficient diet and injected intraperitoneally with corn oil also grew normally and did not develop symptoms of

EFA deficiency. The group of animals maintained on the EFA deficient diet showed all the well recognised symptoms of EFA deficiency and ceased growing at 14–16 weeks of age (Prout & Tring, 1971*a*) (Fig. 1).

The animals given a 5 % corn oil supplement in the diet and those given corn oil by intraperitoneal injection showed normal periodontal membrane structure and the alveolar bone in these animals showed evidence of normal osteoblastic activity. No

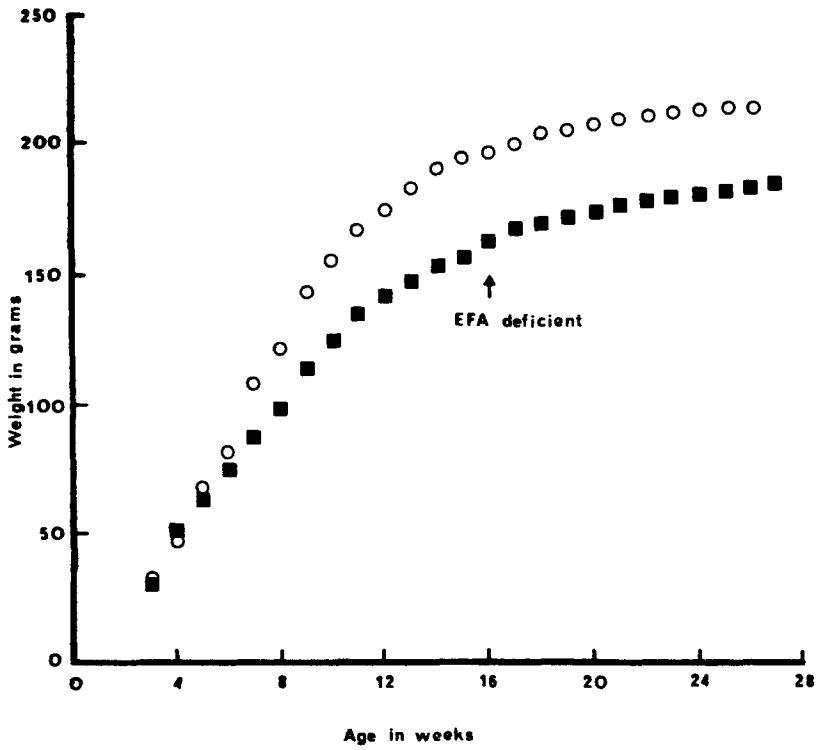
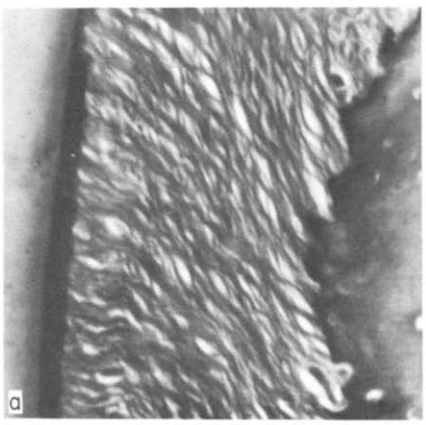


Fig. 1. Growth curves of Wistar rats fed a high sugar, essential fatty acid (EFA) deficient diet (■) and a high sugar EFA deficient diet supplemented with 5·0 % corn oil or interperitoneal injections of corn oil (○). The animals fed the EFA deficient diet showed all the well recognised symptoms of EFA deficiency at 14–16 weeks of age (↑).

osteoclasts were seen. In those animals receiving the EFA deficient diet without corn oil supplement, fibre bundle patterns in the periodontal membrane were irregular and the normal orientation was altered. The cementum was also irregular, occasionally deficient and tended to be easily torn away during sectioning. Alveolar bone showed resorptive changes, increased vascularity and many reversal lines which are usual indicators of active remodelling. The histological appearance of these tissues is illustrated in Figs. 2 and 3.



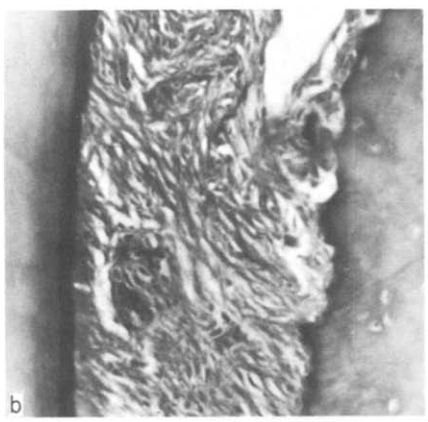
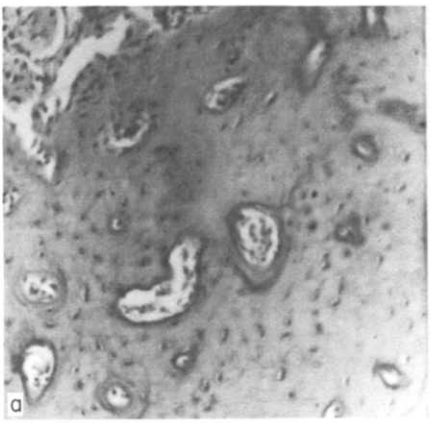


Fig. 2. Periodontal membrane of rats fed (a) diet deficient in EFAs with the addition of 5% corn oil, showing normal fibre bundle pattern (tissues from animals fed the EFA deficient diet and given intraperitoneal injection of corn oil were identical to (a)) (Masson Trichrome ×400), (b) diet deficient in EFAs, showing irregular fibre bundle pattern (Masson Trichrome ×400).



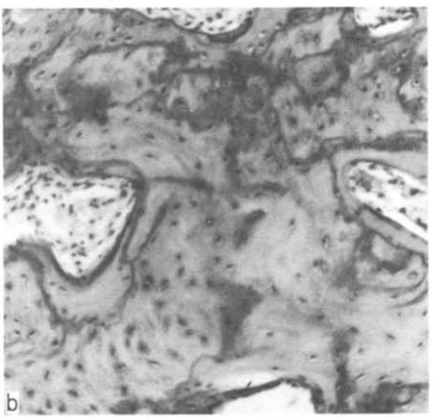


Fig. 3. Alveolar bone from rats fed (a) diet deficient in EFAs supplemented with 5% corn oil, showing normal bone structure (tissues from animals fed the EFA deficient diet and given intraperitoneal injections of corn oil were identical to (a)) (P.A.S. × 250), (b) diet deficient in EFAs, showing reversal lines indicating active remodelling (P.A.S × 250).

DISCUSSION

The extensive degenerative changes seen in the periodontal tissues of EFA deficient rats previously reported (Prout & Tring, 1971*a*) were prevented in the present experiments by giving either a 5% corn oil supplement or twice weekly intraperitoneal injections of corn oil. In the animals receiving corn oil no difference was detected in either the growth rate or general condition; the periodontal tissues appeared to be histologically normal in both groups. Since rats require approximately 40 mg of essential fatty acids per day for normal development (Alfin-Slater & Aftergood, 1968) the 5% corn oil supplement in the diet or twice weekly injections provide an adequate amount of EFAs.

In a previous report of the effect of EFA deficiency on periodontal tissues (Prout & Tring, 1971*a*) it was suggested that though the primary cause of the observed periodontal changes may have been a systemic effect of EFA deficiency, there was also an important local environmental effect due to the high sugar diet in the oral cavity.

One of the major roles of EFAs in the body is as a component of the phospholipids of cell membranes. A deficiency in EFAs in the diet will lead to alteration in the composition of membrane phospholipids and may therefore alter the properties of the membrane making them weaker and more liable to disruption (Biran *et al.*, 1964; Wilson & Leduc, 1963). This effect might be expected to lead to the degenerative periodontal changes seen in the present experiment. Particularly important changes, including increased capillary fragility and permeability in EFA deficiency have been described by Kramar & Levine (1953).

The possible roles of prostaglandins in periodontal disease have been reported in humans (Goodson *et al.*, 1974). In the present experiments prostaglandins might also be involved since they are synthesised in the body from essential fatty acids (i.e. linoleic acid to arachidonic acid to prostaglandin E_2) and in EFA deficiency prostaglandin formation could be impaired. In the presence of inflammation and tissue degeneration EFAs (linoleic and arachidonic acid) are withdrawn from membrane phospholipids for synthesis of prostaglandins (Poyser, 1973; Holdcroft, 1975) and may further disrupt membranes and increase the inflammatory and degenerative changes.

Histological changes in rat periodontal tissues similar to those found in the present study have been reported by Fava-de-Moraes & Villa (1969) in animals subjected to inanition for periods up to ten days. Though these changes are unlikely to result from EFA deficiency in so short a period they might indicate that degenerative periodontal changes can be induced by a number of different causes.

The systemic effect of nutrition on tooth supporting structures indicates that deficiency of a number of vitamins (A, B and C) may have a conditioning, but not necessarily a causative effect on the occurrence of periodontal degenerative changes (McBean & Speckmann, 1974; Peterson, 1971).

The present experiments show that an adequate nutritional intake of EFAs will prevent the periodontal changes seen in EFA deficiency regardless of whether the EFAs are ingested orally or injected intraperitoneally. EFAs may therefore be considered essential for the normal maintenance of periodontal tissues.

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Food Chemistry 6 (1980–81) 295–308

EFFECT OF SWEETENER TYPES ON CHEMICAL AND SENSORY QUALITY OF FROZEN KIWIFRUIT CONCENTRATES

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ABSTRACT

Fresh, mature kiwifruits, not suitable for the fresh market, were used to prepare frozen kiwifruit concentrate. Sucrose, 62 DE corn syrup and high-fructose Cornsweet$_{55}$ were used either alone or in various combinations on the same dry basis to prepare six sweetener types.

The acidity, soluble solids, and ascorbic acid content of the fresh kiwifruit pulp and concentrates are presented. The high performance liquid chromatography method (HPLC) was used to quantitatively analyse the oligosaccharides present in the concentrates. Colour measurements made with a Hunter colour difference meter indicated that the kiwifruit concentrates differed in colour attributes, depending on the holding temperature. Objective measurements with a Brookfield viscometer indicated that the consistency of the concentrates varied with the sweetener types. The concentrate made with 62 DE corn syrup and sucrose (1:2) was thickest in consistency.

Kiwifruit nectars prepared from frozen concentrates sweetened with either sucrose alone or Cornsweet$_{55}$ alone were preferred by the taste panel to the others.

INTRODUCTION

Dadlani *et al.* (1971) reported that Chinese gooseberry (*Actinidia chineneis* Planch) is perhaps the most nutritious fruit known among the group of soft berry fruits. It was first introduced from China into New Zealand in 1906, and the USA began its

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cultivation in 1960. At present, it is known to be growing successfully in Japan, New Zealand, Belgium, France, Germany, England, USSR and USA. According to Beutel *et al.* (1976), California could produce 3000–4000 tons of kiwifruit annually by 1980. There were 6000 tons of kiwifruit produced in New Zealand in 1976. In 1974 kiwifruit became the internationally accepted name, replacing the names Chinese gooseberry and kiwi.

The fruits have a good keeping quality and can be kept for eight weeks or longer under cool storage conditions. The peeled fruits can be sliced and served in a salad. Kiwifruits can also be preserved whole or as sliced segments (Dadlani *et al.*, 1971). Studies carried out by Beutel *et al.* (1976) showed that kiwifruits can be successfully canned, but that frozen, sliced fruits were closest to the fresh fruit in appearance and flavour. Simmons (1978) demonstrated that firm, ripe kiwifruit, too small for the fresh market, can be dried successfully in the form of slices which can be eaten without further treatment. Candied slices retain some green colour and have a pleasant texture and taste.

In this work, fresh mature kiwifruits, not suitable for the fresh market, were used for preparation of frozen kiwifruit concentrates. Sucrose, 62 DE corn syrup and high-fructose corn syrup were used in various proportions but on the same total soluble solids basis. The effect of sweetener types on the chemical properties and sensory qualities of nectars made from the frozen kiwifruit concentrates was investigated. High performance liquid chromatography (HPLC) was used for quantitative determination of sugars in the frozen kiwifruit concentrates.

MATERIALS AND METHODS

Kiwifruits. Kiwifruits (*Actinidia chinensis* Planchon, var. Hayward) were harvested in mid-November 1978, from a commercial ranch. The fruits were harvested at the firm, mature stage when the peeled fruits were at least 7·66 kg on the UCD pressure gauge (0·794 cm plunger). The fruits were stored at 0–1 °C under 90 % relative humidity for three months before processing.

