EDITORIAL

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Whether custom is at the village shop or one of the big supermarkets, certain standards in the foodstuffs purchased are expected. Some of these are not always achieved. The vendor expects his goods to appeal to the would-be purchaser, either by an attractively printed pack, or by a visual display of the goods offered for sale. The purchaser expects the pack to open easily without damaging the goods or frustration to himself. Above all, perhaps, he requires the goods themselves to be in an expected condition, e.g. ready to be eaten, or able to be cooked easily, with little preliminary preparation. A little thought will suggest that there is a certain amount of conflict between these objectives.

The papers presented at this symposium show that there are a good many more problems than at first come to mind. Modern technology offers additives which may either tend to prevent undesirable changes, or to foster desirable ones. But there may also be hidden changes, the nature of which may, as yet, be only suspected. Most foodstuffs are complex mixtures and a slight change in composition, or in the conditions of storage, or in its packaging may well completely alter their behaviour on the shelf, in the refrigerator or deep-freeze.

These papers show that, whereas in some areas much has been done to clarify the problems, in others, there is still very much to do.

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LIPID OXIDATION CATALYSTS AND INHIBITORS IN RAW MATERIALS AND PROCESSED FOODS

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ABSTRACT

This paper is limited to those catalysts and lipid oxidation inhibitors which have been found to be present either in the raw food materials or in processed food as a result of its processing.

Lipoxygenase and haemoproteins are very powerful lipid oxidation catalysts in many foods. Heat treatment usually inactivates lipoxygenase but can, under certain conditions, activate haemoproteins as non-enzymatic metal catalysts. Tocopherol, normally referred to as an antioxidant, can, under certain concentration conditions, shift to pro-oxidative properties. Even volatile lipid oxidation products have been shown to accelerate lipid oxidation.

Inhibitors present in raw materials are found among proteins, amino acids and plant constituents such as flavonoids. Most spices and herbs have antioxidative properties, particularly in darkness. Some microorganisms in their normal metabolism inhibit lipid oxidation or the consequences thereof. Some enzymes, especially superoxide dismutase, are claimed to inhibit lipid oxidation. Among process-induced lipid oxidation inhibitors, Maillard reaction products are those most investigated, together with protein hydrolysates. Smoking generates antioxidative compounds. In most cases the mechanism of lipid oxidation inhibition is still unknown for these materials.

INTRODUCTION

Lipid oxidation is a major cause of deterioration in foods and feeds, both in those containing substantial amounts of fat, like lard and edible oils, and in those where only minor amounts of lipids occur, as in several vegetable products. Several food

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and feed quality attributes can be affected, such as the aroma—also positively—by the formation or modification of volatile odorous compounds (Eriksson, 1979); the taste, by hydroxy acids formed (Baur & Grosch, 1977); the colour, by a Maillard type of reaction between reducing substances originating from lipids and protein (Pokorny, 1981); the texture, e.g. by cross-linking reactions of proteins (Shenouda & Pigott, 1977); the nutritive value, by a decrease of essential fatty acids; and, currently debated, the safety of food by, for instance, oxidation of cholesterol.

There are several levels in lipid oxidation of practical significance in connection with both catalytic and inhibitory action of various chemical compounds and the above consequences of lipid oxidation. One level is the uptake of oxygen by a lipid molecule to produce more highly oxygenated molecules, e.g. fatty acid peroxides. Another level is the one where unstable peroxides undergo reduction or isomerisation to less reactive compounds like hydroxy fatty acids or chain fission to produce smaller molecules, often odorous. Still another level is when lipid oxidation intermediates react with other compounds in the food environment.

Figure 1 summarises the key reactions leading to quality changes in foods and feeds and the various factors which enhance or inhibit them. Strictly, lipid oxidation comprises only the first level, i.e. the reaction between a lipid molecule and oxygen. However, particularly in practical situations, lipid oxidation is noticed through aroma, texture and colour changes, and when these changes are influenced in one direction or the other by a certain treatment, this is often generally referred to as either pro-oxidative or antioxidative, simply because it is often not known at which level of the lipid reactions the treatment is operative. Thus, whether a compound is purely antioxidative or inhibitory depends on the method of measuring. Lingnert *et al.* (1979) have discussed three different methods of monitoring 'lipid oxidation' and defining 'antioxidative effect' from a practical standpoint by the measurement of oxygen consumption, conjugated diene formation and the formation of volatile compounds. In, for instance, the case of some Maillard reaction products, treated further below, and their 'antioxidative' effect, the results obtained by the different methods were not consistent. In this paper I have not tried to make any distinction between antioxidative effect and lipid oxidation inhibition. This paper will be strictly limited to discussion of lipid oxidation catalysts and inhibitors which occur either naturally in raw materials and processed foods or which are formed in procedures commonly used to stabilise foods or make them ready for consumption and which are active during storage of such materials. Cases such as contaminating catalytic trace metals and organic acid used as synergists together with synthetic antioxidants have been omitted both on the above grounds and because their action is too specific because of material, concentration, environmental and technical conditions, etc., to enable simple generalisations and thus they are beyond the scope of this paper. The observations made, particularly on lipid oxidation inhibition, are reported, without any attempt to evaluate their potential for food or feed use.

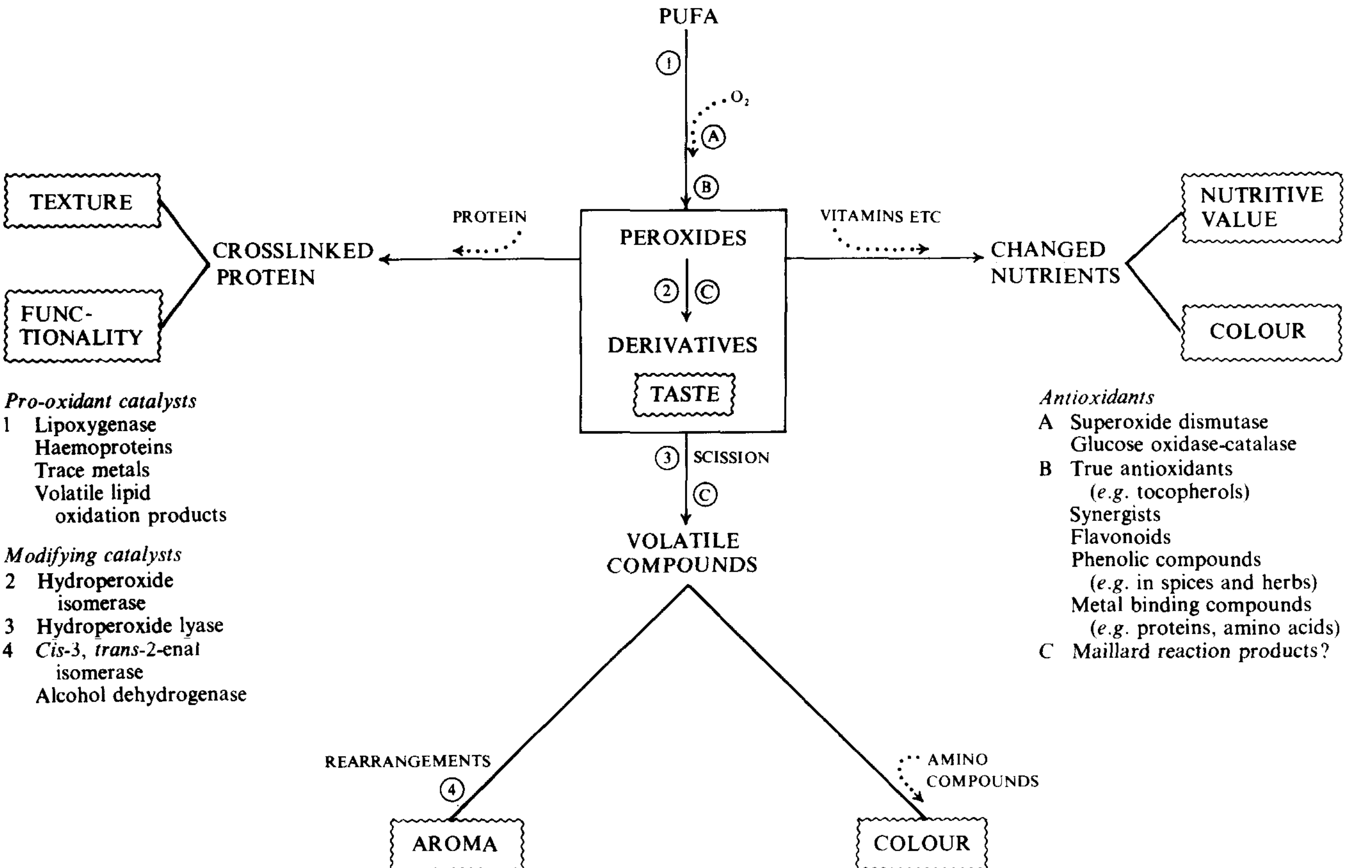

Fig. 1. Reactions of polyunsaturated fatty acids (PUFA) leading to quality and nutritional changes in food. Examples of catalysts (numbers) and antioxidants (letters) are indicated, as well as their site of operation.

Among catalysts, this paper will primarily treat enzymes and haem compounds. Naturally occurring lipid oxidation inhibitory compounds and systems treated are, for example, enzymes, amino acids, peptides and proteins, phospholipids, tocopherols and plant constituents such as pigments, spices and herbs. Process-induced formation of lipid oxidation inhibitors has been shown to arise in fermentation, protein hydrolysis, the common Maillard reaction and smoking.

This paper does not claim to cover all aspects or published data on the topic. Instead it should be looked upon as a collection of examples of catalysts and inhibitors in raw materials and processed food as a framework for the work that has been done and that is still in progress in our laboratory.

CATALYSTS

Enzymes

Lipoxygenase is an enzyme which specifically introduces oxygen into free fatty acids containing methylene-interrupted *cis–cis*-1,4-diene systems to produce hydroperoxides. Most of our present knowledge about lipoxygenase, its occurrence, biochemistry and mechanism of action has been reviewed by Veldink *et al.* (1977), Galliard & Chan (1980) and, recently, by Nicolas & Drapon (1981).

The influence of lipoxygenase on food has been summarised (Davies, 1979). Two isoenzymes, L1 and L2, of lipoxygenase have been demonstrated and characterised. These isoenzymes give rise to two isomers of hydroperoxides, 13-hydroperoxy-9,11-octadecadienoic-*cis* acid and 9-hydroperoxy-10,12-octadecadienoic acid from linoleic acid, one of the most common substrates of lipoxygenase in connection with food. From a flavour point of view, L1 and L2 with pH optima at 9·0 and 6·5, respectively, initiate the formation of varying sets of volatile aldehydes. Fischer & Grosch (1977) found in model systems that when isoenzyme L1 was active, mainly hexanal and *trans*-2, *trans*-4-decadienal were formed, whilst L2 helped produce, in addition to these two compounds, heptanal, *trans*-2-heptenal, *trans*-2-octenal, *trans*-2, *trans*-4-nonadienal and *trans*-2, *cis*-4-decadienal. The sequence of reactions leading to the formation of different C6 and C9 aldehydes and alcohols has been summarised (Eriksson, 1979).

Since active lipoxygenase is a major cause of off-flavour production in stored plant foods during frozen storage, vegetables such as beans and peas are blanched before canning or freezing to inactivate lipoxygenase together with other enzymes. In this case heat treatment is an operation carried out to inhibit lipid oxidation. Such heat treatment, however, introduces other types of change—for example, textural changes—and the inactivation of lipoxygenase should be made under the mildest possible conditions. Svensson & Eriksson (1972*a*,*b*) investigated the influence of environmental conditions on the heat inactivation of lipoxygenase and calculated the activation energy as 479 to 603 kJ/mol, depending on the conditions

(Svensson & Eriksson, 1974*a*). It was demonstrated that lipoxygenase occurs to a larger extent in the cotelydons of peas than in the skins and that the degree of heat inactivation at different depths in the peas could be calculated from time–temperature and thermal diffusivity data as shown in Fig. 2 (Svensson & Eriksson, 1974*b*). Results of this kind enable a better control of lipoxygenase inactivation in order to eliminate this enzyme activity and its consequences for flavour, and at the same time keep other quality attributes of the food as high as possible.

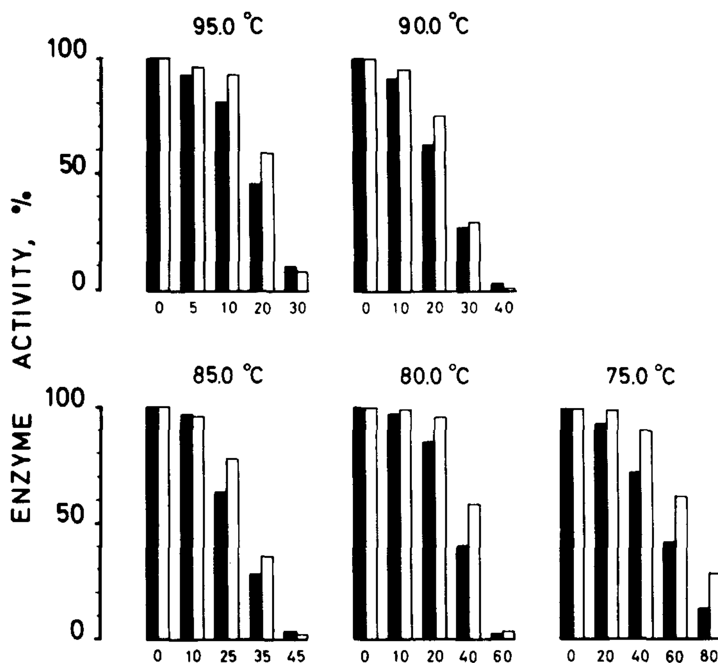

Fig. 2. Residual lipoxygenase activity in blanched whole peas as influenced by temperature and time. Numbers below the black–white bars are seconds at the temperature indicated on top of each set of bars. Black bars: Experimental values. White bars: Computed values. (Svensson & Eriksson, 1974*b*.)