Corn sweeteners. 62 DE corn syrup and Cornsweet_{55} produced by the ADM Milling Co., Cedar Rapids, Iowa 52406 were used. The properties of the corn syrups are shown in Table 1.

Kiwifruit puree. The kiwifruits were sorted to remove defective ones, washed in water containing 10 ppm residual chlorine, and then stored in a cold room at 1 °C overnight. Fifty-five kilogrammes of fruit were run through a Rietz disintegrator at a speed of 1610 rpm, using a 1·2 cm screen. The cold, crushed fruits were run immediately through a Brown juice extractor fitted with a 0·6 mm screen. The plate pressure was set at 6·82 kg and the speed of the screw at 560 rpm. The skin and seeds were removed in the extraction process. The temperature of the crushed fruits coming out of the disintegrator was 6 °C, and that coming out of the finisher was

TABLE 1
PROPERTIES OF CORN SYRUP SWEETENERS

Properties	*62 DE corn syrup*	*Cornsweet$_{55}$*
Solids, %	83·7	77·0
pH	4·8	3·0–4·0
Ash, %	0·28	0·05 (sulphated)
Viscosity, centipoises (26·6 °C)	800	800
Components of the carbohydrate fraction		
Dextrose, %	35·5	41·0
Fructose, %	—	55·0
Maltose, %	30·0	—
Trisaccharides, %	13·0	—
Higher saccharides, %	21·5	4·0

Source: ADM Milling Co., Cedar Rapids, Iowa 52406.

7 °C. The temperature of the kiwi puree was kept at 5 °C or lower in an ice-water bath prior to being mixed with various sweeteners.

Kiwifruit concentrate. The kiwifruit puree had a total soluble solids of 14·8 ° Brix at 20 °C. The cold puree was divided into portions and mixed with various amounts of sweeteners to raise the soluble solids to 40 ° Brix. Six sweetener types were prepared on the dry soluble solids basis as follows:

1. Sucrose only.
2. 62 DE corn syrup only.
3. High-fructose Cornsweet$_{55}$ only.
4. 62 DE corn syrup: sucrose (1:2).
5. 62 DE corn syrup: high-fructose Cornsweet$_{55}$: sucrose (1:1:1).
6. 62 DE corn syrup: high-fructose Cornsweet$_{55}$ (1:1).

The sweetened puree (40 ° Brix) was thoroughly mixed and then pumped through an A.P.V. junior paraflow plate heat exchanger where it was heated to 87·8 °C. The holding time was 1 min. The puree was cooled in the cooling section of the plate heat exchanger to 7 °C, and then filled into enamelled cans (202 × 306), allowing a head space of 0·95 cm. The cans were sealed and frozen in a blast freezer at −37 °C for 3 h. The frozen concentrates were stored at −18 °C for one month prior to chemical analysis and sensory evaluation.

Effect of holding temperature. The kiwi puree was mixed with 62 DE corn syrup and sucrose in the proportion of 1:2 (w/w, dry basis) to a total soluble solids content of 40 ° Brix. The steps for preparing the kiwifruit concentrate were the same as those mentioned previously, except that four different holding temperatures, namely 71·1, 76·7, 82·2 and 87·8 °C were used. The temperature of the product coming out to the cooling section of the heat exchanger ranged from 9 to 12 °C.

Chemical and physical properties

Titratable acidity. A 10–15 g sample of kiwifruit pulp or concentrate was mixed with 100 ml of distilled water. The resulting mixture was titrated with 0·1 N NaOH solution to pH 8 using a Beckman Model K automatic titrator. The results are reported as per cent citric acid (Sherkat & Luh, 1977).

Soluble solids. A Zeiss–Opton refractometer was used to determine the soluble solids of the samples. Results are expressed as Brix at 20 °C (Leonard *et al.*, 1961).

Ascorbic acid. The 2,6-dichlorophenol indophenol titration method was used to determine the ascorbic acid content of the samples (AOAC, 1975).

Consistency. The consistency of kiwifruit concentrate was measured with a Brookfield Synchro-Lectric viscometer, Model RVT at 30 °C (Sherkat & Luh, 1977). A No. 4 spindle was used. The torque readings were converted into centipoises and plotted against the speed (rpm) on a log–log scale.

Colour. A Hunter colour difference meter, Model D25D2 was used for colour measurements (Simmons, 1978). Three porcelain plates were used as references for colour measurements of fruit skin, core, and greenfruit tissue, respectively, as follows:

Yellow plate ($L = 78{\cdot}2$, $a = -2{\cdot}2$, $b = +21{\cdot}7$), white plate ($L = 94{\cdot}1$, $a = -1{\cdot}3$, $b = +1{\cdot}9$) and green plate ($L = 64{\cdot}8$, $a = -14{\cdot}3$, $b = +6{\cdot}4$). The Hunter readings of the kiwifruit pulp and concentrate were made in a cylindrical sample cup 5·6 cm in diameter. The depth of the sample was 5 cm. The sample cup was made of plastic walls, with a colourless bottom plate 1·5 mm thick. A light-trap can coated inside with black coating was used to exclude interfering light from the sample port. The illuminated area was elliptical, 4·1 × 4·4 cm (Sherkat & Luh, 1977).

Texture. Pressure test of the peeled fresh fruit was determined by using a UC Davis pressure tester with a 0·794 cm plunger (Leonard *et al.*, 1953).

Sensory evaluation. The frozen kiwifruit concentrate was thawed and diluted with two parts of water (v/v). For sensory evaluation of the diluted product, 25 selected panellists were asked to score sweetness, flavour, consistency and acceptability on a 1–10 scale: Excellent, 9–10; good, 7–8; fair, 5–6; poor, 3–4; and very poor, 1–2. The experiment was repeated at 10–12 am for three consecutive days. The nectars were served at 25 °C.

RESULTS AND DISCUSSION

Physical and chemical properties. The properties of the kiwifruits used in this study are presented in Table 2. The fruits were comparatively small. They had an average weight of only 36·5 g per fruit. The average pressure reading on a UC Davis fruit gauge (0·794 cm plunger) was 0·75 kg (from 0·5–0·98 kg), indicating a quite uniform ripeness level. The pH of the fresh pulp was 3·5, and the soluble solids 14·8 ° Brix. These data are comparable to those reported by Beutel *et al.* (1976) on

TABLE 2
PHYSICAL AND CHEMICAL PROPERTIES OF FRESH KIWIFRUIT

Properties	*Range*	*Average*
Weight of individual fruit, g	23·8–56·4	36·5
Pressure test (0·794 cm plunger), kg	0·5–0·98	0·75
pH	3·4–3·6	3·5
Acidity as % citric acid	1·46–1·48	1·47
° Brix at 20°C	14·0–15·5	14·8
Ascorbic acid, mg/100 g	93·5–103·2	98·3

kiwifruits grown in California. The soluble solids content was slightly higher than the 12·6° Brix reported by Simmons (1978). The difference could be attributed to the differences in variety and horticultural practices under which the fruits were grown. The acidity of the fresh kiwifruit pulp was 1·47%, expressed as citric acid. The high acidity contributed to the very sour taste of the kiwifruit, especially when they were under-ripe. According to Heatherbell (1975), citric, quinic, and malic acids are the major organic acids present in kiwifruit. The ascorbic acid content of the fresh kiwifruit pulp ranged from 93·5 to 103·2 mg/100 g, comparable to that present in tomato pastes of 26·5% total solids (Liu & Luh, 1979). Thus, kiwifruit contained 4–5 times more ascorbic acid than fresh tomatoes, which usually contain 15–25 mg/100 g in most commercial varieties.

Colour of fresh fruit. Colour readings of the fresh kiwifruits, determined with a Hunter colour difference meter, are presented in Table 3. The instrument gives three integrated readings: L measures lightness and varies from 100 for perfect white to zero for black; a_L measures redness when plus, grey when zero and greenness when minus; b_L measures yellowness when plus, grey when zero and blueness when minus (Grncarevic & Lewis, 1973).

TABLE 3
COLOUR MEASUREMENT OF FRESH KIWIFRUIT

Measured surface	*Range*	*Average*[a]
Outer surface (skin)	L 32·4 to +38·5	35·6
	a_L +4·0 to +6·8	+5·2
	b_L +10·5 to +15·2	+12·6
Green tissue (outer surface)	L 31·4 to 40·6	34·2
	a_L −7·1 to −9·3	−8·1
	b_L +12·2 to +14·6	+13·3
Core (fruit centre)	L 47·0 to +61·1	53·6
	a_L −5·9 to −8·8	−7·5
	b_L +13·4 to +25·3	+20·6

[a] Average of ten measurements

TABLE 4
EFFECT OF SWEETENER TYPES ON pH, ACIDITY, AND ASCORBIC ACID OF FROZEN KIWIFRUIT CONCENTRATES[a]

Sweetener type	*pH*	*Acidity as citric acid %*	*° Brix at 20°C*	*Ascorbic acid mg/100 g*
Sucrose	3·7	0·897	39·5	67·2
62 DE corn syrup	3·7	0·916	40·5	69·3
$Cornsweet_{55}$	3·7	0·911	39·8	68·4
62 DE corn syrup + sucrose (1:2)	3·7	0·892	40·5	61·9
62 DE corn syrup + $Cornsweet_{55}$ + sucrose (1:1:1)	3·7	0·883	40·3	65·2
62 DE corn syrup + $Cornsweet_{55}$ (1:1)	3·7	0·885	40·3	66·4

[a] Sweeteners were added to the fresh fruit puree to adjust the soluble solids to 40° Brix at 20°C.

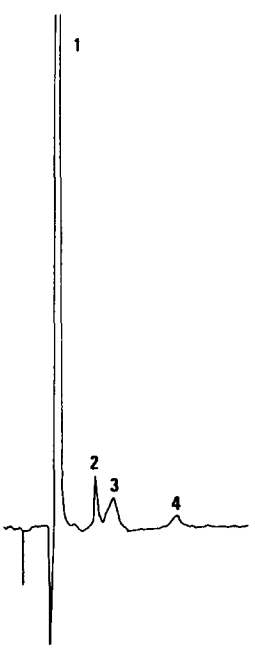

Fig. 1. Elution order of various sugars in fresh kiwifruit. Solvent front (1), fructose (2), glucose (3), sucrose (4).

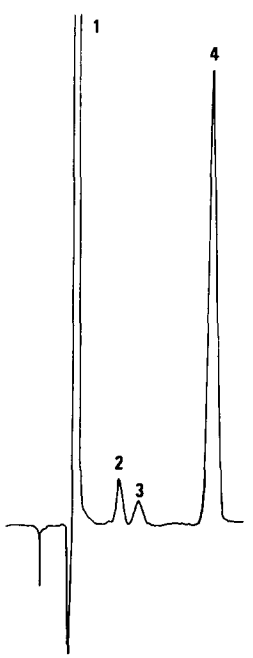

Fig. 2. Elution order of various sugars in kiwifruit concentrates made with sucrose. Solvent front (1), fructose (2), glucose (3), sucrose (4).

The colour of the kiwifruit core was quite different from that of the outer portion of the fruit tissue, as shown by their L, a_L, and b_L values (Table 3).

Chemical properties. The pH, acidity, ° Brix, and ascorbic acid content of the kiwifruit concentrates made with different sweetener types are presented in Table 4. The fresh kiwifruit (Table 2) had pH 3·5; soluble solids, 14·8 ° Brix at 20 °C; acidity, 1·47 % as citric acid; and ascorbic acid, 98·3 mg/100 g. The addition of different sweeteners to the fruit puree to the same soluble solid content of 40 ° Brix resulted in slight difference in pH value, but a lower acidity and ascorbic acid content in the frozen product.

Sugars. Fructose, glucose, and sucrose were found to be present in the fresh kiwifruit (Fig. 1). Sucrose (Peak 4) was the predominant sugar in the kiwifruit concentrate in which sucrose was used as a sweetener (Fig. 2). When 62 DE corn syrup was used as a sweetener, glucose was present in a higher proportion than other sugars in the concentrate (Fig. 3); maltose (Peak 5) and maltotriose (Peak 6) were also present. As shown in Table 1, the 62 DE corn syrup contained glucose, maltose, trisaccharides, and some higher saccharides. Fructose (Peak 2) and sucrose (Peak 4) came from the kiwifruit and were present in smaller amounts as compared with glucose (Fig. 3).

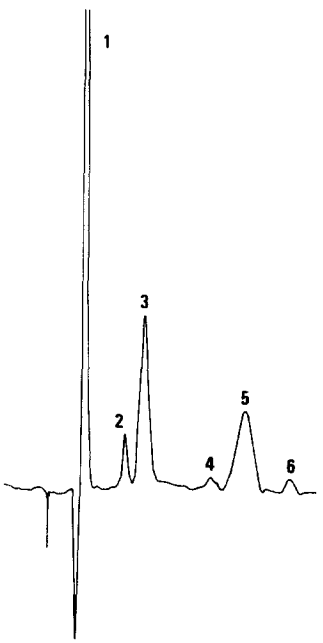

Fig. 3. Elution order of various sugars in kiwifruit concentrates with 62 DE corn syrup. Solvent front (1), fructose (2), glucose (3), sucrose (4), maltose (5), maltotriose (6).

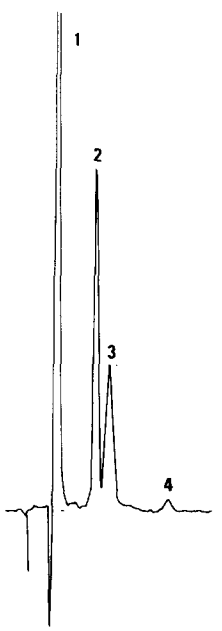

Fig. 4. Elution order of various sugars in kiwifruit concentrates made with Cornsweet$_{55}$. Solvent front (1), fructose (2), glucose (3), sucrose (4).

Results on HPLC of the sugars in the sample sweetened with Cornsweet$_{55}$ are shown in Fig. 4. Fructose (Peak 2), glucose (Peak 3), and small amounts of sucrose (Peak 4) are present.

Quantitative HPLC analyses of sugars present in the kiwifruit concentrates are shown in Table 5. The total sugar content of fresh kiwifruit was 14·62%. Fructose represented the highest proportion, followed by glucose and sucrose. According to Heatherbell (1975), glucose, fructose, sucrose and trace amounts of sorbitol account for the total sugar content of fresh kiwifruit. However, sorbitol was not detected here by the HPLC method.

TABLE 5
SUGARS IN KIWIFRUIT CONCENTRATES MADE WITH DIFFERENT SWEETENER TYPES

Sweetener type	*Fructose* %	*Glucose* %	*Sucrose* %	*Maltose* %	*Total sugars* %
Raw kiwifruit pulp	7·85	4·87	1·90	—	14·62
Sucrose	4·87	2·66	30·34	—	37·87
62 DE corn syrup	4·69	20·88	1·92	11·93	39·42
Cornsweet$_{55}$	28·96	8·99	1·75	—	39·70
62 DE corn syrup + sucrose (1:2)	3·42	10·06	22·13	4·02	39·63
62 DE corn syrup + Cornsweet$_{55}$ + sucrose (1:1:1)	12·85	9·75	10·19	4·43	37·22
62 DE corn syrup + Cornsweet$_{55}$ (1:1)	17·55	14·71	1·29	5·16	38·71

It appears that HPLC can be used as an easy and rapid method to determine the type and amount of sugars present in the kiwifruit concentrate. Incorporation of sucrose, 62 DE corn syrup and Cornsweet$_{55}$ in different proportions resulted in a change in the distribution of sugars in the concentrate. Maltose was always present in the concentrate when 62 DE corn syrup was used as the sweetener.

Effect of holding temperature on colour. The colour of the raw and kiwifruit concentrates heated at different temperatures with a holding time of 1 min was determined with a Hunter colour difference meter. Results are presented in Table 6. The kiwifruit concentrate became more yellow and less green after heating. The L value of the heated kiwifruit concentrates did not differ greatly when they were heated at 71·1–87·8 °C and held for 1 min. The greenness (a_L) decreased with an increase of temperature. This phenomenon may be explained by the thermal degradation of chlorophylls and the formation of yellow-coloured degradation products. The influence of various heat treatments on the colour of green asparagus, green beans and peas has also been studied by Hayakawa (1977), assuming first order reaction kinetics. Thermal treatments result in a decrease in green pigments and an increase in yellow pigments.

TABLE 6
EFFECT OF HOLDING TEMPERATURE ON COLOUR OF KIWIFRUIT CONCENTRATES

Heating temperature °C	*Before heating* Hunter colour values			*After heating*		
	L	a_L	b_L	L	a_L	b_L
71·1	22·1	−4·2	+11·8	28·8	−2·4	+15·0
76·7	22·4	−4·3	+12·1	29·4	−1·7	+15·3
82·2	22·7	−4·4	+12·2	29·4	−1·6	+15·2
87·8	23·1	−4·7	+12·4	29·4	−1·5	+15·1

Effect of sweetener types on colour. A Hunter colour difference meter was used to measure the colour of kiwifruit concentrates made with different sweetener types. The data presented in Table 7 indicate that when the kiwifruit puree is mixed with the sweeteners a decrease in brightness (L) and yellowness (b_L) results. The green colour (a_L) of the pulp was less affected by the presence of sweeteners. Heating the sweetened kiwifruit pulp resulted in a pronounced decrease in the greenness (a_L), a slight increase in the yellowness (b_L) and brightness (L). Generally, the sweetener types had little effect on the colour of the concentrate when used on the same soluble solids basis.

Sensory evaluation

Sweetness. The panel was able to detect differences in sweetness between diluted kiwifruit concentrates (nectars) made with different sweetener types, as shown by the

TABLE 7
EFFECT OF SWEETENER TYPES ON COLOUR OF KIWIFRUIT CONCENTRATES

Sweetener type	*Before heating* Hunter colour difference value			*After heating*[a]		
	L	a_L	b_L	L	a_L	b_L
Raw kiwifruit pulp	32·1	−5·3	+16·8	—	—	—
Sucrose	22·5	−5·5	+12·0	29·5	−1·6	+14·8
62 DE corn syrup	22·6	−5·1	+12·1	29·6	−1·9	+15·0
$Cornsweet_{55}$	22·4	−5·2	+12·0	29·8	−1·8	+14·9
62 DE corn syrup + sucrose (1:1)	22·7	−4·6	+12·1	29·3	−2·0	+15·0
62 DE corn syrup + $Cornsweet_{55}$ + sucrose (1:1:1)	22·3	−5·2	+12·0	29·1	−1·8	+14·7
62 DE corn syrup + $Cornsweet_{55}$ (1:1)	21·9	−4·9	+11·6	29·1	−1·5	+14·7

[a] The sweetened kiwifruit concentrate of 40° Brix was pumped through the A.P.V. junior paraflow heat exhanger at a temperature of 87·8 °C with a holding time of 1 min.

F-test (Table 8). The panel members could tell the difference in sweetness between the samples sweetened with sucrose only and those made with either 62 DE corn syrup only, or 62 DE corn syrup plus Cornsweet$_{55}$, at the 99 % probability level. On the contrary, the panel was not able to tell any difference in sweetness between the sample made with sucrose only and that made with Cornsweet$_{55}$ only; with 62 DE corn syrup and sucrose; or with 62 DE corn syrup, Cornsweet$_{55}$, and sucrose.

TABLE 8
SWEETNESS EVALUATION OF KIWIFRUIT NECTARS MADE FROM FROZEN CONCENTRATES WITH VARIOUS SWEETENER TYPES

Sweetener type	*Mean scores*[a]	*Standard deviation*	*Variance*	*t-test*	*P*	*F-test*	*P*
Sucrose	6·9	1·3	1·8	—		41·52	<0·01
62 DE corn syrup	3·2	1·4	1·9	13·5	<0·01		
Cornsweet$_{55}$	6·7	1·7	2·8	0·88	ns		
62 DE corn syrup + sucrose (1:2)	6·4	1·4	1·9	1·95	ns		
62 DE corn syrup + Cornsweet$_{55}$ + sucrose (1:1:1)	6·2	1·7	2·7	2·58	ns		
62 DE corn syrup + Cornsweet$_{55}$ (1:1)	5·0	1·6	2·5	6·38	<0·01		

[a] The samples were diluted with two volumes of water before serving.
ns = Not significant.

Generally, the nectars receiving the highest sweetness scores were those made with either sucrose or Cornsweet$_{55}$, while the lowest scores were reported for those made with 62 DE corn syrup. These findings can be explained by the higher sweetness attributed to the fructose in Cornsweet$_{55}$ '55 % of the carbohydrate fraction' and the lower sweetness attributed to glucose (35·5 %), maltose (30·0 %), trisaccharides (13·0 %) and higher saccharides (21·5 %) in the carbohydrate fraction of the 62 DE corn syrup.

Flavour. The panel scored a significant difference in flavour between the nectars diluted from the frozen concentrate made with sucrose and those made with either 62 DE corn syrup only or with 62 DE corn syrup: Cornsweet$_{55}$ (1:1), as shown in Table 9. On the contrary, they were unable to differentiate the flavour of the nectars of the diluted concentrate made with sucrose only and those made with Cornsweet$_{55}$ only. There was no significant difference between those made with 62 DE corn syrup: sucrose (1:2), and 62 DE corn syrup: Cornsweet$_{55}$: sucrose (1:1:1). The highest flavour preference was detected in nectars sweetened with sucrose only, followed by Cornsweet$_{55}$ or by 62 DE corn syrup: sucrose (1:2).

Consistency. The panel was able to detect differences in consistency between the kiwifruit nectars made with different sweetener types, as indicated by the *F*-value

TABLE 9
FLAVOUR EVALUATION OF KIWIFRUIT NECTARS MADE FROM FROZEN CONCENTRATES WITH VARIOUS SWEETENER TYPES

Sweetener type	*Mean scores*[a]	*Standard deviation*	*Variance*	*t-test*	*P*	*F-test*	*P*
Sucrose	6·7	1·2	1·4	—		20·0	<0·01
62 DE corn syrup	4·2	1·8	3·2	8·06	<0·01		
$Cornsweet_{55}$	6·4	1·3	1·6	0·91	ns		
62 DE corn syrup + sucrose (1:2)	6·4	1·2	1·4	1·12	ns		
62 DE corn syrup + $Cornsweet_{55}$ + sucrose (1:1:1)	6·1	1·5	2·1	2·01	ns		
62 DE corn syrup + $Cornsweet_{55}$ (1:1)	5·5	1·6	2·5	3·97	<0·01		

[a] The samples were diluted with two volumes of water before serving.
ns = Not significant.

(Table 10). The differences in consistency between the nectar sweetened with sucrose alone, and those sweetened with either 62 DE corn syrup or with 62 DE corn syrup: $Cornsweet_{55}$ (1:1) were significant at 99% and 95% probability levels, respectively.

It appeared that the kiwifruit nectars sweetened with sucrose alone and with $Cornsweet_{55}$ alone were preferred by the panel members, followed by those sweetened with 62 DE corn syrup: sucrose (1:2), and 62 DE corn syrup: $Cornsweet_{55}$: sucrose (1:1:1). The lowest score was obtained by the product sweetened with 62 DE $Cornsweet_{55}$ alone and that sweetened with 62 DE corn syrup: $Cornsweet_{55}$ (1:1).

TABLE 10
SENSORY EVALUATION OF CONSISTENCY OF KIWIFRUIT NECTARS MADE FROM FROZEN CONCENTRATES WITH VARIOUS SWEETENER TYPES

Sweetener type	*Mean scores*[a]	*Standard deviation*	*Variance*	*t-test*	*P*	*F-test*	*P*
Sucrose	6·5	1·5	2·3	—		4·59	<0·01
62 DE corn syrup	5·2	1·8	3·3	4·03	<0·01		
$Cornsweet_{55}$	6·2	1·6	2·7	1·17	ns		
62 DE corn syrup + sucrose (1:2)	6·2	1·5	2·2	1·02	ns		
62 DE corn syrup + $Cornsweet_{55}$ + sucrose (1:1:1)	6·1	1·5	2·1	1·37	ns		
62 DE corn syrup + $Cornsweet_{55}$ (1:1)	5·6	1·7	2·9	2·87	<0·05		

[a] The samples were diluted with two volumes of water before serving.
ns = Not significant.

TABLE 11
CONSISTENCY OF KIWIFRUIT CONCENTRATES MADE WITH DIFFERENT SWEETENER TYPES[a]

Sweetener type	*Viscosity, centipoises rpm*[b]					
	2·5	*5·0*	*10*	*20*	*50*	*100*
Sucrose	10000	6000	3500	2000	940	540
62 DE corn syrup	8000	4800	3450	2100	980	575
$Cornsweet_{55}$	10600	6000	3700	2125	950	540
62 DE corn syrup + sucrose (1:2)	11200	6800	4250	2300	1120	655
62 DE corn syrup + $Cornsweet_{55}$ + sucrose (1:1:1)	8400	4600	3050	1975	900	520
62 DE corn syrup + $Cornsweet_{55}$ (1:1)	8600	5200	3300	1950	900	515

[a] The frozen concentrates were thawed and then evaluated for consistency with a Brookfield viscometer at 30°C.
[b] rpm = Revolutions per minute of the No. 4 spindle.