Haemoproteins

Haemoproteins have in common the fact that they contain iron, located as a central atom in the planar porphyrin structure, together referred to as the haem group. This group is buried in a hydrophobic crevice in native haemoproteins and available for binding of small substrate molecules like oxygen, hydrogen peroxide or phenolic compounds either for transporting oxygen by myoglobin and haemoglobin or for reaction purposes as with hydrogen peroxide in the enzymes, catalase and peroxidase. The association between the protein and the haem group is through co-ordination bonds as in the case of the haemoproteins just mentioned or by

covalent bonds as in cytochrome c. The hydrophobic environment around the haem group also allows larger hydrophobic molecules like unsaturated fatty acids to enter the interior of the protein molecule and, to a limited extent, approach the iron, which can then catalyse the oxidation of the fatty acid (Tappel, 1961). Thus, lipid oxidation catalysed by haemoproteins has nothing to do with the fact that some of them are enzymes; rather, it seems that the non-enzymatic lipid oxidation capacity of the iron is much higher when it is contained in the haem group than when iron is a freely mobile ion. Since lipid oxidation still occurs, even though to a lesser degree in lipoxygenase-inactivated material, the haemoproteins come into focus, and an hypothesis was tested that denatured haemoproteins could be even better lipid oxidation catalysts due to an increased exposure of the haem group, enabling a more rapid and intimate contact between a lipid molecule and the haem iron to be established. In a series of papers it was shown, through a purely biochemical approach, that plant and milk peroxidase, as well as catalase, significantly increased

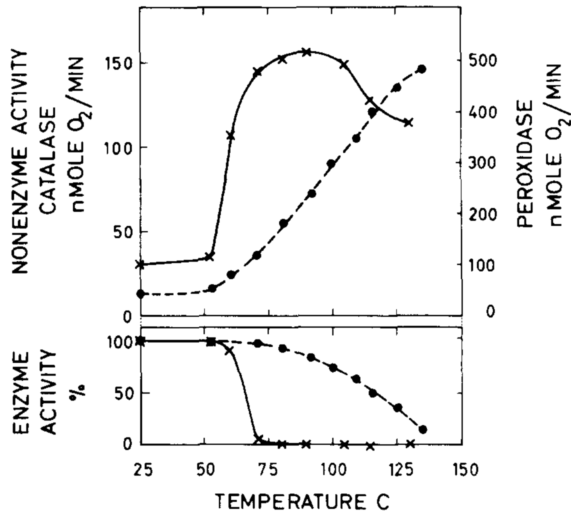

Fig. 3. Influence of temperature on the non-enzymatic lipid oxidation catalysis (upper part) and the enzyme activity on hydrogen peroxide (lower part) of bovine catalase (full line) and horseradish peroxidase (broken line). Heating time was 2 min, at each temperature. (Eriksson *et al.*, 1971.)

their non-enzymatic lipid oxidation capacity after denaturation by urea, guanidinium hydrochloride and heat (see Fig. 3) (Eriksson *et al.*, 1971; Eriksson & Vallentin, 1973) and that the volatile compounds arising from linoleic acid were the same regardless of whether the haemoprotein acted as the catalyst in its native or denatured state (Eriksson, 1970).

Tocopherol

Tocopherols occur in a number of structural forms differing in substituents,

either —H or —CH_3 on the aromatic ring. Tocopherols are primarily known and used as antioxidants, but this effect can be reversed into a pro-oxidative effect on increased concentration (Labuza, 1971). Recently, Cillard *et al.* (1980*a*,*b*) showed that in aqueous media the concentration ratio between α-tocopherol and linoleic acid is critical for the former to act as anti- or pro-oxidant and that α-tocopherol itself is oxidised, acting as an antioxidant. The shift from an antioxidative to a pro-oxidative action of α-tocopherol was found to occur at a concentration ratio of 5×10^{-3} mol α-tocopherol or more per mol linoleic acid.

Lipid oxidation products

An interesting observation was made by El-Magoli *et al.* (1979) that volatile products like hexanal and decadienal from oxidised linoleic acid accelerated the oxidation of unsaturated fatty acids and safflower oil when the latter were in contact with a naturally occurring gas phase containing the volatiles.

INHIBITORS, NATURALLY OCCURRING

Proteins

A number of proteins have been shown to inhibit lipid oxidation; for instance, milk protein, particularly casein (Pokorny *et al.*, 1961; Kajimoto & Kamo, 1964; El-Negoumy & Ku, 1968; Taylor & Richardson, 1980), soyproteins (Pokorny *et al.*, 1961; Pratt, 1972), and serum albumin at low concentration (Yukami, 1972). The latter observation can be of interest to manufacturers of, for example, meat products, who use bovine or porcine blood plasma as a binder.

Amino acids

Most naturally occurring amino acids have been investigated in connection with lipid oxidation, and a number have been reported to act as antioxidants. However, depending on the concentration and pH, the antioxidative effect varies and can even reverse into a pro-oxidative effect. Proline was early found to act as a synergist, together with common antioxidants (Olcott & Kuta, 1959). Linoleic acid oxidation was shown to be inhibited by cysteine, histidine and alanine at pH 9·5, while at pH 7·5 cysteine was strongly pro-oxidative and both histidine and alanine were antioxidative at low, and pro-oxidative at higher, concentrations (Marcuse, 1960). Particularly, histidine was found to act synergistically with α-tocopherol to inhibit oxidation of emulsified herring oil (Marcuse, 1961). Karel *et al.* (1966) found that histidine, alanine, lysine, β-aminobutyric acid, γ-aminobutyric acid and ε-amino caproic acid had an antioxidative effect in a freeze-dried model system, whereas methionine, arginine, phenylalanine and isoleucine had no effect. Histidine and tryptophane had an antioxidative effect at low oxygen partial pressures, whereas added α-tocopherol, BHA or propyl gallate had no effect (Marcuse, 1967; Marcuse & Fredriksson, 1969).

To sum up, the antioxidative effect of amino acids seems to be very complex and to depend on a number of factors such as presence and concentration of trace metals, pH, temperature and the concentration of the amino acid itself.

Phospholipids

Some reports claim that milk phospholipids, lecithin and cephalin, have antioxidative effects; however, it seems that they act more as synergists to antioxidants than as true antioxidants (Olcott & Van der Veen, 1963). Other workers claim that the apparent antioxidative activity of isolated plant phospholipids is due to contaminating phenolic compounds (Pokorny *et al.*, 1976).

Plant constituents

Among plant pigments, especially flavonoids (see Fig. 3) have been shown to have antioxidative effects. Metha & Seshadri (1959) investigated 27 flavonoids and found 17 to have antioxidative properties, particularly 3′,4′-dihydroxy derivatives like robetinine and gossypetine. Jeney *et al.* (1960) found quercitine, dihydrate of quercitine, rutine and quercitine-3-rutinoside to be efficient antioxidants in butter. Pratt & Watts (1964) and Pratt (1965) found that the antioxidative effect of flavones, except rutine, was independent of whether the flavones were glycosidically bound or free. Carotene was found to have an antioxidative effect in the absence, but a pro-oxidative effect in the presence of light (Stanescu & Eisenburger, 1969). Hayes *et al.* (1977) reviewed the antioxidative properties of soybean flour and other soy protein products and concluded that isoflavone glycoside was one of the possible active ingredients. Such an ingredient was later isolated from dry soy beans and identified as isoflavone pentoside *a* (Hammerschmidt & Pratt, 1978). Similar compounds were found in leaves (Hudson & Mahgoub, 1980). The occurrence of flavonoids and flavones in plants was reviewed by Herrmann (1976).

Spices and herbs

Several spices and herbs have been found to have antioxidative properties. Chipault (1957) early observed that 31 out of 32 common spices and herbs were antioxidative, particularly rosemary and sage, the exception being ginger, which was found to be slightly pro-oxidative. However, in lard and bakery products, ginger was found to be antioxidative (Kihara & Inoue, 1962). The antioxidative effect of rosemary and sage is related to their carnosolic acid content (Brieskorn & Dömling, 1969). Herrmann *et al.* (1981) recently reviewed the state of the art in this area and concluded that most spices and herbs have shown varying antioxidative effect on lard in darkness. Particularly large effects were obtained with rosemary, sage, marjoram, thyme, cloves, allspice and mace. In light, green spices and herbs especially were pro-oxidative. The antioxidative effect of these materials may be due to their content of phenols like hydroxycinnamic and hydroxybenzoic acid (Thumann & Herrmann, 1980). Some of these spices and herbs were earlier found to

act strongly synergistically together with BHA or yeast protein hydrolysate (Bishov *et al.*, 1977).

Microorganisms

Growth of bacteria in cod muscle inhibited the onset of oxidative rancidity in it even in the presence of copper. Heat-killed bacteria had no effect (Castell & MacLean, 1964). Antila (1965) found that dried propionic acid bacteria or yeast cells added to milk inhibited the formation of fatty acid hydroperoxides. Grosch *et al.* (1971) and Senser & Grosch (1971) isolated a number of microorganisms from herring, which had the ability to metabolise fatty acid hydroperoxides. In some cases, antioxidative compounds have been extracted from microorganisms; for instance, 2-(6-hydroxy-2-methoxy-3,4-methylene-dioxyphenyl)benzofurane from yeast (Forbes *et al.*, 1958). Methanol or methanol–benzene extracts from antioxidative microorganisms were also found to inhibit lipid oxidation (Smith & Alford, 1969), as was also the case with chloroform–methanol extracts from Torula yeast (Cheeke, 1972).

Enzymes

The combination glucose oxidase–catalase has been reported to act as an antioxidant in mayonnaise (Sarett, 1960), meat (Osadchaya, 1971) and shellfish (Kelley, 1971). Trypsin was shown to protect milk from fat oxidation (Shipe *et al.*, 1972), the possible mechanism being that protein hydrolysis by trypsin increases the copper-binding capacity in milk (Lim & Shipe, 1972). Astrup (1963) reported that xanthine oxidase in milk inhibits lipid oxidation, while other workers have found the opposite (Aurand *et al.*, 1967; Kiermeier & Grassmann, 1966; Smith & Dunkley, 1960). Xanthine oxidase is an enzyme that can generate superoxide and other radicals, which are well known to take part in lipid oxidation. During the last decade much interest has been devoted to the enzyme, superoxide dismutase (SOD), as nature's own means of balancing necessary to toxic concentrations of the superoxide radical, which itself is a very reactive compound, but can also generate other reactive oxygen species such as the hydroxyl radical and singlet oxygen. Kellogg & Fridovich (1975) showed that lipid oxidation increased significantly by superoxide radicals produced by the enzyme, xanthine oxidase, and also by its substrate, hydrogen peroxide. Furthermore, the addition of SOD and catalase effectively inhibited superoxide radical oxidation of lipids. Catalase breaks down hydrogen peroxide into water and ground state (triplet) oxygen, while the spontaneous breakdown of hydrogen peroxide yields water and singlet oxygen. These findings led to much work on the mechanism of lipid oxidation in milk, which contains xanthine oxidase, and this work has recently been critically reviewed by Korycka-Dahl & Richardson (1980), who conclude that SOD can act as an antioxidant under certain conditions. One condition for this effect is that SOD is allowed to act in the early stages of superoxide radical production, since too high a

concentration of the latter can reverse the effect of SOD into a pro-oxidative one. SOD from milk seems to be a relatively heat-stable enzyme, which to a great extent can survive, for example, pasteurisation (Hicks *et al.*, 1977).

Lipids in membranes have also been shown to be protected from oxidation by SOD (Guthridge, 1977). Procedures to protect food from oxidation by SOD have been patented (Michelsen & Monod, 1975).

PROCESS INDUCED INHIBITORS

Maillard reaction products (MRP)

Reactions between sugars and amino compounds were early shown to yield products with an antioxidative effect in food. A British patent (Patent No. 886519, 1962) is based on a method to produce antioxidative compounds by heating sugar and protein together in a fat medium.

Several investigations show that antioxidative compounds are formed from individual amino acids after heating with sugar; for example, lysine to protect bakery products (Yamaguchi & Koyama, 1963), glycine–xylose (Kirigaya *et al.*, 1968), proline and lysine with xylose, ribose, glucose and dihydroxyacetone (Enei & Okumura, 1969) and a range of amino acids and sugars (Kirigaya *et al.*, 1969).

A few attempts have been made to isolate the antioxidative compounds from amino acid–sugar reaction mixtures; for instance, through dialysis, where the antioxidative compounds were non-dialysable and coloured. Yamaguchi & Fujimaki (1970*a*,*b*) fractionated glycine–xylose reaction mixtures on Sephadex into one high molecular and one low molecular weight fraction. However, no strict conclusions could be drawn as to differences in the antioxidative effect between the two fractions.