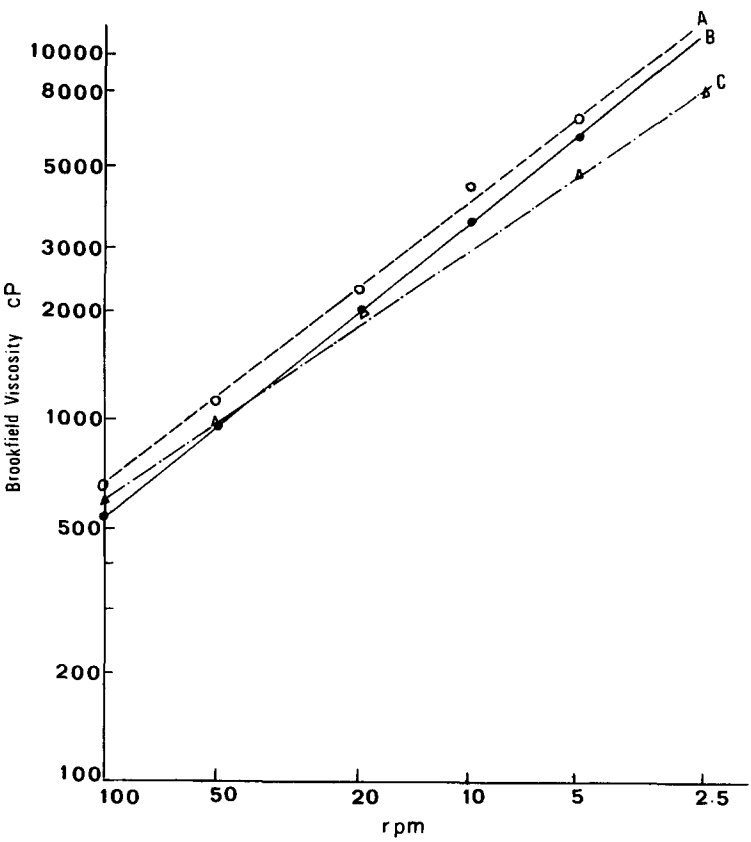

Fig. 5. Brookfield viscometer readings (Model RTV, Spindle No. 4) of kiwifruit concentrates made with 62 DE corn syrup: sucrose (1:2) (A), with sucrose only (B), and with 62 DE corn syrup only (C).

Objective consistency measurement. The Brookfield viscosity readings of the kiwifruit concentrates are presented in Table 11. The highest viscosity was attained in the sample made with 62 DE corn syrup: sucrose (1:2). The log–log plot of the Brookfield viscometer readings v. spindle speed yields a linear relationship, as illustrated in Fig. 5.

The Brookfield viscosity reading of the kiwifruit concentrate decreased as the speed of the spindle increased. The phenomenon may be explained by the orientation of the water-insoluble particles as the spindle speed increases. The higher speed of rotation results in a higher centrifugal force which tends to push the insoluble particles towards the circumference of the spindle, resulting in a lower Brookfield reading.

There were some indications that the nectar prepared with 62 DE corn syrup solids was lowest in the consistency score, and the one with sucrose was highest (Table 10). However, no general conclusion can be drawn between the sensory scores of the nectar and the Brookfield readings of the concentrates.

CONCLUSIONS

Fresh, mature kiwifruits can be made into attractive frozen, sweetened concentrates of 40% soluble solids. The frozen concentrates can be converted into nectars after dilution with two volumes of water. It was necessary to stabilise the frozen concentrates during processing by heating the sweetened products in a plate heat-exchanger to 87·8 °C for 1 min to inactivate the peroxidase and polyphenoloxidase enzymes present in the kiwifruit.

Sensory evaluation of the kiwifruit nectars of 13% soluble solids was carried out by a panel. The nectars sweetened with sucrose only, and with Cornsweet_{55} only were preferred by the panel over those made with 62 DE corn syrup solids or a combination of 62 DE corn syrup with Cornsweet_{55} (1:1). The difference in preference scores between the samples with various sweetener types was related to the type and quantity of oligosaccharides present as determined by the high performance liquid chromatography method.

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Food Chemistry 6 (1980–81) 309–322

A HEAT DENATURATION STUDY OF THE 11S GLOBULIN IN SOYBEAN SEEDS

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ABSTRACT

When the 11S globulin, one of the major storage proteins in soybean seeds (Glycine max), *was heated at 0·5 ionic strength, the denaturation temperature was biologically estimated to be about 10 degrees higher than that at 0·1 ionic strength. The results also coincided well with those obtained by differential scanning calorimetry. The heat denaturation temperatures of the protein obtained by differential scanning calorimetry were estimated to be 78·1 °C and 89·6 °C at 0·1 and 0·5 ionic strength respectively. The enthalpies of heat denaturation were 2·0 cal/g and 3·2 cal/g at 0·1 and 0·5 ionic strength, respectively. Correlation was not observed between the heat stability at high ionic strength and the content of the ordered secondary structure or a dissociation–association reaction of the protein with change of ionic strength. However, increase of the hydrophobic region at high ionic strength indicated the possibility of stabilisation of the quaternary structure of the 11S globulin by hydrophobic bonding during heat denaturation.*

INTRODUCTION

Most soybean foods are heated during processing or in preparation for eating. The importance of the heat-treatment has been practically recognised for a long time, because the treatment raises the nutritional value of soybean proteins by inactivation of potent inhibitors of proteinases thus facilitating digestion of the denatured storage protein by means of proteases. Little, however, is known about

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the physical and chemical changes of the proteins that occur as a result of heating. About 10 years ago the changes were first studied at the molecular level.

Wolf and Tamura (1969) found that the 11S globulin was converted into a fast sedimenting aggregate and a slow sedimenting fraction by heating. Catsimpoolas *et al.* (1969) reported that glycinin (the 11S globulin) aggregated at temperatures above 70 °C and precipitated almost completely at 90 °C. However, the antigenic activity of the protein was not entirely destroyed even by heating up to 90 °C. They (Catsimpoolas *et al.*, 1970) followed the changes in turbidity of the protein solution as a function of heating time under various conditions. Results showed that rate and extent of the aggregation were favoured by low ionic strength with mercaptoethanol. The maximum rate of the aggregation was observed between pH 4·0 and 6·0. A combination of ionic and hydrophobic bonding was proposed as the basis for the aggregation.

Subsequently, Catsimpoolas *et al.* (1971) showed that a rapid loss in antigenicity of glycinin was observed in the temperature range between 70 and 90 °C at an ionic strength of about 0·15. The soluble glycinin subunits did not react with anti-glycinin serum as judged by immunodiffusion. These results suggested that loss in antigenicity was strongly associated with conformational changes involving destruction of the quaternary structure of the protein.

More recently, Hashizume & Watanabe (1979) reported that four sedimenting patterns of the acid precipitated soybean proteins, 2S, 7S, 11S and 15S, were almost lost by exposure to temperatures of 80 °C and 90 °C at low (0·001 μ) and high (0·1 μ) ionic strengths, respectively. However, changes of ultraviolet difference spectra, turbidity, reactive sulphydryl groups and disulphide bonds were still observed at temperatures higher than 80 °C or 90 °C at the respective ionic strengths.

This paper describes the effects of ionic strength on heat denaturation of the 11S globulin at ionic strengths of 0·1 and 0·5. The effects were examined by biological methods, and the thermal denaturation temperature and the denaturation enthalpy of the protein were determined by differential scanning calorimetry. The heat stability of the protein at the high ionic strength is also discussed.

MATERIALS AND METHODS

Preparation of the 11S globulin

A simple method is outlined in Fig. 1. The characteristic of this method is that the precipitate at pH 5·8 from the water-extractable fraction is used as the crude 11S fraction. The precipitate was composed of 11S sedimenting fraction with a slight contamination of 7S sedimenting fraction.

Immunochemical methods

Antiserum to the 11S globulin was prepared by immunisation procedures described previously (Koshiyama & Fukushima, 1976*a*). The experimental

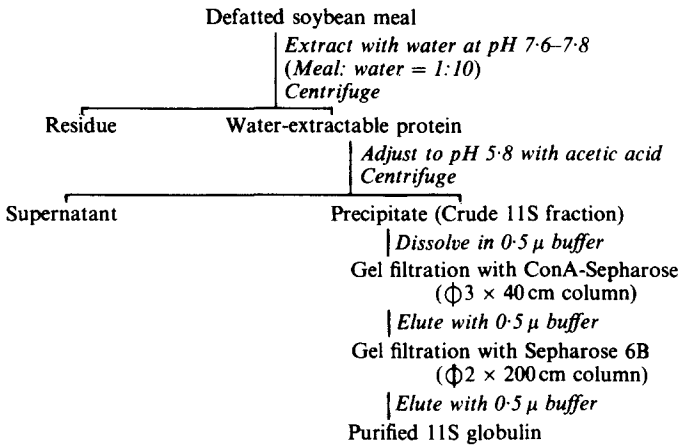

Fig. 1. Preparation of the 11S globulin.

conditions for double gel immunodiffusion of the protein have been reported (Koshiyama & Fukushima, 1976*a*).

Heating of the 11S globulin

The heat treatment of the protein was done at temperatures from 50 °C to 100 °C for 1 h in 0·1 μ and 0·5 μ buffer.

Differential scanning calorimetry (DSC)

The measurement was made with a Daini Seikosha SSC/560 differential scanning calorimeter. Determination of the thermal denaturation temperature and the enthalpy of denaturation were essentially as done by the method of Donovan & Beardslee (1975). The denaturation thermogram was obtained with a silver or aluminium pan sealed with the samples of 10–15 μl and the buffers as reference. The enthalpy of denaturation (ΔH_d) was determined by integrating the area between the denaturation curve and a base-line drawn under the peak. This value was virtually independent of the heating rate within experimental error.

Optical rotatory dispersion and circular dichroism

Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements were made using a Japan Spectroscopic model ORD/UV-5 and J-20 recording spectropolarimeter as described in a previous paper (Koshiyama & Fukushima, 1973).

Sedimentation analysis

Ultracentrifugation was carried out using a Hitachi UCA-1 centrifuge.

Determination of hydrophobic region

For estimation of the hydrophobic region in the 11S globulin the maximum amount of 1-anilino-8-naphthalene-sulphonate (ANS) as a hydrophobic probe bound to the protein was determined by the following two methods. ANS was obtained from Tokyo Kasei Kogyo Co.

(*a*) *Equilibrium dialysis:* Cellulose tubes (Visking Company) were filled with 5 ml of 0·2% protein solution in 0·1 μ and 0·5 μ buffer. The tubes were immersed in 20 ml of each ANS solution adjusted to various concentrations in both buffers and placed in a cold room at 4 °C for 5 days with a reciprocal agitation of 80 rpm. The tubes were then removed and the external solutions analysed spectrophotometrically with a Hitachi model 139 UV-VIS spectrophotometer. The value of $5{\cdot}46 \times 10^3 \text{M}^{-1}\,\text{cm}^{-1}$ at 350 nm obtained from this experiment was used as the molar absorption coefficient of ANS.

(*b*) *Fluorescence spectrum titration:* The determination of fluorescence intensity was carried out using a Japan Spectroscopic model FP-550 spectrofluorimeter with fluorescence exitation at 375 nm and emission spectrum at 475 nm. The measurements were made at room temperature.

Determination of native protein

Native protein was determined by the modified method of Fukushima (1959). For the digestion of the heated 11S globulin, 2 ml (1·75%) of each heated protein solution was transferred into L-shaped tubes (ϕ1·5 × 15 cm). Two ml of 0·005% protease solution (alkaline protease of *Aspergillus sojae*) dissolved in the both buffers was added to the tubes. The mixed solutions were kept at 30 °C for 24 h with reciprocal shaking of 60 rpm. After digestion, the tubes were filled up to 7 ml with 0·25M phosphate buffer, pH 7·2. The resulting mixtures were centrifuged at 12,000 rpm for 20 min. To 1 ml of the supernatant were added 6 ml of water, 2 ml of 2% gum arabic solution and 1 ml of trichloroacetic acid. After keeping the mixtures at 30 °C for 20 min, the turbidity produced was measured at 420 nm with a Klett–Summerson photoelectric colorimeter.