Eichner (1981) showed that Maillard reaction intermediates were antioxidative in freeze-dried systems.

Lingnert & Eriksson (1980*a*) showed that the basic amino acids, arginine, histidine and lysine, gave strongly antioxidative compounds after reaction with simple sugars. The results were, however, somewhat dependent on the measuring method used. By means of a polarographic method, which records the oxygen consumption in a forced oxidation system, reaction mixtures from histidine with glucose, fructose or xylose, respectively and from lysine or arginine with glucose or fructose were obtained, that were much less antioxidative than the corresponding products from lysine or arginine and xylose (Fig. 4). When a gas chromatographic method was used, all of the histidine– and lysine–sugar reaction products were found to be highly antioxidative (Fig. 5). Only in the case of arginine did the two methods give consistent results. If MRP are to be used as antioxidants in foods, a number of conditions must be fulfilled. First, the reactants must be inexpensive; secondly, the colour formation must be kept at a minimum and, lastly, the reaction

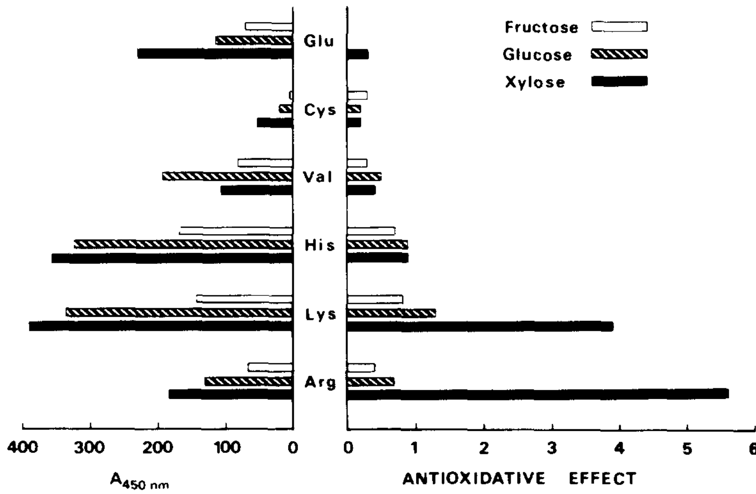

Fig. 4. Antioxidative effect and colour of amino acid–sugar reaction mixtures measured by an oxygen consumption method. Substrate: linoleic acid emulsion, pH 6·5. Catalyst: haemin. (Lingnert & Eriksson, 1980*a*.)

should preferably take place during the normal processing of food at suitable time–temperature conditions.

The first condition, the use of inexpensive raw materials, is primarily associated with the choice of amino compound. If, for instance, protein hydrolysates were to be used, the degree of hydrolysis and proportion of free amino acids to peptides becomes important. Basic amino acids are more or less abundant in proteins, but

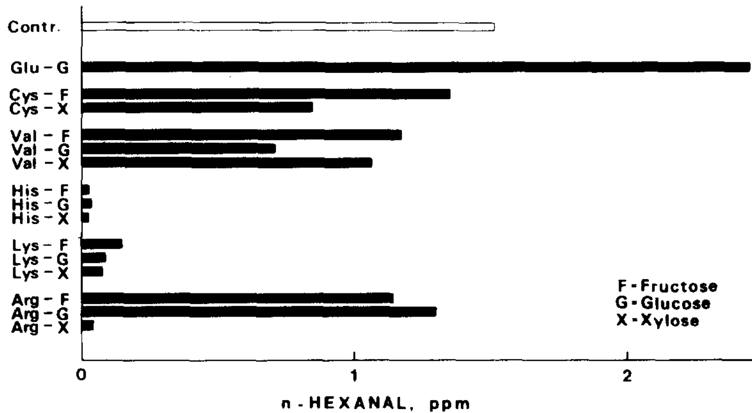

Fig. 5. Inhibition of *n*-hexanal formation from linoleic acid emulsion, pH 6·5, in the presence of different amino acid–sugar reaction mixtures as antioxidants and denatured horseradish peroxidase as a catalyst. (Lingnert & Eriksson, 1980*a*.)

TABLE 1

INHIBITION OF *n*-HEXANAL FORMATION FROM LINOLEIC ACID EMULSION pH 6·5 IN THE PRESENCE OF MAILLARD REACTION PRODUCTS (MRP) FROM GLYCINE, L-HISTIDINE, GLYCYL-L-HISTIDINE AND L-HISTIDYL-GLYCINE. THE AMINO ACIDS AND PEPTIDES WERE REACTED WITH 2 mmol D-XYLOSE FOR 5 h AT pH 7 (INITIAL)

MRP added	n-*Hexanal* (*ppb*)
None	1512
Glycine (2 mmol)	2656
L-Histidine (2 mmol)	193
Glycine (1 mmol) + L-histidine (1 mmol)	305
Glycyl-L-histidine (1 mmol)	308
L-Histidyl-glycine (1 mmol)	185

(Lingnert & Eriksson, 1980*b*.)

rarely account together for more than 20 % of the total amount of amino acids. It is not yet known if amino acids—for example, glutamic acid and glycine, which do not give any antioxidative effect—compete too much as sugar reaction partners or yield possible pro-oxidants. In order to shed some light on the latter question the two dipeptides of histidine and glycine were investigated. Both gave antioxidative xylose reaction products, of which the one from *N*-terminal histidine was found to be equal to comparable free histidine–xylose reaction mixtures and slightly superior to the one from the *N*-terminal glycine dipeptide which had the same antioxidative effect as the reaction products from a mixture of free histidine and free glycine together with xylose (Table 1). Also, MRP derived from an enzymatic protein hydrolysate of bovine erythrocytes, rich in histidine, were found to be strongly antioxidative (Lingnert & Eriksson, 1980*b*) (Fig. 6).

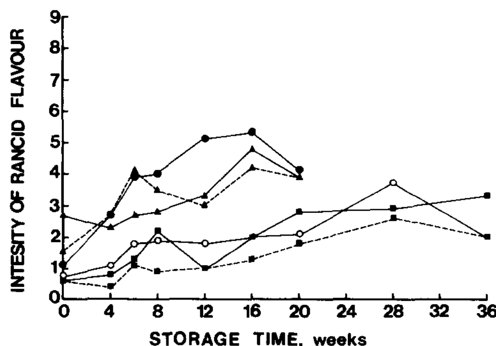

Fig. 6. Rancid flavour development in freezer stored sausage containing no added antioxidant (●——●), 0·08 % L-histidine and 0·32 % D-glucose (▲——▲); 0·16 % L-histidine and 0·32 % glucose (▲- - -▲); 0·08 % reaction mixture of L-histidine and D-glucose (■——■); 0·16 % reaction mixture of L-histidine and D-glucose; (■- - -■) 0·16 % of a reaction mixture of enzymatic haemoglobin hydrolysate and D-glucose (○——○). (Lingnert & Lundgren, 1980.)

In application studies it was found that free histidine and free glucose added to a cookie dough retarded the development of rancidity and the formation of hexanal in the stored cookies baked from the dough. The effect was superior to that obtained by added synthetic antioxidants. Analysis of retained histidine in the cookies suggests that about 70% of the added histidine had reacted with glucose to form antioxidative compounds in the cookies during the baking (Lingnert, 1980), showing that the time–temperature–water activity relationships favoured the formation of antioxidative compounds. Preformed mixtures of histidine–glucose and bovine erythrocyte protein hydrolysate–glucose were found to retard the development of rancidity of frozen stored sausage (Lingnert & Lundgren, 1980) (Fig. 6). A histidine–glucose reaction mixture added to whole milk before spray-drying increased the initial flavour score of the dried milk and retarded the development of negative flavour characteristics during storage of the dried milk (G. Hall to be published).

Fermentation products

Asian fermented foods, particularly those like tempeh and miso, have been shown to contain antioxidative principles—for example, flavonoids of the type 4′,6,7-trihydroxyisoflavone (Gyorgy *et al.*, 1964; Packett *et al.*, 1971; Ebine, 1971).

Antioxidative compounds were extracted from the fungi *Aspergillus niger* and fractionated into one yellow and one brown pigment fraction, both antioxidative. The yellow pigment seemed to have a linear naphthopyrone structure (Zaika & Smith, 1975).

Protein hydrolysates

There are numerous observations that peptides and protein hydrolysates act inhibitorily on lipid oxidation, often in combination with other active compounds such as spices and herbs (Bishov *et al.*, 1977). Also, spontaneous and induced protein hydrolysis can introduce antioxidative effects in food; for example, in milk (Shipe *et al.*, 1972), possibly due to the greater metal-chelating capacity of peptides and amino acids (Lim & Shipe, 1972).

Recently, Yamaguchi *et al.* (1980*a*,*b*) showed that, particularly, acid hydrolysed, but also enzymatically hydrolysed, egg white protein had antioxidative properties in biscuits. For enzymatic soy bean protein hydrolysates the antioxidative effect in biscuits was found to increase with an increased degree of hydrolysis.

Wood smoke

Earlier, Linko (1951) found that wood smoke for food preservation purposes contained antioxidative compounds. Later, Polish workers investigated a smoke flavouring material and found it antioxidative, protecting lard from being oxidised (Chomiak & Goryn, 1977). The choice of smoke generation parameters, such as the temperature, had an influence on the antioxidative effect of the particle fraction of

wood smoke. The vapour phase of wood smoke showed no, or very little effect (Daun & Tilgner, 1977).

CONCLUSION

Catalysts of lipid oxidation are mostly well known. The inactivation of enzymes to decrease lipid oxidation has been studied in detail and is commonly applied to food. However, heat inactivation of lipid oxidation enzymes can be partly counteracted by increased non-enzymatic activity of haemoproteins. Trace metals, especially copper, cobalt and iron, are well known catalysts, but their catalytic action is dependent on a variety of factors.

Antioxidative properties or lipid oxidation inhibition are observed for many raw materials or their constituents, as well as antioxidative effects introduced by different common food processes. In most cases, except for amino acids, tocopherol and organic acids, the nature of the antioxidative effects is less well understood.

There is a tendency in society to accept 'natural' protecting agents rather than synthetic ones. It seems reasonable to assume that antioxidative effects or lipid oxidation inhibition capacity can be introduced into foods by the proper use of ingredients and process parameters. However, much more work remains to be done in order to identify the responsible compounds and to explain their mechanisms of action.

ACKNOWLEDGEMENT

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OXIDATIVE REACTIONS OF UNSATURATED LIPIDS

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ABSTRACT

Monohydroperoxides, which are the products of free radical autoxidation of unsaturated fatty acids, can undergo further free radical chain reactions initiated by abstraction of hydrogen to reform peroxy radicals. In the autoxidation of methyl linolenate, the peroxy radicals corresponding to 'inner' hydroperoxides may cyclise and, after further oxygenation, yield diperoxides. The mixtures of the two peroxidic species, monohydroperoxides and diperoxides, obtained from linolenate can be identified and determined chromatographically. Mechanistic studies showed that the oxygenation of pentadienyl radicals to form peroxy radicals—an important propagation step in autoxidation—is reversible. H-donors can be used to control the relative rates of the forward and reverse reactions and so determine the relative proportions of different peroxidic species formed. α-Tocopherol was shown to be an effective H-donor and drastically altered the distribution of products formed in linolenate autoxidation. Such control of peroxide formation may be a factor determining the manifestations that are specific to the oxidative deterioration of a food product.

INTRODUCTION

Autoxidation is a natural chemical reaction occurring in the earth's environment between atmospheric oxygen and unsaturated compounds. In food systems, the autoxidation of unsaturated fatty acid moieties, especially those with more than one double bond, causes the rapid deterioration of foods containing unsaturated lipids. The major effect of autoxidation in food that causes rejection and waste is the development of off-odours generally associated with oxidative rancidity. Much of

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the basic understanding of the mechanism of autoxidation has been provided by earlier studies (Bateman, 1954) largely in model systems. An important outcome of this work was the establishment of hydroperoxides as the primary products of autoxidation. Adaptation of this to food systems led to an understanding of autoxidation in foods as a two step process (Fig. 1), i.e. (1) the primary autoxidation reaction leading to the formation of hydroperoxides and (2) the further reactions of hydroperoxides which lead to the manifestations of autoxidation in foods. Thus, the

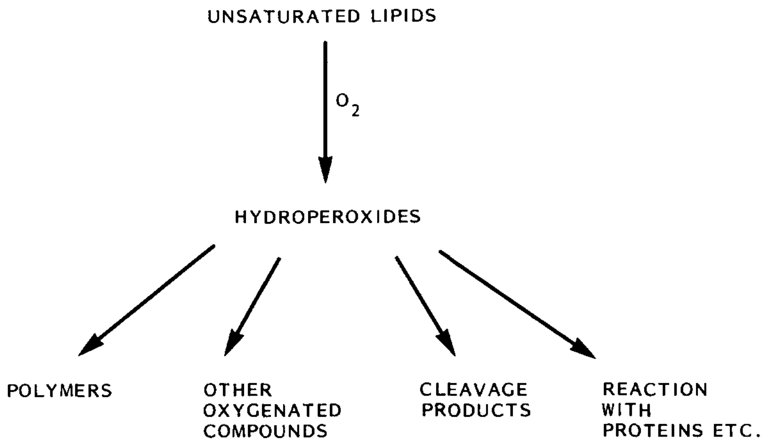


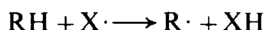
Fig. 1.

decomposition of hydroperoxides by C—C bond cleavage, to form volatile compounds, forms the basis of the understanding of oxidative rancidity in molecular terms. Such a simple analysis is, however, not adequate to explain the many different facets of the consequences of autoxidation in food which vary in both intensity and molecular complexity. The origins of this complex situation lie in the fact that both the primary autoxidation and the decomposition of hydroperoxides are themselves complex reactions that are affected by physical and chemical factors. In the last decade, attempts have been made by different groups of workers to simplify the system into its two components and to study them in isolation. Some of this work, which has been made possible by the development of methods for the preparation and rapid analysis of pure hydroperoxides, has been reviewed recently (Gardner, 1982; Grosch, 1982; Frankel, 1979). Work at the Food Research Institute has been concentrated on primary autoxidation, i.e. the formation of hydroperoxides, as this may lead to the better control of the whole process of autoxidation. The results of this and related work which are reported here reveal new details of the mechanism of autoxidation and of the products formed.

THE MECHANISM OF MONOHYDROPEROXIDE FORMATION

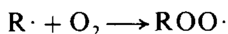
The classical studies (Bateman, 1954) established autoxidation as a free radical chain reaction which could be described in terms of initiation, propagation and termination processes as follows:

Initiation:

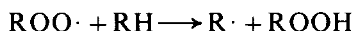


$$RH + X\cdot \longrightarrow R\cdot + XH$$

Propagation:

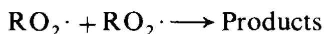


$$R\cdot + O_2 \longrightarrow ROO\cdot$$



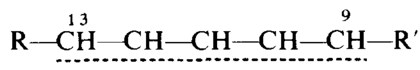
$$ROO\cdot + RH \longrightarrow R\cdot + ROOH$$

Termination: (at atmospheric levels of O_2)

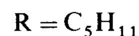


$$RO_2\cdot + RO_2\cdot \longrightarrow \text{Products}$$

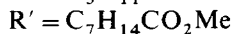
The steps leading to hydroperoxide formation are the propagation reactions which include the important step of the formation of a *peroxy radical* ($RO_2\cdot$) by oxygenation of R· which, in the case of an unsaturated lipid with more than one double bond, such as methyl linoleate, is the *pentadienyl radical*



$$R\text{—}\overset{13}{C}H\text{—}CH\text{—}CH\text{—}CH\text{—}\overset{9}{C}H\text{—}R'$$



$$R = C_5H_{11}$$



$$R' = C_7H_{14}CO_2Me$$

(in the case of methyl linoleate)

Oxygenation of the pentadienyl radical can occur at either extremity of the radical to give, in the autoxidation of methyl linoleate, the 9-OOH and the 13-OOH hydroperoxides. Detailed study (Chan & Levett, 1977*a*) of the monohydroperoxides formed by linoleate autoxidation made possible by the development of HPLC for product analysis showed that the following four monohydroperoxides are formed:

$$R\text{—}CH\overset{c}{=}CH\text{—}CH\overset{t}{=}CH\text{—}CH(OOH)\text{—}R' \quad (1)$$

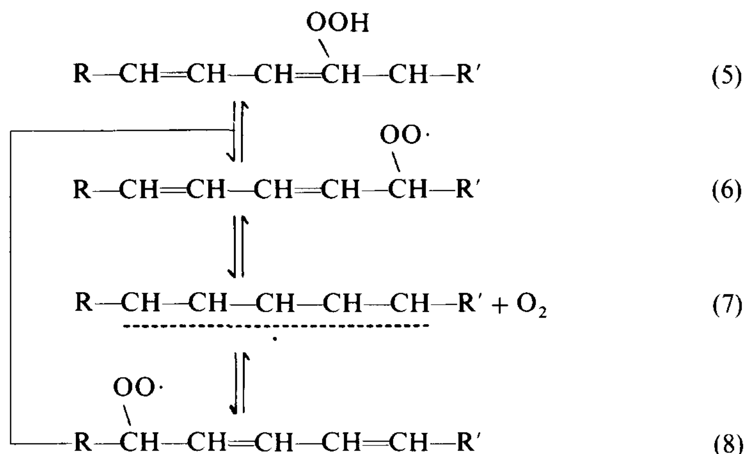
$$R\text{—}CH\overset{t}{=}CH\text{—}CH\overset{t}{=}CH\text{—}CH(OOH)\text{—}R' \quad (2)$$

$$R\text{—}CH(OOH)\text{—}CH\overset{t}{=}CH\text{—}CH\overset{c}{=}CH\text{—}R' \quad (3)$$

$$R\text{—}CH(OOH)\text{—}CH\overset{t}{=}CH\text{—}CH\overset{t}{=}CH\text{—}R' \quad (4)$$

Thus, two isomers of each of 9-OOH and 13-OOH monohydroperoxides are obtained, all containing a *trans* (*t*) double bond adjacent to the OOH group. The isomers with the OOH group at the same position differ in the configuration of the other double bond (they will be referred to as the *cis–trans* and *trans–trans* isomers). Equal proportions of the 9-OOH ((1) and (2)) and 13-OOH ((3) and (4))

hydroperoxides are produced by autoxidation. Although preparative HPLC could yield individual isomers of monohydroperoxides, in practice it is more convenient to prepare large quantities of single hydroperoxides for further study by enzymic oxidation of linoleate (Chan *et al.*, 1976; Matthew *et al.*, 1977). Using isolated single monohydroperoxides, it was shown (Chan *et al.*, 1979) that the monohydroperoxides undergo a novel free-radical chain reaction which resulted in the isomerisation of a single isomer of monohydroperoxide to all four isomers. The isomerisation is:



A free-radical rearrangement proceeds by the abstraction of H from a monohydroperoxide (5) to form a peroxy radical (6). Deoxygenation to a pentadienyl radical (7) followed by re-oxygenation can yield a different radical (8). Abstraction of hydrogen from another hydroperoxide molecule will yield the rearranged monohydroperoxide as well as propagating the chain. The novel feature inherent in this reaction is the deoxygenation of the peroxy radical to form the pentadienyl radical—a step which is the reversal of the oxygenation reaction in the propagation step of autoxidation. That pentadienyl radicals can undergo reversible oxygenation was demonstrated by the exchange of the oxygen atoms of the OOH group in the monohydroperoxide with molecular oxygen in the atmosphere.

One of the difficulties encountered in understanding the autoxidation of linoleate has been an adequate explanation of the distribution of isomeric monohydroperoxides obtained. In particular, the pathway for the formation of *trans–trans* isomers has been unclear for, if the stereochemistry of the *cis–cis* 1,3-diene system is retained in the pentadienyl radical because of the double bond character in the delocalised system, then only *cis–trans* isomers should be formed. The reversible oxygenation of pentadienyl radicals was used by Porter *et al.* (1980) to explain the formation of *trans–trans* as well as *cis–trans* isomers. In the scheme of Porter *et al.* (Fig. 2) only *cis–trans* peroxy radicals (for example, (10)) are formed *initially*.

Direct hydrogen abstraction by (10) will lead to *cis–trans* monohydroperoxide isomers. *Trans–trans* isomers are formed by (i) conformational change via rotation of a single bond (10)→(11); (ii) de-oxygenation to a pentadienyl radical with a configuration different from that formed initially (11)→(12); (iii) re-oxygenation at the other extremity of the pentadienyl radical to form a *trans–trans* peroxy radical (13) (re-oxygenation at the same position will reform the original peroxy radical) and (iv) hydrogen abstraction. The validity of this scheme was shown by the effect of

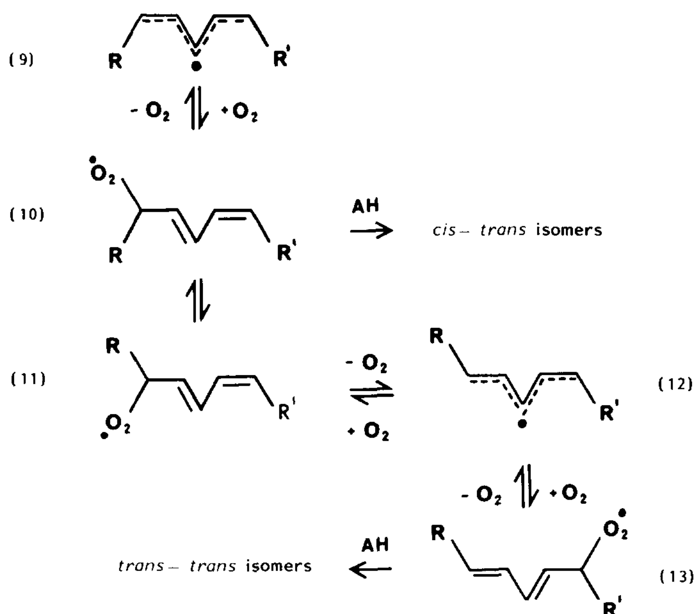


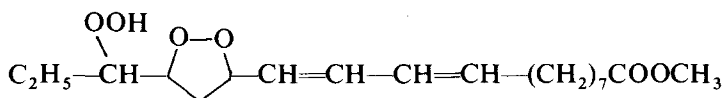
Fig. 2.

H-donors on the *cis–trans* and *trans–trans* isomeric ratio of the hydroperoxides. Addition of the synthetic H-donor *p*-methoxyphenol in sufficient quantities quenched the initially formed peroxy radical and resulted in the formation of only *cis–trans* isomers.

THE 'DIPEROXIDES' OF METHYL LINOLENATE

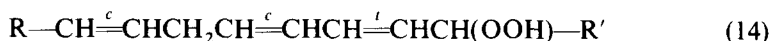
Autoxidation of unsaturated lipids containing more than 2 methylene-interrupted double bonds, e.g. methyl linolenate gives rise not only to isomeric monohydroperoxides but also to a mixture of hydroperoxy-epidioxides (diperoxides) in which

two molecules of oxygen are incorporated to form a cyclic peroxide function as well as a hydroperoxide function.

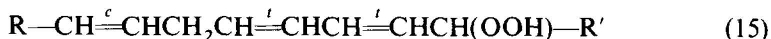


A methyl linolenate diperoxide.

The two types of peroxidic compounds (monohydroperoxides and diperoxides) are readily separated by HPLC (Fig. 3). However, analysis of the mixture of monohydroperoxides is better carried out using the hydroxy derivatives, as these are separated completely into the expected eight constituent isomers. The monohydroperoxides are the *cis–trans* and *trans–trans* isomers of the 9-, 12-, 13- and 16-OOH compounds (Chan & Levett, 1977*b*). Autoxidation of methyl linolenate is therefore analogous to that of methyl linoleate as far as the oxygenation of pentadienyl radicals is concerned except that two 1,3 diene systems (the 9,13 and the 12,16 systems) are involved instead of one. However, unlike the autoxidation of methyl linoleate which resulted in equal proportions of the 9-OOH and 13-OOH hydroperoxides, autoxidation of methyl linolenate yielded far higher (nearly threefold) proportions of the 9- and 16-OOH isomers. There is, therefore, a difference in the amounts of isomers with the OOH group inside the system of three double bonds (the 'inner' isomers, 12- and 13-OOH, i.e. (16) to (19) and those with the OOH outside (the 'outer' isomers 9- and 16-OOH, i.e. (14), (15), (20) and (21).



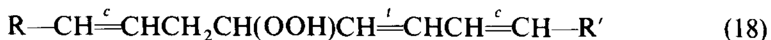
R—CH$\overset{c}{=}$CHCH$_2$CH$\overset{c}{=}$CHCH$\overset{t}{=}$CHCH(OOH)—R′ (14)



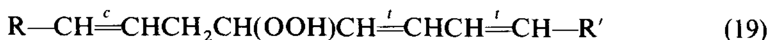
R—CH$\overset{c}{=}$CHCH$_2$CH$\overset{t}{=}$CHCH$\overset{t}{=}$CHCH(OOH)—R′ (15)

R—CH$\overset{c}{=}$CHCH$\overset{t}{=}$CHCH(OOH)CH$_2$CH$\overset{c}{=}$CH—R′ (16)

R—CH$\overset{t}{=}$CHCH$\overset{t}{=}$CHCH(OOH)CH$_2$CH$\overset{c}{=}$CH—R′ (17)



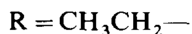
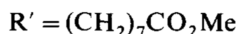
R—CH$\overset{c}{=}$CHCH$_2$CH(OOH)CH$\overset{t}{=}$CHCH$\overset{c}{=}$CH—R′ (18)



R—CH$\overset{c}{=}$CHCH$_2$CH(OOH)CH$\overset{t}{=}$CHCH$\overset{t}{=}$CH—R′ (19)

R—CH(OOH)CH$\overset{t}{=}$CHCH$\overset{c}{=}$CHCH$_2$CH$\overset{c}{=}$CH—R′ (20)

R—CH(OOH)CH$\overset{t}{=}$CHCH$\overset{t}{=}$CHCH$_2$CH$\overset{c}{=}$CH—R′ (21)

R = CH_3CH_2— R′ = $(CH_2)_7CO_2Me$

This difference in the proportions of the inner and outer isomers arises because diperoxides are formed by the cyclisation of the peroxy radicals corresponding to