Buffers

Potassium phosphate buffer (0·0325M K_2HPO_4, 0·0026M KH_2PO_4, pH 7·60) was used as 0·1 μ buffer and the buffer containing 0·4M sodium chloride as 0·5 μ buffer. The ionic strength (μ) of the buffers was calculated from the equation

$$\mu = \Sigma r Z^2/2$$

where r is the molar concentration of each ion dissociated and Z the atomic value of each ion.

RESULTS AND DISCUSSION

Effect of ionic strength on heat denaturation of the 11S globulin

In general, as protein dispersions often turn turbid on heat treatment, spectrophotometric methods are difficult to use for heat denaturation of proteins. So, two biological methods were used for the heat denaturation of the 11S globulin.

(*1*) *Loss of antigenicity by heating:* As shown in Fig. 2, it is clear that the inactivating temperature of the antigenicity of the 11S globulin was strongly dependent on ionic strength of the protein solution. There was apparently a difference of at least 10 degrees between the two ionic strengths, because the

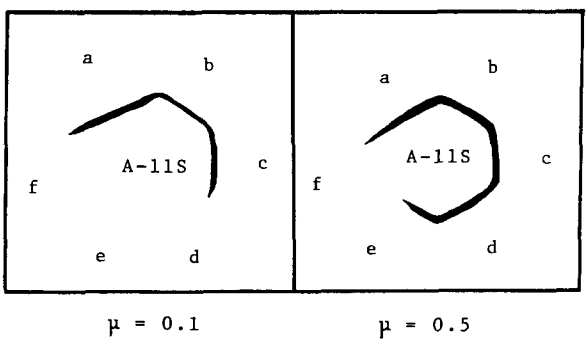

Fig. 2. Double gel immunodiffusion of the 11S globulin heated at 50 °C (a), 60 °C (b), 70 °C (c), 80 °C (d), 90 °C (e), and 100 °C (f) for 1 h in 0·1 μ and 0·5 μ buffer. A-11S; antiserum against the 11S globulin. Each well was filled with 25 μl of the heated protein at the concentration of 1·8 mg per 1 ml of each buffer.

temperature was found to be between 70 and 80 °C in 0·1 μ buffer and between 80 and 90 °C in 0·5 μ buffer. Catsimpoolas *et al.* (1969) showed that a significant immunoprecipitin band of the protein occurred at the treatment of 90 °C in 0·5 μ buffer, but only a very slightly obscured band was observed in this experiment.

(*2*) *Decrease of native protein by heating:* In Fig. 3, the decrease of the native protein by heating is shown. A marked decrease occurred between 70 and 80 °C in 0·1 μ buffer, but, in 0·5 μ buffer there was a marked decrease between 80 and 90 °C. It was also shown in this experiment that the heat stability of the protein in 0·5 μ buffer was about 10 degrees higher than in 0·1 μ buffer.

Differential scanning calorimetric studies of the 11S globulin in relation to its heat denaturation

DSC is an interesting alternative method, where the physical state of the system is unimportant (Privalov & Khechinashvilli, 1974; Donovan *et al.*, 1975; Hägerdal & Martens, 1976). Figure 4 shows typical thermograms for the thermal denaturation of the 11S globulin in 0·1 μ and 0·5 μ buffer. Each thermogram was observed to have

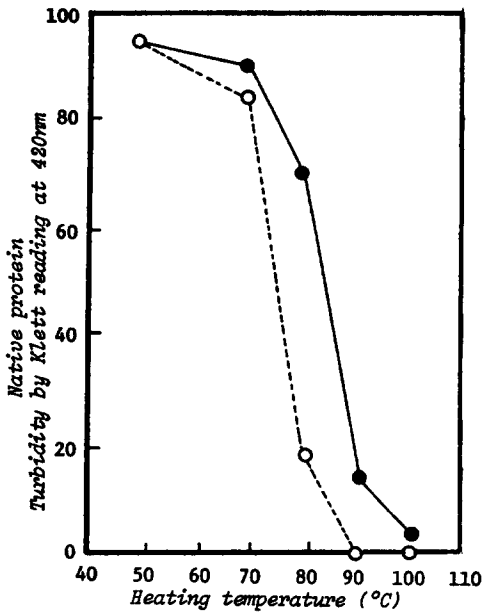

Fig. 3. Effect of heating temperature on changes of native protein concentration in the 11S globulin. ○, 0·1 μ buffer; ●, 0·5 μ buffer.

only one peak and was nearly symmetrical with no detectable shoulders or minor peaks. This fact was considered to indicate that the protein was thermally homogeneous.

The denaturation process starts at the point where the curve begins to deviate from the base-line. However, as this temperature is difficult to identify, the intercept of the extrapolated slope of the peak and base-line is taken as a start of the denaturation temperature (T_d). But, as biopolymers such as proteins have a wide range of thermal transition temperatures, the temperature at the peak maximum (T_{max}) is also generally used to indicate heat denaturation (Donovan *et al.* 1975; Hagerdal & Martens, 1976). The denaturation temperature was dependent on the heating rate of the DSC (Fig. 5). The extrapolated temperature of heat denaturation to zero degrees of the heating rate (T^0_{max}) was calculated to be 78·1 °C in 0·1 μ buffer and 89·6 °C in 0·5 μ buffer by regression analysis.

The enthalpy changes for heat denaturation (ΔH_d) are proportional to the area under the peak in the DSC thermogram. ΔH_d for the protein was 608 kcal in 0·1 μ buffer and 1010 kcal per mole in 0·5 μ buffer at the molecular weight of 312000 (Koshiyama & Fukushima, 1976*b*).

These values seem to be somewhat lower than those of enzyme proteins. Specific enthalpies of denaturation have been reported to be 4·8–6·5 cal/g for ribonuclease

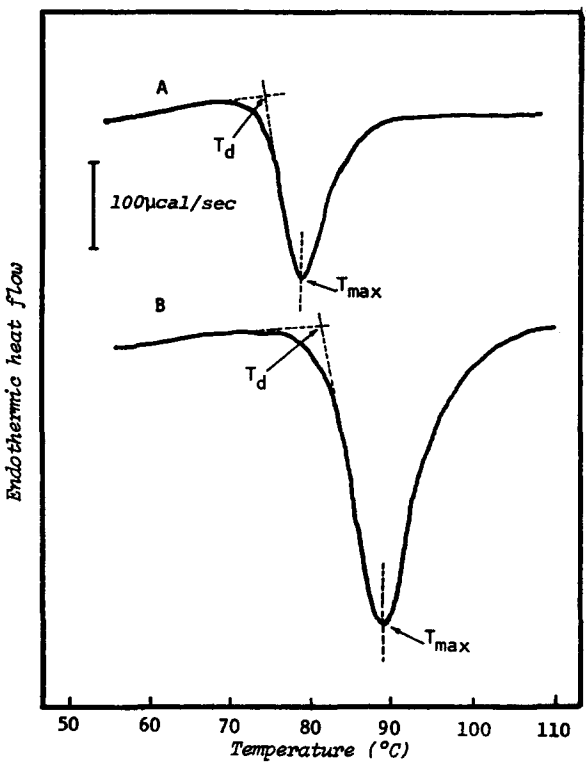

Fig. 4. Differential scanning calorimetric thermograms for thermal denaturation of the 11S globulin. Curve A, 11 μl of 1% 11S globulin in 0·1 μ buffer; Curve B, 15 μl of 1% 11S globulin in 0·5 μ buffer. Heating rate, 2·5 °C per min.

(Donovan & Ross, 1973), 8·8 cal/g for lysozyme (Donovan & Ross, 1973) and 3·7–5·7 cal/g for chymotrypsinogen (Jackson & Brandts, 1970; Biltonen *et al.*, 1971). In contrast, the value for the 11S globulin was 3·2 cal/g in 0·5 μ buffer and 2·0 cal/g in 0·1 μ buffer. These low values may possibly be attributed to the low content of the ordered secondary structure of the protein (Koshiyama, 1972; Koshiyama & Fukushima, 1973).

Some approaches to the heat stability of the 11S globulin at high ionic strength

Apparently the 11S globulin was more stable by about 10 degrees in 0·5 μ buffer than in 0·1 μ buffer during heat denaturation, from the results mentioned above. What differences in the structure are there at the two ionic strengths?

(1) ORD and CD experiments: At first, it was considered that a high content of the ordered secondary structure at the high ionic strength could be responsible for the high heat stability. However, no detectable differences in the secondary structures at

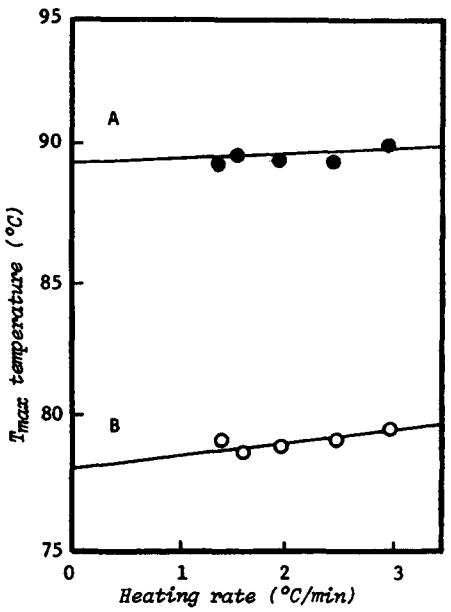

Fig. 5. The dependence of the peak temperature of the thermogram on the heating rate. A, 0·1 μ buffer; B, 0·5 μ buffer.

the two ionic strengths could be found from ORD and CD results as shown in Table 1 and Fig. 6.

(*2*) *Sedimentation analysis:* Recently, Stellwagen & Wilgus (1978) suggested that by increasing the internal area of proteins at the expense of the surface area, the resulting solvent-accessible surface area decreases their heat stability. To increase thermostability in larger polypeptide chains two alternative ways are suggested. One involves using globular surfaces to contribute to conformational stability through association of globular subunits to form a polymer. A second way is folding a single chain into multiple globular domains.

In the former case, to increase the surface area of the 11S globulin will in particular cause the protein to dissociate when the ionic strength increases, into two

TABLE 1

COMPARISON OF THE SECONDARY STRUCTURE OF THE 11S GLOBULIN IN 0·1 μ AND 0·5 μ BUFFER BY ORD (300–600 nm)

I	a_0	b_0	*α-helix*	*β-structure*	*random coil* (%)
0·1	−260	−32	5·1	33·2	61·7
0·5	−246	−33	5·2	34·8	60·0

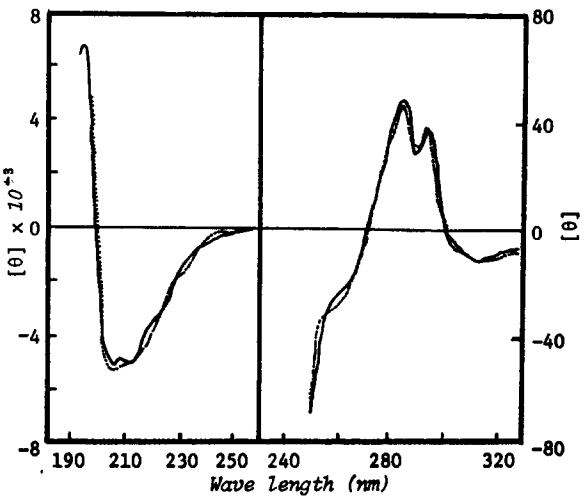

Fig. 6. CD spectra of the 11S globulin in 0·1 μ (——) and 0·5 μ (– – –) buffer.

globular $^{7}S_{t}$ units with a cooperative loss in the quaternary structure. However, no dissociation and association reaction with the change of ionic strength was found, as shown in Fig. 7.

(*3*) *Determination of hydrophobic region:* Recently, Takagi *et al.* (1979) reported that the hydrophobic region in the soybean globulin denatured by heating increased to twice that in the native protein (by measuring with ANS).

As shown in Fig. 8, the fluorescence emitted by the hydrophobic region of ANS absorbates on the 11S globulin increased also by heating up to 80 °C at 0·1 ionic strength and 90 °C at 0·5 ionic strength. The fluorescence polarisation was higher

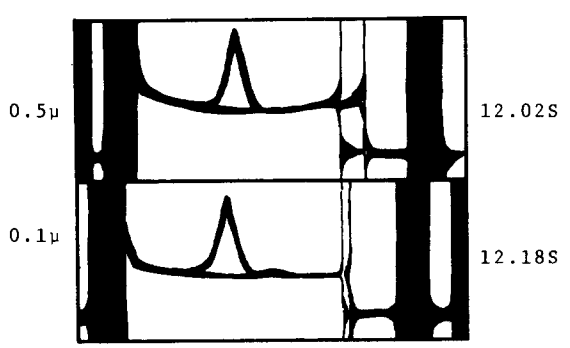

Fig. 7. Ultracentrifugal patterns of the 11S globulin in 0·1 μ and 0·5 μ buffer. The protein concentration was 0·72 %. Bar angle was 60° at 20 °C. Photographs were taken at 40 min. after reaching a full speed of 51 200 rpm. Direction of sedimentation is from right to left.