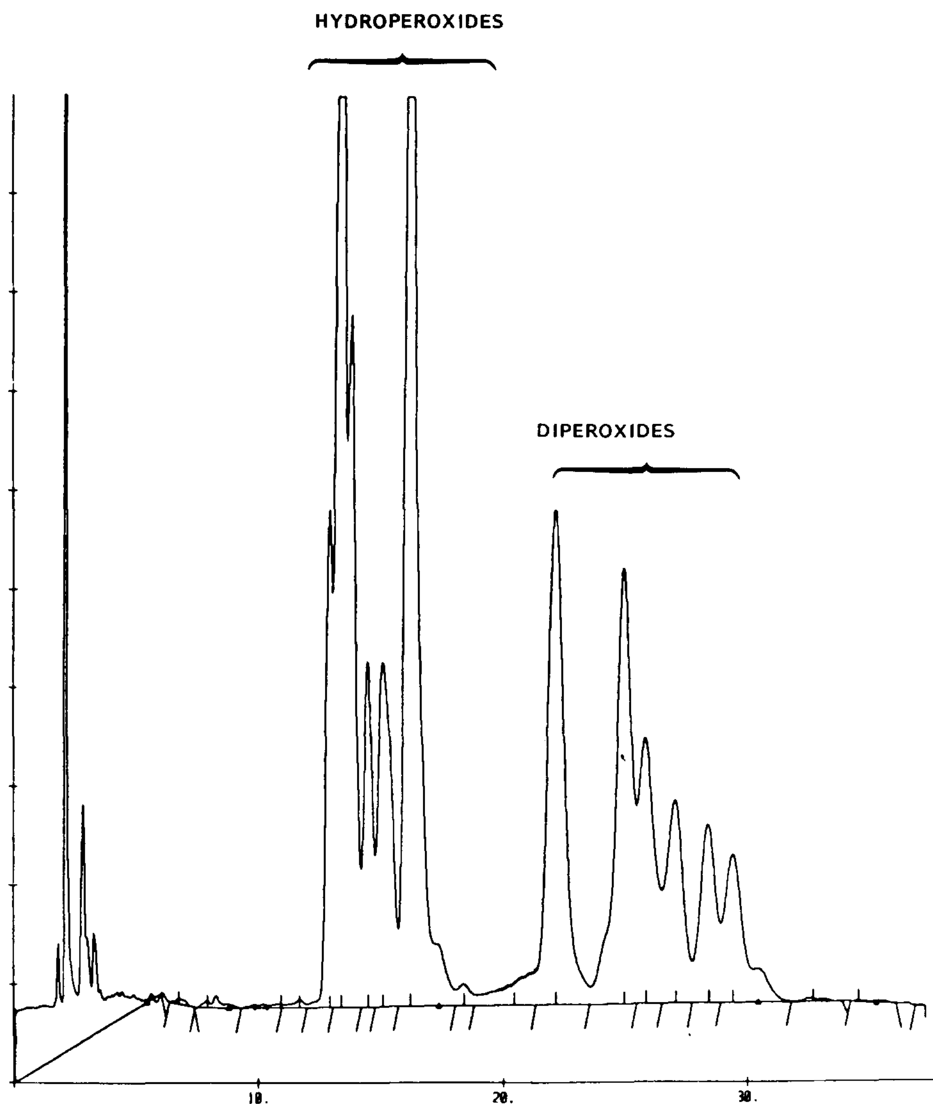


Fig. 3. Autoxidation of methyl linolenate. Analysis of peroxidic products (monohydroperoxides and diperoxides) by HPLC (see Coxon *et al.*, 1981).

inner isomers (Fig. 4) while outer peroxy radicals cannot cyclise in this way. With no cyclisation reaction to deplete the amounts of outer peroxy radicals, larger amounts of these hydroperoxides are formed.

As two different positional isomers of inner peroxy radicals are formed in the autoxidation of linolenate, two distinct 'sets' of diperoxides are formed, differing in

stereochemistry as well as positions of oxygenation. Although the mixtures of diperoxides are readily separated from the hydroperoxides, the resolution of the mixture of diperoxides was too poor to permit their separation and detailed analysis easily. Taking advantage of the fact that a *single* peroxy radical can be generated from an isolated *hydroperoxide* by hydrogen-abstraction, as was shown in the case of linoleate hydroperoxides, and the fact that cyclisation (22)→(23) is preferred to isomerisation in the case of linolenate, simpler mixtures of diperoxides were obtained using single linolenate hydroperoxides obtained by enzymic oxidation.

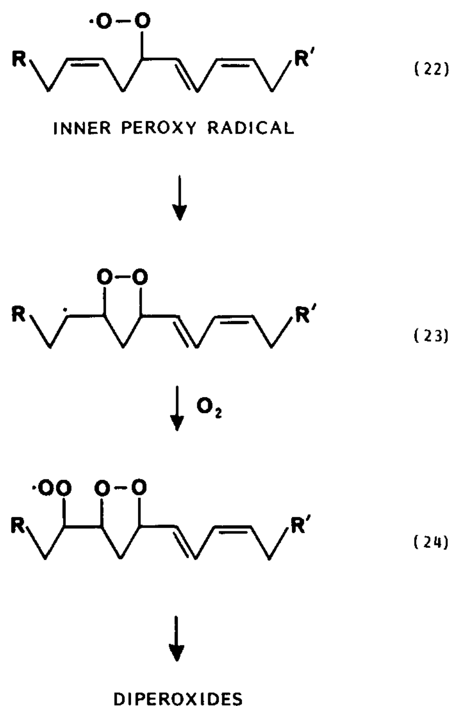


Fig. 4.

Thus, the diperoxides were obtained in two distinct steps, each with a single starting material: the oxidation which yields a single hydroperoxide which is then *autoxidised* (with conversion of a monohydroperoxide to (22) as a first step) to a diperoxide. This procedure enabled the isolation and structural determination of the six major diperoxides obtained by oxidation of methyl linolenate (Coxon *et al.*, 1981). Two groups of diperoxides are formed (Fig. 5): the 9-hydroperoxy-10, 12-peroxy derived from the 12-peroxy radical and the 16-hydroperoxy–13, 15-peroxy derived from the 13-peroxy radical.

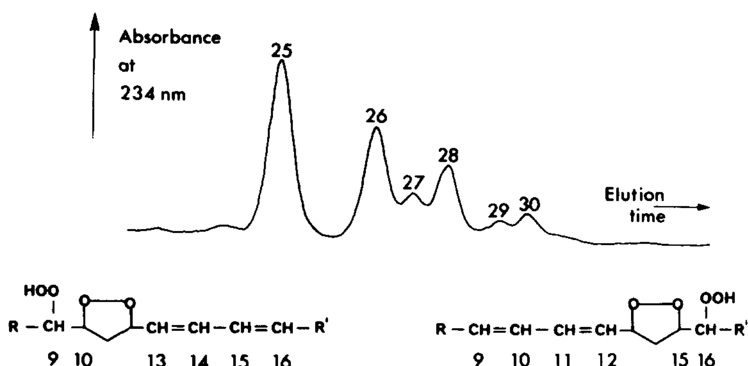


Fig. 5. The structures and HPLC separation of the six major diperoxides (25) to (30) obtained by autoxidation of methyl linolenate. R = CH_3CH_2—; R' = $(CH_2)_7CO_2Me$. (25) 9,10-threo,13-trans,15-cis; (26) 15,16-threo,9-cis,11-trans; (27) 9,10-erythro,13-trans,15-cis; (28) 15,16-threo,9-trans,11-trans; (29) 15,16-erythro,9-cis,11-trans; (30) 15,16-erythro,9-trans,11-trans.

CONTROL OF PRODUCT DISTRIBUTION BY ANTIOXIDANTS

In the above discussion, mention has already been made of the effects of an H-donor on the ratio of the *cis–trans* to *trans–trans* isomers in the autoxidation of linoleate. Many antioxidants are H-donors but, depending on the conditions and system under investigation, antioxidants may not always be effective inhibitors of oxidation. This applies especially to tocopherols which, under some circumstances, act as 'pro-oxidants' (Heiman *et al.*, 1957; Cillard *et al.*, 1980*a,b*) despite their H-donor capacity. This apparent duality of the rôle of natural antioxidants may arise because they control the complex oxidative reactions of unsaturated lipids discussed above in a differential manner. The first evidence that this may be the case was the observation of the control of product distribution in autoxidation by a natural antioxidant reported by Peers *et al.* (1981). The presence of α-tocopherol drastically affected the relative ratios of monohydroperoxides formed from linoleate oxidation. While autoxidation without α-tocopherol yielded the following percentage compositions of isomers: (1) 21 %; (2) 28 %; (3) 23 %; (4) 28 %; the presence of 5 % of α-tocopherol resulted in the formation of only *cis–trans* isomers (49 % of (1); 51 % of (3)). This is presumably a result of the quenching of the initially formed *cis–trans* peroxy radicals (e.g. (10)) by the action of α-tocopherol as an H-donor. The effect of α-tocopherol on the autoxidation of methyl linolenate (Fig. 6 and Table 1) is more complex and affects both types of peroxidic compounds. As the concentration of α-tocopherol is increased, the effects are: (i) a decrease in the ratio of diperoxides to hydroperoxides; (ii) an increase in the proportion of *cis–trans* isomers (as in linoleate autoxidation) and (iii) the ratio of the four positional isomers of monohydroperoxides approaches unity—i.e. the bias towards the formation of outer isomers is diminished. At the concentration of 5 % α-tocopherol,

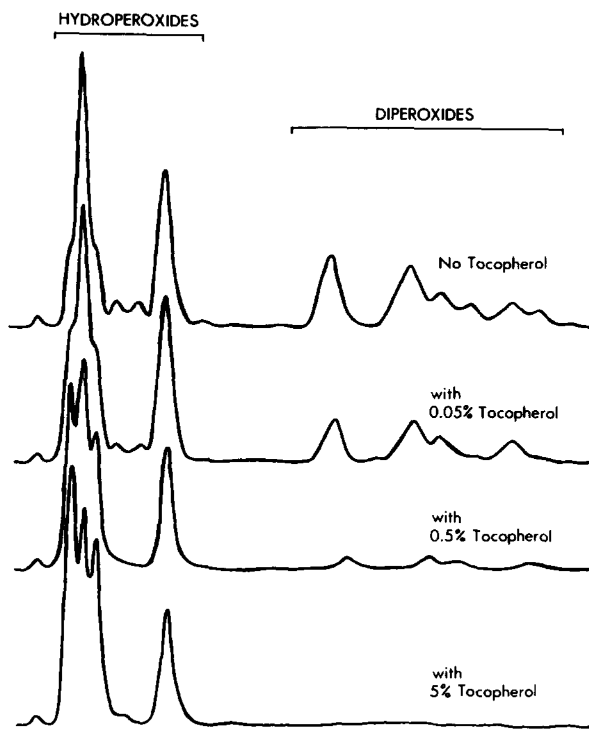


Fig. 6. Effect of α-tocopherol on the distribution of peroxidic products obtained from methyl linolenate autoxidation.

the mixture of peroxide compounds simplified to yield only the four *cis–trans* monohydroperoxides which were formed in nearly equal amounts. Thus, quenching of the *cis–trans* peroxy radicals (e.g. (22)) in this case prevents formation of diperoxides as well as *trans–trans* monohydroperoxides.

Although inhibition of *trans–trans* isomer and diperoxide formation is only complete at the 5 % level, the effects of α-tocopherol are observable at much lower levels. This is in contrast to the effects of *p*-methoxyphenol obtained by Porter *et al.* where comparable effects were observed only at much higher molar concentrations. Apart from indicating that α-tocopherol is a more effective H-donor than *p*-methoxyphenol, these results suggest that α-tocopherol may have significant effects in some foodstuffs. This applies particularly if a deleterious effect is due to the further reactions of an inner hydroperoxide, since the greatest increase as a result of the presence of α-tocopherol is in the proportions of inner isomers as a percentage of total peroxidic species (Table 1). The pro-oxidant effect of α-tocopherol is therefore very selective and is in favour of the formation of inner isomers. The effect on the

TABLE 1

EFFECT OF α-TOCOPHEROL ON THE AUTOXIDATION OF METHYL LINOLENATE—DISTRIBUTION OF PEROXIDIC PRODUCTS

α-Tocopherol added (% *w/w*)	*Extent of*[a] *oxidation* (μM/g)	% *composition of peroxidic compounds*		*Sub-classes of HPO as* % *of total peroxidic comps* (% *change based on control in parentheses*)			
		DPO[b]	*HPO*[c]	*ct*[d]	*tt*[e]	*Inner*	*Outer*
0 (Control)	665	38·2	61·8	43·9 (—)	17·9 (—)	11·1 (—)	50·7 (—)
0·05	42	23·7	76·3	62·6 (+42)	13·7 (−23)	24·4 (+120)	51·9 (+2)
0·5	74	7·5	92·5	87·0 (+98)	5·5 (−69)	42·6 (+284)	49·9 (−3)
5	469	—	100	100 (+128)	0·0 (−100)	53 (+377)	47·0 (−7)

[a] Determined as conjugated diene (Peers *et al.*, 1981).
[b] Diperoxides (DPO).
[c] Monohydroperoxides (HPO).
[d] Total *cis–trans* isomers.
[e] Total *trans–trans* isomers.

total amount of inner hydroperoxides formed is marked, even at fairly low concentrations of α-tocopherol, i.e. when its overall action is antioxidative. Although the total extent of oxidation in 0·5 % α-tocopherol is only just over one-tenth of that of the sample without tocopherol (the control sample) the amount of inner hydroperoxides formed is nearly half of that of the control, because of the much higher (nearly threefold) proportions of inner hydroperoxides in the mixture. An example where the selective pro-oxidant effect of α-tocopherol caused the development of a fishy (metallic) taint in caramels has been reported (Swoboda & Peers, 1977). This effect of α-tocopherol may now be understood as the selective formation of inner hydroperoxides since the tainting compound, octa-1,5-dien-3-one, is believed to be derived from an inner, rather than an outer, hydroperoxide (Swoboda & Peers, 1979).