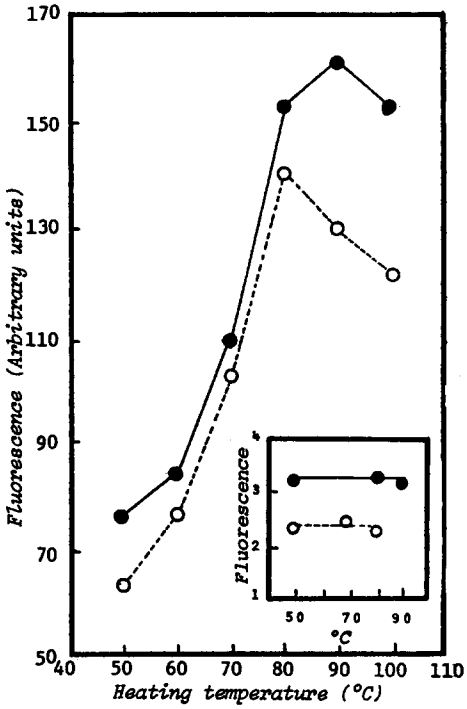

Fig. 8. Effect of heating temperature on changes of hydrophobic region of the 11S globulin in 0·1 μ (○) and 0·5 μ (●) buffer.

than in the former at their maximum points. However, turbidity simultaneously developed by heating the protein solution, especially at the high ionic strength and high temperature, so the protein concentration of the supernatant was adjusted after the heated protein solutions were centrifuged at 45,000 rpm for 30 min. The fluorescence polarisations of the supernatants are compared in the small frame of Fig. 8 which shows that the fluorescence polarisation at 50 °C at both ionic strengths was constant even at higher temperature. The increased fluorescence intensity, due to the newly formed hydrophobic region, was considered to have been transferred to the precipitates. Thus, the hydrophobic regions at both ionic strengths were determined by the following two methods.

(*a*) *Equilibrium dialysis:* The absorption of ANS on the 11S globulin is characterised by the quantities of the maximum number of moles of ANS absorbed per mole of the protein (n) and the dissociation constant of the ANS/11S globulin complexes (K) according to the equation (1) of Klotz *et al.* (1946) if all the dissociation constants are identical,

$$1/r = K/nC + 1/n \qquad (1)$$

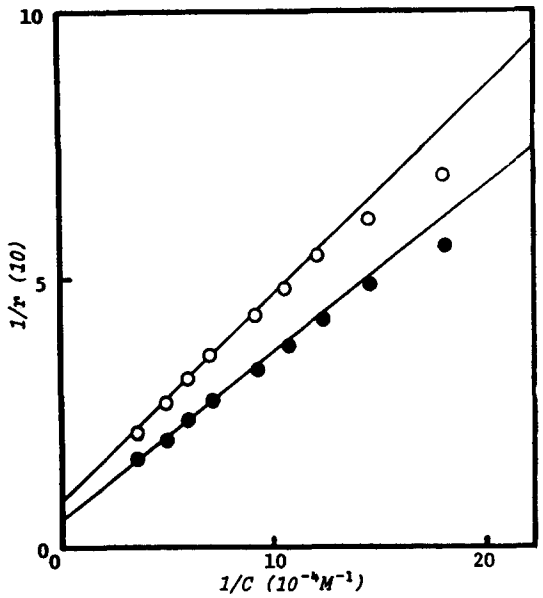

Fig. 9. A plot of reciprocal of the ANS bound to the protein against the free ANS concentration○, 0·1 μ buffer; ●, 0·5 μ buffer.

where r is the moles of ANS bound to the total moles of the protein and C the moles of free ANS. A reciprocal plot of $1/r$ against $1/C$ is shown in Fig. 9. For $1/C$ smaller than $1{\cdot}20 \times 10^{3}\text{M}^{-1}$, the linear relationship was fulfilled at both ionic strengths. The values of K and n were evaluated over the range of the linear relationship by the method of least squares. The value of n was obtained as 13·2 and K as $5{\cdot}10 \times 10^{-5}$M at 0·1 ionic strength whereas n was found to be 21·3 and K to be $6{\cdot}82 \times 10^{-5}$M at 0·5 ionic strength.

(*b*) *Fluorescence spectrum titration:* Weber & Young (1964) rearranged eqn. (1) to give eqn. (2) for fluorescence spectrum titration:

$$np/(D_0 + K) = x = F/F_0 \qquad (x < 1) \tag{2}$$

where p is the total protein concentration, D_0 is the ANS concentration and x the fraction of ANS bound. The value x equals F/F_0 where F_0 is the actual absorbed fluorescence efficiency when all the ANS in solution has been absorbed. F_0 was determined by a reciprocal plot of $1/F$ aginst $1/p$ as shown in Fig. 10. For determination of F_0, high ($2{\cdot}68 \times 10^{-4}$M) and low ANS concentrations ($2{\cdot}68 \times 10^{-5}$M) were used. The values of F_0 in high ANS concentration were 263 and 294, and those in low ANS concentration 24 and 32 at 0·1 and 0·5 ionic strength, respectively.

Using eqn. (2), the relationship between p and x is shown in Fig. 11. In low

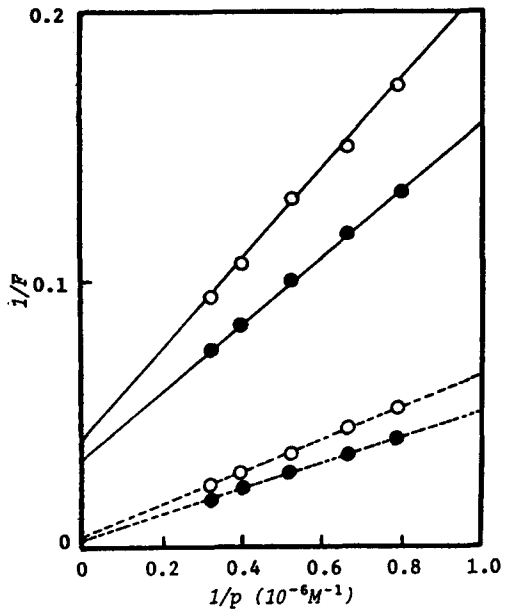

Fig. 10. A reciprocal plot of $1/F$ against $1/p$. ○, 0·1 μ buffer; ●, 0·5 μ buffer; ——, $2{\cdot}68 \times 10^{-5}$M ANS; ------, $2{\cdot}68 \times 10^{-4}$M ANS.

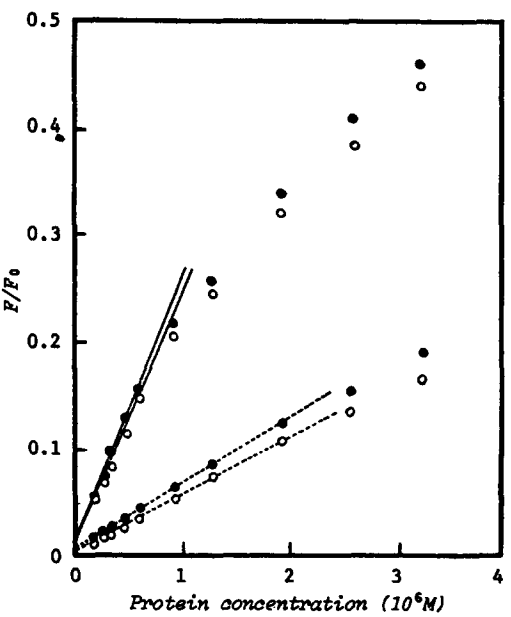

Fig. 11. Plot of ANS bound to the globulin as a function of protein concentration. ○, 0·1 μ buffer; ●, 0·5 μ buffer; ——, $2{\cdot}68 \times 10^{-5}$M ANS; ------, $2{\cdot}68 \times 10^{-4}$M ANS.

concentration of the protein, the linear relationship was obtained in all cases investigated. The values of n and K obtained by using these linear ranges were 18·4 and $6{\cdot}75 \times 10^{-5}$M, and 23·2 and $9{\cdot}09 \times 10^{-5}$M at 0·1 and 0·5 ionic strength, respectively. Although these values did not always agree with those obtained by the dialysis experiment, the amounts of ANS bound to the protein were larger at the high ionic strength than at the low ionic strength in both experiments.

The observed denaturation data are in good agreement with the literature data on the 11S globulin (Catsimpoolas *et al.*, 1970; Hashizume *et al.*, 1975; Hashizume & Watanabe, 1979). Dissociation into subunits has been found to be a necessary step prior to thermal aggregation of the 11S globulin (Wolf & Tamura, 1969). Considering this fact, high ionic strength (by adding salt) seems to prevent the dissociation of the quaternary structure, that is, to stabilise the structure against dissociation and denaturation. Also, hydrophobic bonding must strongly contribute to the stabilising of the quaternary structure at 0·5 ionic strength.

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Food Chemistry 6 (1980–81) 323–335

FRACTIONATION OF POTATO JUICE PROTEINS INTO ACID-SOLUBLE AND ACID-COAGULABLE FRACTIONS*

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ABSTRACT

Potato juice proteins were fractionated by acidification to pH 3 into acid-coagulable and acid-soluble fractions. Both fractions are heterogeneous. They differ in molecular weight, conformation in solution and composition of amino acids. The acid-coagulable fraction consists of units of molecular weight between 32,000 and 87,000, while the acid-soluble fraction consists of units of molecular weight between 17,000 and 27,000. Circular dichroism measurements show that the acid-coagulable fraction is in helical and beta conformations whilst, for the acid-soluble fraction, a substantial contribution of the random conformation is observed. The acid-coagulable fraction has a higher content of methionine than the acid-soluble fraction. It is shown that this type of fractionation is more meaningful than fractionation in terms of albumins and globulins by solubility in water or dilute salt solutions.

INTRODUCTION

The potato starch industry produces copious amounts of potato juice which has a high BOD value and therefore cannot be discharged in public waterways (Anon., 1968). Potato juice can be considered as a dilute solution containing about 2–5% solids having approximately the following composition: 35% proteins and amino acids, 35% sugar, 20% minerals, organic acids and other minor components (Knorr *et al.*, 1977; this laboratory, unpublished). The interest in the recovery of potato proteins from potato juice increases not only because of their contribution to the BOD value of the effluents of potato-processing industries (Anon., 1968), but also because of their potentially high nutritive value. Potato proteins are considered to be amongst the most valuable vegetable proteins, with a high content of lysine and with the sulphur-containing amino acids, methionine and cystine, as their limiting amino acids (Markakis, 1975; Thompson, 1978; Knorr, 1978).

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Potato proteins consist of a heterogeneous mixture, with thirty fractions resolved by Loeschke & Stegeman (1966). Classification of potato proteins by classical methods gave ambiguous results. Based on fractionation by solubility in $(NH_4)_2SO_4$ solutions of various degrees of saturation (Lindner *et al.*, 1960), potato proteins were considered to consist mainly of a globulin (76 %) named tuberin and other minor protein components, among them the albumin, tuberinin (de Groot *et al.*, 1947; Jirgensons, 1946). However, by classical fractionation of potato proteins according to solubility in solvents, water, dilute salt, aqueous alcohol and dilute alkali (which classifies them in terms of albumins, globulins, prolamins and glutelins), tuberin was not resolved as a single component but was shown to be a mixture of albumins and globulins (Kapoor *et al.*, 1975). In the course of studies of protein recovery from potato juice conducted in our laboratory (1978, unpublished), we observed that only part of the proteins soluble in potato juice coagulate by acidification at room temperature. A similar observation was reported by Knorr *et al.* (1977).

The purpose of this investigation was to study the fractionation of potato juice proteins into acid-soluble and acid-coagulable fractions and to see whether a relationship can be established between this fractionation and fractionation in terms of albumins and globulins.

EXPERIMENTAL

Preparation of potato juice

Potato juice was prepared from tubers of the variety Désirée from the preceding season, stored at 8 °C for 2–4 months.

Potatoes were washed, peeled and disintegrated in a laboratory Waring Blendor with 25 % added water and 1000 ppm $NaHSO_3$. The slurry was pressed through a cloth and the juice was centrifuged for 10 min at 8000 *g*. The centrifugation removed the remaining starch granules and other coarse particles.