CONCLUSIONS

The results of recent investigations on the autoxidation of unsaturated lipids described briefly above indicate several additions to the conventional view of autoxidation. In the first instance, the oxygenation of pentadienyl radicals is reversible and the relative rates of the reversible oxygenation of pentadienyl radicals and the cyclisation of peroxy radicals determine the distribution of peroxidic products. Secondly, hydroperoxides can undergo free radical chain reactions as a result of H-abstraction to form a peroxy radical. Since peroxy radicals are intermediates in autoxidation, the study of autoxidation may be carried out via the 'backdoor' by using a hydroperoxide. Finally, antioxidants may affect the relative rates of the reactions in autoxidation and so control the distribution of products. These observations may, in turn, lead us to a better understanding of the factors controlling the manifestations of lipid oxidation in foods. It is generally recognised that the 'higher' unsaturated fatty acids (i.e. those containing more than two double bonds) are more important in causing the oxidative deterioration of fatty foods even though these fatty acids may be minor constituents. Apart from hydroperoxides, the higher unsaturated lipids form other peroxidic species such as diperoxides. There is as yet little information on the reactions of diperoxides, especially on their decomposition reactions. Preliminary studies by the authors suggest that the reactions of hydroperoxides and diperoxides are qualitatively and quantitatively dissimilar. For example, thermal decomposition of diperoxides can yield cleavage products not obtained by the decomposition of hydroperoxides. In the quantitative sense, hydroperoxides and diperoxides decompose at different relative rates in different solvents. Generally, the controlling factors determining which products are formed in the oxidation of fatty foods may be classified broadly (Fig. 7) as (a) those factors affecting the differential formation of peroxidic species, e.g. α-tocopherol, and (b) the factors affecting the relative rates of decomposition of the

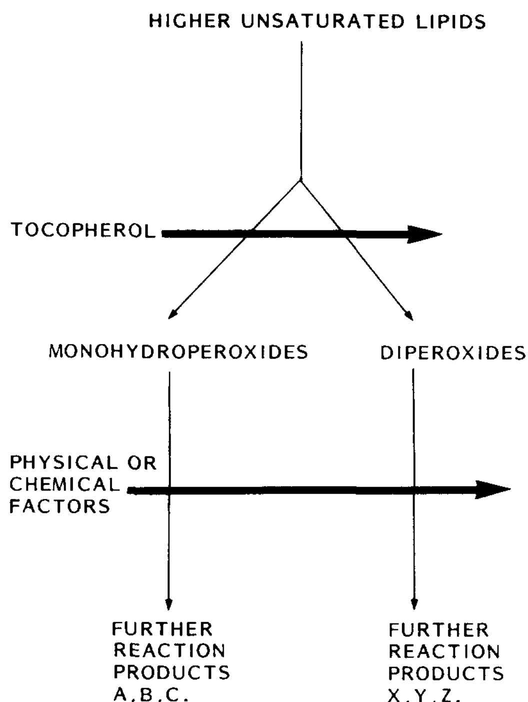


Fig. 7.

different peroxidic species to specific products. The different products formed by autoxidation in different foods may therefore be determined by other food constituents acting as the controlling factors.

Although detailed analysis of such controlling factors will be the subject of further research, a simple analysis shows that the most significant effects of H-donors are on the levels of diperoxides which are suppressed and on inner hydroperoxides, the concentrations of which are dramatically increased. Judging from such major effects alone, there may be two distinct types of oxidative rancidity which are controlled by H-donors, depending on whether the flavour compounds are derived from diperoxides or from inner hydroperoxides.

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THE MOLECULAR BASIS OF LONG-TERM CHANGES IN POLYSACCHARIDE BASED SYSTEMS

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ABSTRACT

Polysaccharides are important in terms of their ability to impart texture in a wide variety of systems ranging from dilute solutions to low moisture solids. The effects reported in the literature vary from the modification on storage of the flow properties of polysaccharide solutions, syneresis, swelling and annealing of gel systems to the slow rearrangement of chains in concentrated materials. Examples are considered over this whole concentration range. The equation derived by Smoluchowski to describe the aggregation of colloidal systems is shown to be relevant to such ageing processes while the annealing of thermolabile gel systems is described in thermodynamic terms (Borchard et al.*, 1980).*

INTRODUCTION

To the food manufacturer, the differential time dependence of changes occurring during processing is one of the most valuable inbuilt features of a foodstuff which, with the requisite knowledge and skill, can be used to attain a desired product or quality. Such processes have, typically, time constants of seconds or minutes at processing temperatures. However, if such changes continue at ambient temperature, or even others appear under these conditions, they may be considered an embarrassment. These reactions usually have time constants several orders of magnitude greater than those observed during processing.

Post-processing changes in polysaccharide based systems have received, for the most part, far less attention than they deserve. In some cases their very tardiness has dissuaded attention; in other instances the possible molecular basis of changes has been obscure, while in other situations research workers have been satisfied with

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describing the effect without determining the cause. Bellet *et al.* (1979), for example, have shown that there are irreversible changes on ageing in the rheological properties of aqueous solutions of carboxymethyl cellulose with an attendant substantial modification of the velocity profile, but they give no information on the molecular basis for such changes.

GENERAL THEORETICAL FRAMEWORK FOR AGEING EFFECTS

The majority of the effects which will be described in this paper arise from the enhancement of polymer interactions rather than scission of polymer chains. Those that occur under normal storage conditions will be non-covalent in character and arise from random molecular motion either of whole molecules or particular segments of a polymer chain. The formalism to describe the kinetics of such changes had previously been developed to treat the coagulation problem considered by Smoluchowski (1918). Although Flory (1936) and Stockmayer (1943, 1944) had both considered the kinetics of this type of gelation process, only recently has a general treatment been formulated (Ziff, 1980). If we assume that (fragments of) one polymer (i-mers, concentration v_i) react with (those of) another polymer (j-mers, concentration v_j) to give a product (k-mers, concentration v_k) and that the concentration of k-mers is increased by all reactions between i-mers and j-mers such that $i + j = k$, and reduced by subsequent reactions between k-mers and all the rest, then the development of v_k is described by the equation:

$$\frac{dv_k}{dt} = \frac{1}{2} \sum_{i+j=k} K_{ij} v_i v_j - v_k \sum_{j=1} K_{kj} v_j \qquad (1)$$

K_{ij} is, in fact, a collision matrix which reflects the mechanism of particle motion and tendency for adhesion. Such a matrix can take into account both the effects of random collision and aggregation and the more specific organisation required in the development of crystallites (and indeed all shades of behaviour between those extremes).

We shall consider a number of situations, looking firstly at solutions, then by contrast at semi-solid systems and, finally, at gels.

LONG-TERM CHANGES IN SOLUTION

Non-specific aggregation in solution

Southwick *et al.* (1980) have reported investigations aimed at resolving the questions that have surrounded the molecular weight of xanthan. Various groups of workers had reported values varying from 2×10^6 to 62×10^6, depending not only

on the source but also on the treatment immediately prior to molecular weight measurements. Using quasielastic light scattering, they were able to show (in the absence of contaminating bacteria and after either centrifugation or filtration) a progressive reduction in the diffusion coefficient of the polysaccharide molecules and a corresponding increase in the hydrodynamic radius as calculated by the Stokes–Einstein equation. No such time-dependent aggregation was observed in 4M urea and therefore they postulated a progressive aggregation of xanthan molecules as the explanation for the observed variation in molecule weight, the primary value being 2×10^6. Although Southwick *et al.* (1980) represented the change in diffusion coefficient as being linear with time, the data could equally be fitted to a non-linear process.

Aggregation and organisation in solution

The aggregation kinetics of amylose have also been extensively investigated. Pfannemuller *et al.* (1971) showed, by using almost monodisperse amyloses which had been enzymatically synthesised, that molecules of intermediate length, with a degree of polymerisation (DP) of about 80, are particularly unstable and precipitate rapidly. Shorter and longer chains are much more stable in solution (Fig. 1). Measurements of the radii of gyration and the second virial coefficient (A_2) of amyloses of different DP had indicated that A_2 shows a maximum at a DP for which *inter*molecular association is inhibited by the coiling of the chains while no change of the radii of gyration was found using retrograding solutions (Husemann *et al.*,

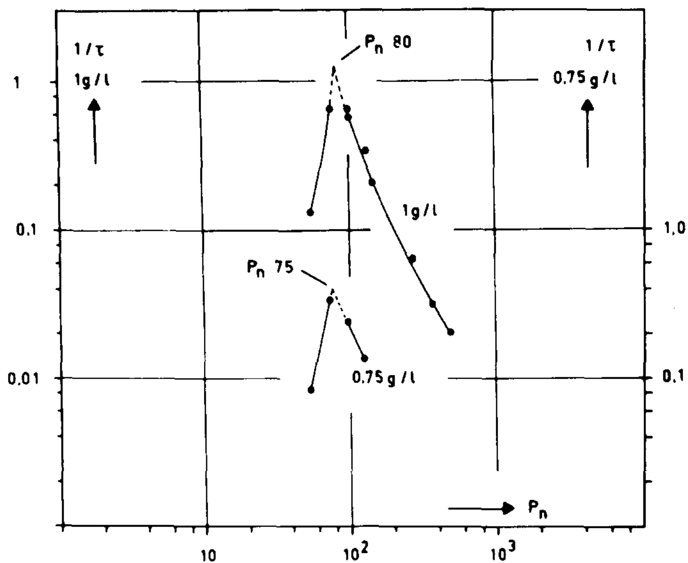

Fig. 1. Dependence of association rate on the degree of polymerisation of synthetic amyloses.

1963; Burchard, 1963). Pfannemuller & Ziegart (1981) conclude that, because of the known stiffness of the amylose chain, *inter*molecular hydrogen bonding is favoured in that critical DP range which displays a maximal retrogradation rate. Longer chains, however, show a larger coiling tendency and a stabilisation can occur predominantly by intramolecular hydrogen bonds. Pfannemuller *et al.* (1971) have also found that admixing an amylose of DP97 with one of DP ~2000 led to a significant reduction in the rate of retrogradation (Table 1).

TABLE 1
ASSOCIATION RATE IN A MIXTURE OF SYNTHETIC AMYLOSES DP97 AND ~2000

DP97 (*c, g/litre*)	*DP~2000* (*c, g/litre*)	*Units of τ* (*h*)
0·75	0·25	17
0·75	0	4·5
1·0	0	1·5

These workers initially proposed a solubilising process. In view of the fact that retrogradation occurs to form a double helix (Wu & Sarko, 1978) and that chains of different DP may associate (Kodama & Noda, 1978), it seems quite possible that the larger, more stable, chains delay precipitation of the smaller chains through a process of associating with them.

Crystallisation from solution

A further development on the theme of aggregation and organisation is illustrated by the work of Barham *et al.* (1974) who have reported on the growth of dextran spherulites from water and other solvents. This phenomenon is of particular interest because of the use of dextran as a blood plasma filler. The rate of growth of the dextran spherulites was followed microscopically under varying conditions of temperature and solvent and employed dextrans of different molecular weights. The formation of distinctive, banded and birefringent spherulites was observed. The authors also investigated the activation energy of spherulitic growth as a function of solvent environment, initial concentration of the solution and the molecular weight of the particular dextran used. Some of these results are shown in Table 2.

Two principal trends can be seen in the activation energies, although it should be noted that the energies do lie within experimental error of each other. They are (i) increasing length of the molecular chains results in an increased activation energy and (ii) that this varies with the solvent used. The lower activation energy for spherulites with an MW ~2400 may be related to the fact that whereas a chain-folded configuration is well established in spherulites of synthetic polymers—and there is no reason to expect a different situation with dextran—the short chains of

TABLE 2
ACTIVATION ENERGIES (IN kJ/mol) FOR SPHERULITIC GROWTH OF DEXTRANS FROM A 10%w/v SOLUTION

Solvent	*MW of dextran*	*Activation energy (kJ/mol)*
DMSO	2400	24 ± 5
	40000	30 ± 2
Formamide	2400	29 ± 5
	40000	33 ± 5
Water	2400	38 ± 9
	40000	41 ± 8

dextrans of MW ~2400 (~15 monomer units) are very unlikely to crystallise in the chain-folded configuration and thus a lower activation energy is to be expected.

CRYSTAL GROWTH IN SEMISOLID SYSTEMS—THE AVRAMI EQUATION

The development of a new phase within the mother phase, as with a crystal in a liquid, involves both the initiation of the phase and its subsequent development. The former process is termed nucleation and the latter, growth. For most cases of interest, isothermal crystallisation can be described in terms of the nucleation frequency, N, and the growth rates of each of the crystallographic planes. We may describe the number of nuclei generated in time, dt, as:

$$dn = \dot{N}[1 - \theta]\, dt \tag{2}$$

where $\dot{N}$ is the steady-state nucleation frequency per unit of untransformed mass and θ is the fraction of material transformed at time t. Building on this foundation, Avrami (1941) was able to develop a general equation to describe the progress of isothermal crystallisation, as follows:

$$\ln(1 - \theta) = Kt^n \tag{3}$$

where K is a constant containing the nucleation and growth parameters while n is an integer whose value depends on the mechanism of nucleation and the form of crystal growth. Values of n calculated for different situations are given in Table 3.