Preparation of acidified potato juice

Part of the juice was acidified to pH 3 with 25 % H_2SO_4 v/v. After holding for 1 h at room temperature, the precipitate was removed by centrifugation for 15 min at 27,000 *g*. A clear yellowish supernatant was obtained.

Solubility versus pH of native potato juice proteins

Aliquots of 40 ml potato juice were acidified to various levels of pH with 25 % H_2SO_4 v/v, held for 1 h at room temperature, and centrifuged for 15 min at 27,000 *g*. Clear supernatants were obtained, from which three 8-ml aliquots were withdrawn for determination of the TCA-coagulable protein (see below).

Dialysis

Potato juice was dialysed at 4 °C against 20 volumes of the following solutions: (a) deionised water; (b) 50 mM buffer phosphate at pH 6·35 (ionic strength, $I = 0{\cdot}07$); (c) 5% K_2SO_4 in 10 mM buffer phosphate, pH 6·15 (ionic strength, $I = 0{\cdot}88$).

Dialysis was continued for five days, during which period the dialysing solutions (a), (b) and (c) were changed at least five times.

Acidified potato juice was dialysed under the same conditions against solutions (a) and (b). Before dialysis against deionised water the pH was raised to its original value (5·8). The content of the dialysis tubes was centrifuged for 20 min at 27,000 *g* and the supernatants were analysed for their contents of nitrogen and TCA-coagulable nitrogen. The pellets were dissolved in 0·2N NaOH and the solution was analysed for nitrogen content (see below).

Gel filtration

The dialysed preparations were fractionated at room temperature on a Sephadex G-100 (Pharmacia Fine Chemicals) column with dimensions of 62 × 2·0 cm, or on a G-200 column with dimensions of 54 × 2·0 cm. The column was eluted at a flow rate of 12 ml/hour with 50 mM phosphate buffer, pH 6·35, to which 0·02% of NaN_3 was added to prevent microbial growth. Fractions of 2 ml were collected and their absorbance at 280 nm was measured. The protein content of the various tubes was determined by the absorbance at 280 nm or according to the method of Lowry *et al.* (1951).

SDS polyacrylamide gel electrophoresis

SDS gel electrophoresis was carried out on slabs of 10% polyacrylamide gel containing SDS and tris HCl buffer according to the method of Weber & Osborn (1975). Samples with and without mercaptoethanol treatment were applied to the gels. The samples subjected to electrophoresis were dialysed potato juice, dialysed acidified potato juice and the fractions separated by gel filtration on the Sephadex columns.

BSA, ovalbumin, trypsin and myoglobin (Sigma) were used as marker proteins. The plot of log molecular weight versus the migration distance of the markers was used to assign molecular weights to the various bands. The results presented in Fig. 5 (see the section *Gel electrophoresis*), show the molecular weight of the bands resolved from the various samples.

Circular dichroism (CD) measurements

For each peak the contents of a few tubes with the highest absorbance were combined and dialysed against 50 mM phosphate buffer, pH 6·35, to remove the azide ions which interfere with the CD measurements due to their high absorbance below 230 nm.

After dialysis the protein content was assayed according to the method of Lowry *et al.* (1951). Circular dichroism spectra were measured on a Cary 60 spectropolarimeter working on the CD mode, using cells with a path length of 1 mm. The results were transformed to molar ellipticities (degree $\times$ cm^2 $\times$ decimole^{-1}), assuming a mean residue weight of 110 for potato proteins.

Nitrogen content

Nitrogen content was determined by micro-Kjeldahl.

Trichloroacetic acid (TCA)-coagulable nitrogen

Nitrogen coagulable by TCA was considered as protein nitrogen. Protein was precipitated at room temperature by TCA at a final concentration of 15%. The pellet, after centrifugation (15 min at 10,000 *g*), was washed with 5% TCA and dissolved in 1N NaOH. From this solution a sample was taken for micro-Kjeldahl determination of nitrogen.

Protein

Protein was estimated as coagulable N $\times$ 6·25 or by the method of Lowry *et al.* (1951).

Carbohydrates

Carbohydrates were determined by the method of Dubois *et al.* (1956).

Amino acid analysis

Amino acid analysis, of dialysed potato juice and the fractions separated by gel filtration (after removal of the azide ions by extensive dialysis), was carried out by the standard methods used in the Division of Animal Nutrition of the Agricultural Research Organisation, Israel. Aliquots of the samples were lyophilysed and hydrolysed for 22 h with 6N HCl at 110°C in a nitrogen atmosphere. The hydrolysates were analysed on a Technicon TSM 1 analyser (Chertsey, Surrey, Great Britain).

RESULTS AND DISCUSSION

Only about 25% of the total nitrogen content (usually termed crude nitrogen) of potato juice is coagulable by TCA and can therefore be considered as protein nitrogen.

pH dependence of the precipitation of proteins from potato juice

Precipitation of potato juice proteins versus pH is shown in Fig. 1. Maximum precipitation, usually 35% to 40% of the protein content, is obtained at pH values

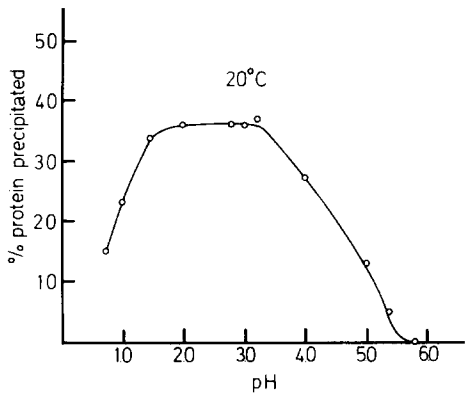

Fig. 1. Precipitation of potato juice proteins as a function of pH.

between 3 and 2. The precipitation curve is valid for relatively non-diluted potato juice and resembles that obtained by Knorr *et al.* (1977) for potato juice produced from Russet Burbank potatoes.

As is the case with other proteins, the solubility of potato proteins is a function of both pH and ionic strength. Waste water from potato chip factories is more diluted than the potato juice considered here and therefore a different dependence of protein solubility on pH was obtained in the former case by Meister & Thompson (1976).

Dialysis experiments

The results of a typical dialysis experiment are presented in Table 1. After dialysis the values of the TCA-coagulable and the total nitrogen contents were about the same, as expected. From Table 1 it can be seen that at pH values above 5·3, potato juice proteins were soluble in 50 mM phosphate buffer and in 5 % K_2SO_4, but only partially soluble at low ionic strength (deionised water). The small amounts of nitrogen precipitating on dialysis against 50 mM phosphate or 5 % K_2SO_4 (Table 1) seem to us to represent non-soluble nitrogen, bound to particles which were not removed by centrifugation during the preparation of the potato juice. A total of 36 % of the potato juice proteins precipitated on dialysis against deionised water. Of the proteins soluble in acidified potato juice, 78 % precipitated on dialysis against deionised water. Therefore, this fraction consists mainly of globulins. Precipitation was rapid (a few hours) when the acidified juice (pH 3) was dialysed against deionised water without raising the pH to its original value (5·8). When the pH value was raised to 5·8 prior to dialysis against deionised water, precipitation appeared only after extensive dialysis of at least 48 h.

The increased solubility of potato proteins at higher ionic strength is demonstrated in the last column of Table 1, which gives the lowest pH value to which

TABLE 1
DISTRIBUTION OF NITROGEN BETWEEN PRECIPITATE AND SUPERNATANT IN DIALYSED POTATO JUICE

	Total N, mg/ml[b] *(±0·02)*	*TCA-coagulable N, mg/ml*[b] *(±0·01)*	*% N precipitated*	*pH of precipitation*[c]
Potato juice				
Not dialysed	3·17	0·81	—	5·4
Dialysed against deionised water				
Supernatant	0·36	0·33	36	
Precipitate	0·21			
Dialysed against 50 mM phosphate buffer, pH 6·35 ($I^a = 0·07$)				
Supernatant	0·64	0·62	8	5·3
Precipitate	0·05			
Dialysed against 5% K_2SO_4 in 10 mM phosphate buffer, pH 6·15 ($I = 0·88$)				
Supernatant	0·69	0·65	4	3·3
Precipitate	0·03			
Acidified potato juice (pH 3)				
Not dialysed	2·75	0·43	—	—
Dialysed against deionised water				
Supernatant	0·07		78	
Precipitate	0·24			
Dialysed against 50 mM phosphate buffer, pH 6·35 ($I = 0·07$)				
Supernatant	0·38	0·34	—	4·2
Precipitate	—			

[a] Ionic strength.
[b] Concentration refers to 1 ml of supernatant after dialysis.
[c] Highest pH at which precipitation from the juice or from the supernatant of the dialysed preparations starts.

the supernatant of the dialysed preparation could be adjusted before flocculation started. This pH value is 5·4 for potato juice prepared as described in the section above headed 'Experimental'; we estimate the ionic strength to be in the range 0·1–0·2 (this estimate is based on concentrations of 90 mM K^+ and 25 mM Na^+). At higher ionic strength, after dialysis against 5% K_2SO_4 ($I = 0·88$), precipitation appears at pH values below 3·3.

Potato proteins also contain small amounts of prolamines and glutelins (Kapoor *et al.*, 1975), but these are probably not extracted in the procedure used for the preparation of the potato juice.

Gel filtration

The elution profiles from the Sephadex G-100 column of potato juice and acidified potato juice, dialysed against phosphate buffer, are shown in Figs 2 and 3, respectively. The elution profile of potato juice consists of three peaks of absorbance at 280 nm. By applying the Lowry *et al.* (1951) protein assay to the content of various

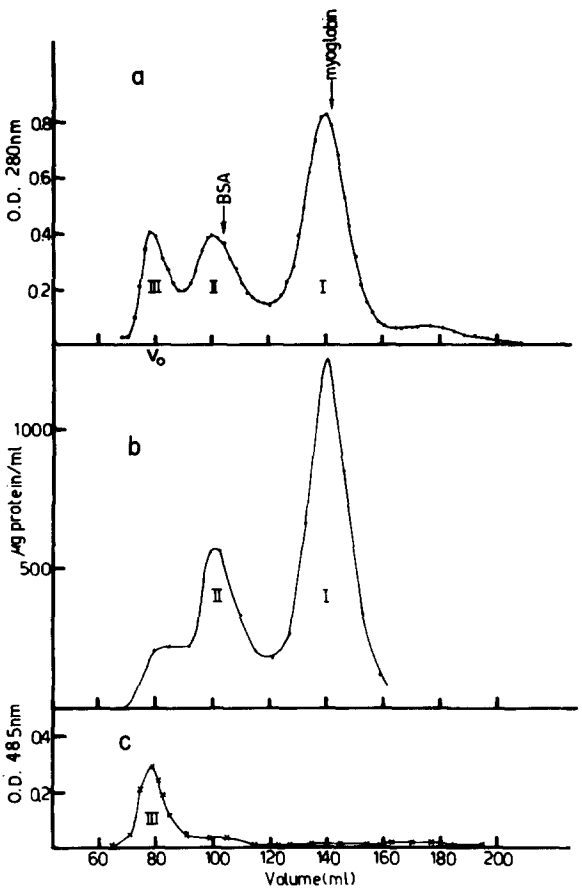

Fig. 2. Gel filtration of potato juice proteins on a Sephadex G-100 column. Elution profiles: (a) absorbance at 280 nm; (b) protein (Lowry *et al.*, 1951); (c) carbohydrates (Dubois *et al.*, 1956). Sample: potato juice dialysed against 50 mM phosphate buffer, pH 6·35. Elution buffer: 50 mM phosphate buffer, pH 6·35 + 0·02% sodium azide. V_0 = void volume.

tubes, it was demonstrated that only two of them—I and II—are protein peaks. Peak III, which also appears at the void volume of G-200, is not a protein peak, although it contains a certain amount of protein (see Fig. 2(b)). Peak III was slightly turbid and had a ratio of 1·1 between the absorbance at 280 nm and 260 nm. After boiling for 1 min with 1% SDS, a normal protein spectrum was obtained with a ratio of 1·6 and a maximum at 278 nm. The extinction at 260 nm is thus due both to turbidity and to absorbance of the small amounts of protein eluted under this peak but is not due to nucleic acids. By applying the phenol sulphuric acid assay (Dubois *et al.*, 1956) to the various tubes, a carbohydrate peak was found under peak III (Fig. 2(c)). This carbohydrate did not give a blue colour with iodine.

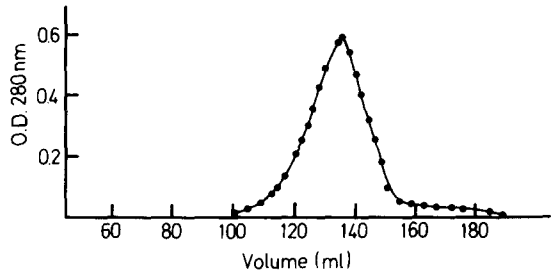

Fig. 3. Gel filtration of potato juice proteins on a Sephadex G-100 column. Sample: acidified potato juice after dialysis against 50 mM phosphate buffer, pH 6·35. Elution buffer: see legend to Fig. 2.

As can be seen from Fig. 2(b), peaks I and II represent two protein fractions. From Figs 2 and 3 it is seen that the high molecular weight fraction II is quantitatively precipitated on acidification, and that potato juice acidified to pH 3 contains only low molecular weight proteins (fraction I). The elution profiles of the same samples from the Sephadex G-200 column show the same main peaks with the same positions relative to the marker proteins. No additional peak was resolved between peaks II and III.

The elution profile of the supernatant of potato juice dialysed against deionised water (see Table 1) is shown in Fig. 4. The ratio between the estimated areas (height of the peak × width at half of its height) of peaks I and II is about 2·0 in Fig. 2 and 1·8 in Fig. 4. This means that on dialysis against deionised water, the decrease in the solubility of both fractions was about the same. In the absence of fraction II proteins, 78% of fraction I proteins were found to precipitate on dialysis against deionised water (Table 1, acidified potato juice). When both fractions were dialysed together against deionised water, only 36% of the total protein precipitated (Table 1, potato juice). Therefore, from the results of the dialysis experiments, we expect the elution profile in Fig. 4 to consist mainly of peak II. The fact that fraction I proteins are partially retained in solution when both fractions are dialysed together against water (Fig. 4) can be explained by assuming that, at low ionic strength, a certain degree of association between fraction I and fraction II takes place.

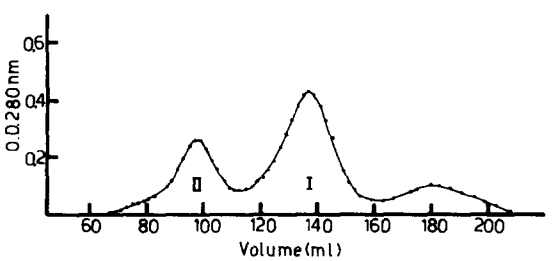

Fig. 4. Gel filtration of potato juice proteins on a Sephadex G-100 column. Sample: supernatant of potato juice dialysed against deionised water (see Table 1). Elution buffer: see Fig. 2.

By definition, proteins soluble in dilute salt solutions (5 % K_2SO_4) and in water are classified as albumins, while proteins soluble in dilute salt solutions but insoluble in water are classified as globulins. The dialysis experiments suggest that 64 % of the potato juice proteins are albumins whilst 78 % of the proteins remaining soluble in acidified potato juice (fraction I) are globulins. From Fig. 4 it can be seen that globulins are retained in solutions after dialysis of potato juice against water. We therefore conclude that the results of our dialysis experiments cannot be used to classify potato juice proteins in terms of albumins and globulins.

Gel electrophoresis

The electrophoretic results are presented in Fig. 5. Ignoring the numerous weak bands, we observe in the pattern of the dialysed potato juice a strong band which migrates like ovalbumin and a group of bands—two of them strong, two of medium intensity and one weak—distributed between trypsin and myoglobin (Fig. 5,

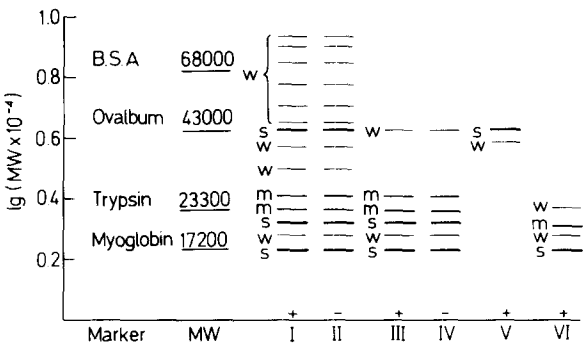

Fig. 5. SDS-polyacrylamide gel electrophoresis of potato juice proteins. Patterns I and II: potato juice dialysed against 50 mM phosphate buffer, pH 6·35. Patterns III and IV: acidified potato juice dialysed against 50 mM phosphate buffer, pH 6·35. Pattern V: fraction II, see Fig. 2. Pattern VI: fraction I, see Fig. 2. +: Treated with mercaptoethanol; −: not treated with mercaptoethanol; s = strong; m = medium; w = weak.

patterns I and II). The pattern obtained from dialysed acidified potato juice consists of the five low molecular weight bands and a weak band migrating like ovalbumin (Fig. 5, patterns III and IV). The latter represents a residual amount of protein of higher molecular weight protein which was left in the pH 3 supernatant.

The electrophoretic patterns of the two protein fractions resolved by gel filtration from dialysed potato juice are shown in Fig. 5, patterns V and VI. The pattern of fraction II consists mainly of the strong band moving like ovalbumin, which is observed in the pattern of whole potato juice. The samples obtained from the Sephadex column were not concentrated prior to electrophoresis. Therefore, due to the dilution effect introduced by the gel filtration, the patterns obtained from these fractions were less intense than those obtained from samples not subjected to gel

filtration. For this reason most weak bands observed in potato juice patterns do not appear in the patterns of fractions I and II. Samples treated or not treated with mercaptoethanol show the same patterns (Fig. 5, compare pattern I with II and pattern III with IV). We therefore conclude that, in their native state, the units resolved by gel electrophoresis are not intermolecularly crosslinked by disulphide bonds.

From the patterns of fractions I and II we conclude that fraction II proteins are not aggregates or oligomers of fraction I proteins. We also note that the size estimated by gel filtration for fraction I proteins agrees roughly with the molecular weights of the bands resolved by electrophoresis (between trypsin and myoglobin). In gel filtration fraction II proteins have an apparent molecular weight greater than BSA (molecular weight = 68,000), whilst in electrophoresis they migrate like ovalbumin (molecular weight = 42,000). The apparent molecular weights estimated by gel filtration in the absence of SDS or a strong hydrogen bond breaker (like a guanidine salt) in the elution medium depend very much on the degree of aggregation and specific conformation assumed by the protein molecules in the elution medium. In the electrophoresis medium the SDS disaggregates hydrogen-bonded aggregates into their units. It therefore seems reasonable that, in the elution medium and probably also in the natural potato juice, fraction II consists of dimers of units with a molecular weight of 42,000. Although the pattern of fraction II consists essentially of one strong band, this fraction is not necessarily homogeneous and even the strong band may represent a mixture of different molecules of about the same size.

Circular dichroism

Circular dichroism of fractions I and II (see Fig. 2) are shown in Fig. 6. Fraction II exhibits a negative ellipticity with a minimum at 218 nm and a positive ellipticity below 200 nm. Fraction I exhibits a completely different CD spectrum. It has a positive ellipticity with a maximum at 228 nm, and a negative ellipticity below 219 nm. Using the reference spectra of Chen *et al.* (1974) for the three basic conformations—helix, beta and random—we conclude that fraction II proteins are mainly in helical and beta conformations, whilst fraction I protein is mainly in the beta and random conformations. Since both protein fractions are heterogeneous, we see no advantage to a more detailed conformational analysis and are content with the conclusions that the high-molecular weight fraction has a more ordered conformation than the low-molecular weight one.

Amino acid analysis

The amino acid analysis of non fractionated potato juice proteins, fractions I and fraction II proteins are shown in Table 2. The results are expressed as milligrammes of amino acid per 1 g N and as number of residues per 1000 residues. For the latter

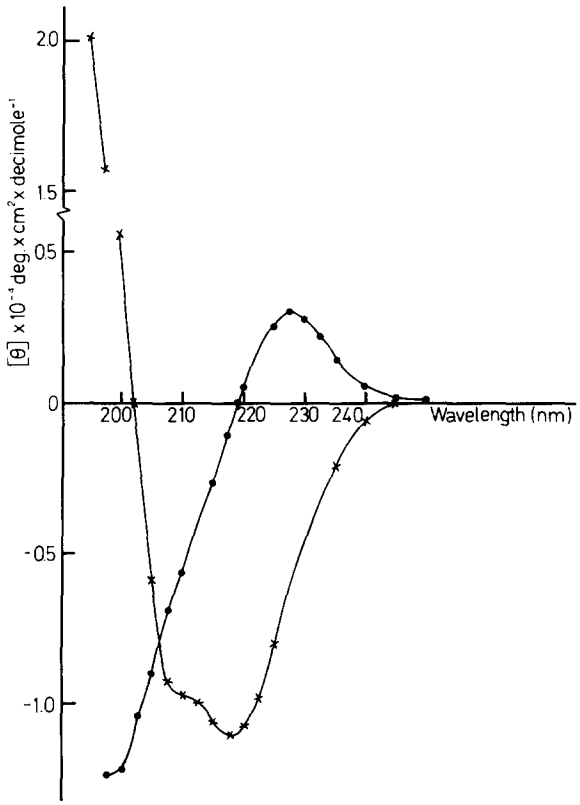

Fig. 6. Circular dichroism of potato juice protein. ●—●: Fraction I; ×—×: fraction II.

purpose we ignore the relatively small contribution of cystine, cystein and tryptophan—which are destroyed during the hydrolysis—and consider the sum of the micromoles of the seventeen measurable amino acids as 100% micromoles of residues. The error introduced by such a procedure is estimated to be less than 5%. The presentation of the results as number of residues per 1000 residues gives more direct structural information than the usual presentation as milligrammes of amino acid per 1 g N.

It is of interest to know whether the difference in amino acid composition between fractions I and II can be related to the difference in conformation observed in their CD spectra. Generally, a high proportion of hydrophobic residues favours the formation of a helical structure. In Table 2 it is seen that the content of the hydrophobic residues (6–11) is about the same for both fractions: 40·6% and 42·3% of the residues for fractions I and II, respectively. Thus, a higher degree of hydrophobicity cannot be ascribed to one of the fractions. Fraction I is somewhat

TABLE 2
AMINO ACID ANALYSIS OF POTATO JUICE PROTEINS

Amino acid	*Fraction I residues per 1000[a] mg a.a[b] per 1 g N*		*Fraction II residues per 1000[a] mg a.a[b] per 1 g N*		*Non-fractionated proteins[c] residues per 1000[a] mg a.a[b] per 1 g N*	
1. Aspartic acid	121	953	97	768	112	874
2. Glutamic acid	71	617	105	913	87	756
3. Lysine	72	619	68	584	72	622
4. Arginine	35	360	34	352	38	384
5. Histidine	15	137	19	175	16	146
6. Threonine	50	350	74	517	56	394
7. Valine	106	737	70	482	91	625
8. Alanine	45	234	95	502	64	320
9. Leucine	90	700	95	737	90	694
10. *Iso*-leucine	59	462	42	325	54	414
11. Phenylanaline	56	543	48	474	50	485
12. Serine	69	429	67	407	63	388
13. Glycine	97	431	76	340	90	397
14. Tyrosine	43	462	40	435	40	420
15. Methionine	12	106	22	201	17	150
16. Proline	59	404	50	336	59	389
17. Hydroxyproline	Traces		Traces		Traces	

[a] Number of residues per 1000 residues.
[b] Amino acid.
[c] Potato juice proteins dialysed against 50 mM phosphate buffer, pH 6·35 (see Table 1).

richer in proline than fraction II. Proline cannot be incorporated in an alpha helix and is known as a helix breaker.

From the nutritive point of view, we note the higher level of methionine in fraction II compared with fraction I. Methionine is considered as the limiting amino acid in potato proteins (Kapoor *et al.*, 1975).

CONCLUSIONS

By acidification of potato juice to pH 3, potato juice proteins are resolved into two major distinct groups—a low molecular weight (<27,000) group which remains soluble in the juice and a high-molecular weight (32,000–87,000) group which precipitates. These two protein groups have different conformations in solution and different amino acid compositions.

Classification of potato juice protein in terms of albumins or globulins by dialysis against the appropriate solutions gave ambiguous results; therefore, we prefer classification in terms of acid-soluble and acid-coagulable fractions.

The classification of potato proteins in terms of acid-coagulable and acid-soluble proteins is meaningful and also relevant from the practical point of view, since the fractionation can be carried out easily on an industrial scale. It is expected that

separate recovery of each fraction will yield products with different nutritional and functional properties.

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Food Chemistry 6 (1980–81) 337–339

INDEX TO VOLUME 6

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