In the classical studies of McIver *et al.* (1968), this expression was applied to the study of the retrogradation of gelatinised starch by differential thermal analysis (DTA). The value of the Avrami exponent, n, was unity and was unaffected by the rate of cooling after gelatinisation, from which was deduced the fact that the nucleation process in starch crystallisation is instantaneous in these systems and thereafter the crystal growth was rod-like. Similar behaviour has been reported by Kim *et al.* (1976) for cassava starch. Subsequently, Kim & D'Appolonia (1977) have also investigated the effect of pentosans on starch retrogradation using rheological

TABLE 3
EXPONENTS OF THE AVRAMI EQUATION FOR VARIOUS MECHANISMS OF CRYSTALLISATION (MEARES, 1965)

Form of growth	*Type of nucleation*	*Exponent* n
Fibrillar	Predetermined	1
Fibrillar	Sporadic	2
Discoid	Predetermined	2
Discoid	Sporadic	3
Spherulitic	Predetermined	3
Spherulitic	Sporadic	4
Sheaf-like	Predetermined	5
Sheaf-like	Sporadic	6

techniques. Both soluble and insoluble pentosans delayed the retrogradation process, the soluble pentosans particularly so, and indeed there is some indication that the Avrami exponent is significantly lower than 1 (Table 4) for these instances.

Maxwell & Zobel (1978) have examined the effect of added sugars and varying water contents on the ageing processes in wheat starch gels, as measured by gel rigidity. The time constant of the ageing process was unaffected by sucrose, but

TABLE 4
EFFECT OF PENTOSANS ON THE AVRAMI EXPONENT AND THE TIME CONSTANT OF 50 % STARCH GELS STORED AT 21 °C AND 30 °C

Gels	*Storage temperature (°C)*	*Avrami exponent*	*Time constant units (days)*	
			Overall	*Over the first day storage*
Starch	21	0·98	3·80	3·70
	30	1·01	5·47	5·64
Starch + soluble pentosan	21	0·70	5·33	3·29
	30	0·75	6·80	3·49
Starch + insoluble pentosan	21	0·83	7·50	5·75
	30	0·89	9·65	8·14

reduced by dextrose and markedly so by fructose. On the other hand, the final rigidities of the gel showed a maximal value for starch/water by itself and then, progressively, lower values for fructose, dextrose and sucrose. The water content also had a significant effect upon the time constant of the retrogradation process, these falling from 85 h at 70 % water (w/w) to 20 h at 40 % (w/w).

LONG-TERM CHANGES IN GELS

In practice, of course, most foodstuffs cannot be considered as simple solutions or as polymer units, but, particularly where polysaccharides are present, display

rheological characteristics typical of crosslinking and therefore may be considered to be gels. Gels may vary widely in their water content and are characterised by organised crosslinked regions frequently referred to as crystallites. Indeed, gels may be thought of as the product of a hindered crystallisation process, the ordered regions being separated and linked by less organised chains in a state very much as found in solution. We may anticipate that the phenomena of aggregation and crystallisation are important in the ageing of these systems also. Some results of the rheological effects of ageing in pectate gels (Mitchell & Blanshard, 1976*b*) are shown in Fig. 2.

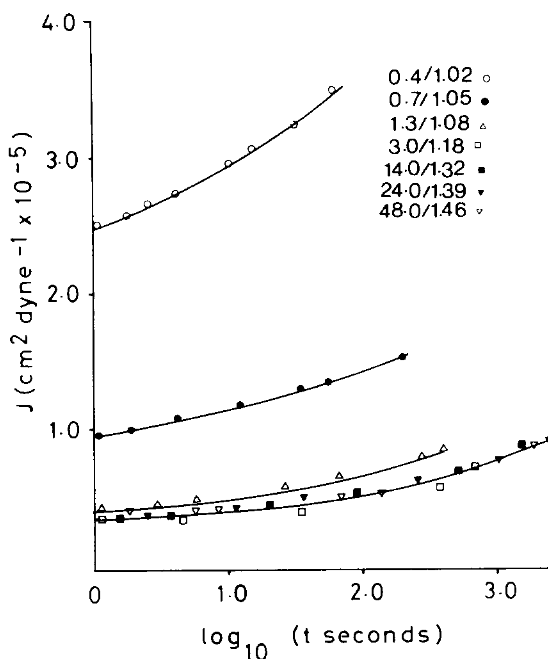

Fig. 2. Creep compliance response of pectate gels aged for different times. Numbers refer to the time after formation of the gelling mixture (h)/% polysaccharide in the gel.

THE THERMODYNAMICS OF GEL STABILITY

In general, polysaccharide gels are in metastable equilibrium and, as such, changes in their rheological properties and water-holding capacity are commonplace. We can discuss the factors contributing to the stability of such thermolabile materials along the same lines as that used by Borchard *et al.* (1980) in an investigation of time- and temperature-dependent changes in gelatin gels. We shall assume that the initial formation of the mini-crystallites which constitute the crosslinks of gel systems is closely akin to the process of nucleation in synthetic polymers.

In such synthetic polymers two types of nuclei are discussed, lamellar and bundle-like crystals, and these two forms may be conceived as the extremes of what is found in the nuclei and crystallites in natural polymer gel systems. Suppose that the volume and surface of such crystallites can approximately be described by a cylinder of length, l, and radius, r, in which the chains are oriented in the direction of the cylinder axis, then the change in the free energy ΔG when a cylindrical crystallite is formed from solution can be described by three expressions:

A volume term $\Delta G_1 = -\pi r^2 l \Delta G_u^{(v)}$ (4)

A surface term $\Delta G_2 = 2\pi[r^2\sigma_e$ and $rl\sigma_l]$ (5)

A solution term $\Delta G_3 = \dfrac{-\pi r^2 l}{V_u/N} \cdot \dfrac{kTV_u}{V_{0_1}} \left[\dfrac{\ln \phi_2}{x} - \left(1 - \dfrac{1}{x}\right)(1-\phi_2) + \chi(1-\phi_2)^2\right]$ (6)

where ϕ_2 is the volume fraction of the polymer in solution, x the ratio of the molar volumes of the polymer and solvent, χ the polymer–solvent interaction coefficient, T = temperature absolute, V_u and V_{0_1} the molar volumes of the polymer unit and solvent, respectively, N Avogadro's number, σ_e and σ_l the end and lateral surface free enthalpies and $\Delta G_u^{(v)}$ is the Gibbs free energy of melting per unit volume of a polymer. It can be shown (see Appendix) by the usual derivation of the Gibbs–Thomson equation that:

$$T_m = \left(\frac{R}{V_0}\left[\frac{\ln \phi_2}{x} - \left(1 - \frac{1}{x}\right)(1-\phi_2) + \chi(1-\phi_2)^2\right] - \frac{\Delta H_u^{(v)}}{T_m^\circ}\right)^{-1} \cdot \left(\frac{2\sigma_l}{r} - \Delta H_u^{(v)}\right) \quad (7)$$

where T_m is the melting point of a crystallite of radius r in the mixture. At first sight this is rather complex, but we can simplify it by taking the following two cases:

when $\phi_2 = 1$, then:

$$\frac{T_m^\circ - T_m}{T_m^\circ} = \frac{2\sigma_l}{r} \cdot \frac{1}{\Delta H_u^{(v)}} \quad (8)$$

where T_m° is the melting point of a crystallite of infinite size into the pure polymer melt. This is the Gibbs–Thomson equation for a cylinder of radius, r:

when $r = \infty$

$$\frac{1}{T_m^\circ} - \frac{1}{T_m^\infty} = \frac{R}{\Delta H_u^{(v)} \cdot V_0}\left[\frac{\ln \phi_2}{x} - \left(1 - \frac{1}{x}\right)(1-\phi_2) + \chi(1-\phi_2)^2\right] \quad (9)$$

T_m^∞ is the melting point of a crystallite of infinite size in the mixture (which Flory simply terms T_m as he neglected surface effects). Using this definition of T_m, eqn (7) can be put in the simpler form:

$$\frac{T_m^\infty - T_m}{T_m^\infty} = \frac{2\sigma_l}{r} \cdot \frac{1}{\Delta H_u^{(v)}} \quad (10)$$

It is evident from both eqn (8) and eqn (10) that if r increases, then T_m also increases (since $T_m < T_m^\circ$ and T_m^∞). Processes therefore which result in an effective increase of the crystalline size will lead to an elevation of T_m not only for pure melt but also for mixtures as found in gels.

ANNEALING OF GEL SYSTEMS

Annealing is such a process. Borchard *et al.* (1980) have applied these principles to gelatin gels and have quite clearly shown developments in the structure of a 40% gelatin gel as the sample is held at 302 K. Whereas at zero time a broad peak occurred with a maximum at 300 K, on annealing at 302 K, a second maximum developed which shifted to higher temperatures (310–314 K). Similarly, the thermograms of 60% gelatin gels annealed at temperatures between 278 K and 312 K for 8 h differed vastly, obviously reflecting the differences in gel structure.

Such changes have not been studied in polysaccharide gels, but Ainsworth (1974) has observed a strong time dependent increase in the specific rotation at 400 nm of κ-carrageenan gels after gelation, which indicates continuing changes in the conformation and interactions of the macromolecular chains.

A related phenomenon is the observation (Marchant & Blanshard, 1978) that the degree of gelatinisation, as measured by birefringence and small angle light scattering, is dependent on the previous thermal history. Annealing of starch granules had already been reported but at first sight it appeared surprising that successive ~2 °C jumps in temperature (for details see the paper) had only resulted in a loss of 42% of the birefringence at 58·0 °C, whereas ~7 °C temperature jumps had resulted in the total destruction of birefringence by 58·4 °C. This observation, which has been separately confirmed (A. H. Muhr, personal communication), suggests that the annealing process may occur quite rapidly. Such changes, taking place much more slowly at room temperature and in relatively dry conditions, may be partly responsible for changes in the properties of cereal raw materials.

SYNERESIS

The loss of water by polysaccharide gels is a serious problem with which food manufacturers and raw material suppliers are constantly faced. It reflects an imbalance between entropically originating free energy changes which would encourage mixing, i.e. absorption of solvent and swelling of gels, and the elastic free energy changes arising from crosslinking which, if predominating, lead to contraction of the gel mass and expression of solvent (the so-called syneresis). At the molecular level it is clearly a case of progressive aggregation and organisation of polymer chains when thermodynamically favoured.

From a theoretical point of view several interesting theories have been developed and applied principally to polyacrylamide gels. Tanaka (1979), for example, has found evidence of a phase transition in the polymer chain in the pronounced network collapse observed as an aged polyacrylamide gel is shifted from a good to a poor solvent and has formulated an equation to take this behaviour into account based on the Flory-type mean field theory. Janas *et al.* (1980) have recently pointed out, however, that such a phenomenon may have an alternative explanation arising from the heterogeneity of such gel structures as has been demonstrated by Weiss & Silberberg (1977, 1979). Obviously, it will be of interest to study syneresis of polysaccharide gels in the light of these and other theories.

From a practical point of view the amount and rate of water loss may be critically affected by the nature and concentration of the polysaccharide, the method of gel preparation and what ions are present, if any. The following unpublished results (Mitchell, 1974) (Table 5) illustrate this point. All gels were prepared as described by Mitchell & Blanshard (1976*a*).

TABLE 5
PERCENTAGE OF WATER REMAINING IN ALGINATE AND PECTATE GELS AFTER AGEING FOR 24 h

$CaHPO_4$ (*molarity*)	*0·010*		*0·150*	
Polysaccharide conc. (%)	*1·0*	*0·50*	*0·75*	*1·0*
% *water remaining after 24 h:*				
Alginate (high mannuronic acid)	96	45	54	66
Alginate (high guluronic acid)	80	53	60	68
Sodium pectate	87	61	67	72

In these gels it is evident that syneresis is enhanced by increasing calcium concentration and decreasing polysaccharide concentration, and in general syneresis was somewhat less pronounced for the calcium pectate gels. There is little doubt that the degree of syneresis of gels prepared under comparable conditions reflects the stiffness of the chains. Bailey *et al.* (1977) have shown, from theoretical calculations, that the stiffness of the polysaccharide chains increases through the series high mannuronic alginate, high guluronic alginate, pectate. It is not surprising, therefore, at high calcium concentrations ($\geq$0·50M $CaHPO_4$) where the distance between crosslinks is small, that the chains are non-Gaussian in behaviour and those that are stiffer are less predisposed to syneresis. (On the other hand, at low calcium ion levels (0·010M $CaHPO_4$) the high guluronic alginate and pectate bind calcium ions more tightly than high mannuronic alginate, thereby favouring crosslinking and hence syneresis.) In accordance with this view Smidsrod & Haug (1972) have also shown that the volume of calcium alginate gels prepared by dialysis increases with an increasing proportion of the stiff polyguluronic acid.

MECHANICAL ENHANCEMENT OF AGEING EFFECTS

Recent investigations in synthetic polymers (Struik, 1980) have shown that the application of repeated mechanical stresses may enhance the ageing as determined by measuring changes in the tensile creep compliance, but only when the resultant strains were larger than 0·3–0·5%. It is, however, quite conceivable that food products are exposed to such strains during transportation and this aspect deserves examination.

APPENDIX

Since the derivation of eqn (7), given in Borchard *et al.* (1980), is erroneous, the correct derivation of the Gibbs–Thomson equation is given here.

At equilibrium, there is no change in free energy on making small changes in the size r, l of the crystallite. Thus:

$$\frac{\partial}{\partial l}\Delta G = 0$$

$$\frac{\partial}{\partial r}\Delta G = 0$$

Applying these equations to the expression for $\Delta G\ (=\Delta G_1 + \Delta G_2 + \Delta G_3)$ in eqns (4), (5) and (6) we obtain at equilibrium (which we denote by $T = T_m$, where T_m is a function of r, l):

$$r = \frac{2\sigma_l}{\frac{RT_m}{V_0}\left[\frac{\ln \phi_2}{x} - \left(1 - \frac{1}{x}\right)(1-\phi_2) + \chi(1-\phi_2)^2\right] + \Delta G_u^{(v)}} \tag{11}$$

and also the necessary condition $l = 2r\sigma_e/\sigma_l$.

To obtain eqn (7), it is necessary to express $\Delta G_u^{(v)}$ as a function of T. Assuming $\Delta H_u^{(v)}$ and $\Delta S_u^{(v)}$ to be temperature independent, it follows from the relationship $\Delta G_u^{(v)} = \Delta H_u^{(v)} - T\Delta S_u^{(v)}$ and the fact that $\Delta G_u^{(v)} = 0$ when $T = T_m^\circ$ that:

$$\Delta G_u^{(v)} = \Delta H_u^{(v)} \frac{T_m^\circ - T}{T_m^\circ} \tag{12}$$

Substitution of eqn (12) into eqn (11) yields, after algebraic manipulation, eqn (7).

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THE CHEMISTRY OF TEXTURAL CHANGES IN FRUIT DURING STORAGE

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ABSTRACT

Cells in plant tissue are surrounded by cell walls which are comparatively rigid and give mechanical support to the tissue. The walls of pome fruits, strawberry and tomato contain a high proportion of galacturonic acid, galactose and arabinose residues which are typical of pectic polysaccharides. The bonding between the polymers in the middle lamella, the region of the wall between adjacent cells, is thought to be ionic in nature involving Ca^{2+} and carboxyl groups of the pectic polysaccharides. Structural changes occur in the middle lamella and primary cell wall during ripening which lead to cell separation and softening of the tissue.

Softening is characterised by an increase in the concentration of soluble pectic polysaccharide. In apple, the molecular weight of this fraction remains unchanged and endo-polygalacturonase (endo-PG), a random cleavage enzyme, is absent. Exo-polygalacturonase (exo-PG), a terminal cleavage enzyme, is present and approximately 10% of the galacturonic acid residues of the cell wall are lost during ripening. In other fruits, such as pear and peach, both exo- and endo-PG activities develop and the molecular weight of the soluble pectic polysaccharide decreases. It is concluded that the softening observed in ripening fruits derives from the synthesis and transport to the cell wall of wall degrading glycosidases.

INTRODUCTION

Softening of the fleshy tissues of fruits is one of the most important changes occurring during storage and has a major influence on customer acceptability. The texture of living plant tissue is affected by its cellular anatomy, the water relations of the cells and the composition of the cell walls. Fruit cells retain their normal osmotic

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properties during ripening (Simon, 1977) and there is probably little loss of turgor pressure although this may be affected by dehydration if fruit is kept in a low humidity. Turgor pressure probably provides a driving force for cell separation following structural changes in the middle lamella and primary cell wall.

CELL WALL STRUCTURE

Plant primary cell walls

The primary wall consists of cellulose microfibrils embedded in a matrix of pectic and hemicellulosic polysaccharides and hydroxyproline-rich glycoprotein (Northcote, 1972). The model structures established by Albersheim and his co-workers (Talmadge *et al.*, 1973; Bauer *et al.*, 1973 and Keegstra *et al.*, 1973) for rhamnogalacturonan with its side chains of galactose and arabinose residues (Fig. 1) and for xyloglucan have gained general acceptance. However, the proposed

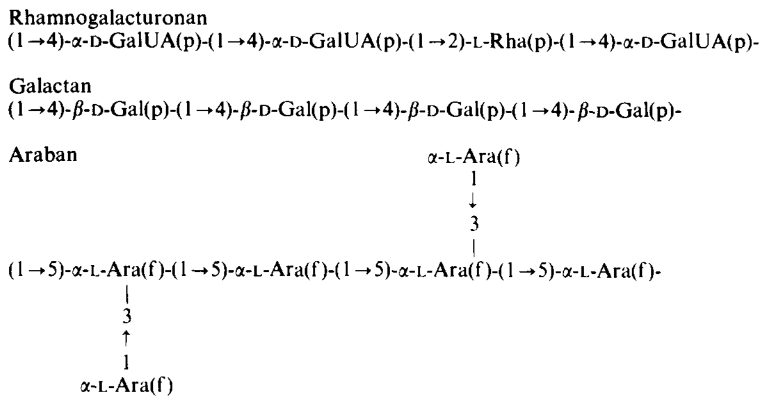

Fig. 1. General features of pectic polysaccharides. Standard abbreviations are used to represent the monosaccharides and (p) and (f) denote pyranose and furanose forms, respectively.

covalent linkages between these polymers and others in the cell wall have proved more controversial. Keegstra *et al.* (1973) presented evidence for a linkage of xyloglucan to rhamnogalacturonan through a galactose chain, and of rhamnogalacturonan to protein through arabino-galactan linked to serine. The existence of these linkages has not been substantiated and other authors (for example, Monro *et al.*, 1976) have preferred structures based on non-covalent bonding.

Water is an important constituent of the cell wall (Northcote, 1972). The quantity of water within the wall matrix can be controlled to some extent by the proportions of the polymers as pectic polysaccharides are able to bind much more water than the hemicelluloses (Cook & Stoddart, 1973). Some of the water associated with the

pectic polysaccharides is tightly bound and is required for the maintenance of the conformation of the polyuronide chains. Water also acts as a solvent within the wall for the presence and transport of salts and low molecular weight organic compounds, and provides a suitable environment for the function of enzymes such as the glycosidases which hydrolyse cell wall polymers.

The galacturonan backbone of pectic polysaccharides exists as an extended ribbon-like chain with a threefold screw axis (Rees & Wight, 1971). Rhamnose residues occur within the chain and at these points the chain is bent or kinked (Rees & Wight, 1971). The rhamnose residues are the points of attachment for side chains of galactose and arabinose (McNeil *et al.*, 1980). The gel is the state most typical for polysaccharides in biological systems, the polymer chains usually forming an interconnecting network which gives rise to characteristic texture and properties (Rees, 1969). Molecules of water are held within the interstices of the network. The galacturonan molecules of the plant cell wall are thought to form an interconnecting network through the formation of non-covalent bonds at regions of chain association, known as junction zones. Galactose residues occur as β-1,4-linked linear polymers whilst the arabinose residues occur as branched arabans. The arabinofuranose residues form a hydrophilic network so that these molecules can also hold water within the gel structures formed by the galacturonan (Northcote, 1972).

Analyses of cell wall pectic polysaccharides using chemical (Cook & Stoddart, 1973) and enzymic methods (Talmadge *et al.*, 1973; Knee *et al.*, 1975; McNeil *et al.*, 1980) indicate that the neutral sugar side chains occur in blocks interspersed in the rhamnogalacturonan. The pectic polysaccharides of the primary wall are thought to be more highly substituted than the polymers of the middle lamella (Knee *et al.*, 1975). The rhamnose residues which cause kinking in the galacturonan would prevent alignment of chains, whilst regular packing could be hindered by the side chains (Rees, 1969). The pectic polysaccharides of the primary wall might therefore be expected to form a less extensive gel network than the middle lamella polymers.

Whereas genes control the assembly of peptide chains by an accurate template mechanism, the synthesis of the matrix polysaccharides of the cell wall is catalysed by a large number of glycosyl transferases coded in the genetic material of the cell (Northcote, 1972); the polysaccharides are thus secondary gene products assembled by the concerted action of multiglycosyl transferase systems. Little is known about the regulation of matrix polysaccharide biosynthesis (Delmer, 1977) but, by analogy with the synthesis of the carbohydrate moiety of glycoproteins, control of polymer size and composition could be mediated by a number of factors including the concentration and structural features of donor and acceptor molecules and the concentration and specificity of the transferases (Pazur & Aronson, 1973). Polysaccharides are polydisperse in nature. For example, the pectic polysaccharides, although essentially similar in structure, may have different proportions and distributions of sugar residues and also display variation in the

frequency of branch points (Reid & Wilkie, 1969). Polysaccharides are also polymolecular, showing a range of molecular weights (Reid & Wilkie, 1969), although they generally tend towards an upper limit of molecular weight which is often very high (Cook & Stoddart, 1973).

Structure of the middle lamella

Histological studies of ripening fruit reveal extensive cell separation and it is natural to suppose that changes in the intercellular matrix, the middle lamella, are responsible for this. Electron microscope studies of the cell walls of apple and pear during ripening support this hypothesis (Ben-Arie *et al.*, 1979). Traditionally, the middle lamella has been thought to be rich in pectic polysaccharides and this has been confirmed by use of ferric hydroxamate staining prior to electron microscopy (Albersheim & Killias, 1963). The observation that cells of non-woody plants can be dissociated using chelating agents (Ginsburg, 1961; Letham, 1962; Linehan & Hughes, 1969) suggests that cohesion of the middle lamella depends on ionic rather than covalent bonds. Divalent cations, especially calcium, are the obvious candidates, but, as Rees (1969) pointed out, single calcium ions would only form weak bonds between isolated carboxyl groups. More stable bonding occurs by co-operative effects when sequences of uronic acids lie parallel and each pair of residues encloses a calcium ion; the so-called 'egg box' junction zones (Grant *et al.*, 1973; Morris, 1980).

Fruit cell walls

Apart from the vascular tissue and specialised cells, such as the stone cells in pears, the cell walls of the fleshy parts of most fruits are unlignified. They also contain a low proportion of hydroxyproline-rich protein and small amounts of xylose and mannose residues which are characteristic of hemicellulosic polysaccharides; on the other hand, they contain a high proportion of galacturonic acid, galactose and arabinose residues which are typical of pectic polysaccharides (Knee *et al.*, 1975).

Purified glycosidases, mainly from fungal sources, have been used in detailed structural studies of apple fruit cell walls (Knee *et al.*, 1975). The pectic polysaccharides in the walls are (a) a simply substituted methyl esterified galacturonan which is probably localised in the middle lamella and (b) a branched methyl esterified rhamnogalacturonan probably concentrated in the primary wall. Branched araban chains are covalently linked to the rhamnose residues of the rhamnogalacturonan, and linear galactan chains are attached to galacturonosyl residues of the polymer (Barrett & Northcote, 1965). The branched rhamnogalacturonan shares most of the features of the rhamnogalacturonan of sycamore suspension culture cell walls (Talmadge *et al.*, 1973) and forms the main constituent of the primary wall.

Subsequent chemical studies showed that the simply substituted galacturonan in

apple cell walls could be solubilised by partial methylation of its free carboxyl groups with diazomethane (Knee, 1978*a*). Diazomethylation or treatment with the chelating agent, sodium polyphosphate, also caused loss of cell to cell cohesion in ethanol-extracted tissue (Knee, 1978*a*). This suggested that at least some of the 20 % of non-esterified carboxyl groups in the cell wall are important in maintaining cell cohesion by cross linkage through divalent metal ions.

Single cross links are weak and a stable structure would require the alignment of non-esterified sequences of uronic acid in otherwise esterified chains; as yet nothing is known about the distribution of methyl ester groups in the galacturonan.

Chromatographic purification of an alkaline extract of apple cell walls (Knee, 1973*a*) yielded a polysaccharide of similar gross composition to the xyloglucan of sycamore cell walls extensively studied by Bauer *et al.* (1973). Although xylose and glucose residues are often present in pectic polysaccharide fractions obtained by enzymic (Knee *et al.*, 1975) or chemical (Knee, 1978*a*) degradation of apple fruit cell walls, there is no direct evidence of linkage between the hemicellulosic and pectic polysaccharides.

ENZYMES ASSOCIATED WITH CELL WALL DEGRADATION

A number of glycosidases have been implicated in the degradation of cell wall polysaccharides, together with an esterase, pectin esterase, which catalyses de-esterification of methyl esterified pectic polysaccharide (Table 1). β-Galactosidase and exo-polygalacturonase (exo-PG) are terminal cleavage enzymes, releasing monosaccharides from the non-reducing end of the substrate. Endo-polygalacturonase (endo-PG) and endo-β-1,4-glucanase are random cleavage enzymes. The pH optimum of the exo-PG and endo-PG, determined in *in vitro* assay, is in the range 4·0–5·5, whilst for fruit pectinesterases, assayed with NaCl (0·05–0·20M), the

TABLE 1

MOLECULAR WEIGHTS OF ENZYMES ASSOCIATED WITH THE HYDROLYSIS OF CELL WALL POLYMERS OF RIPENING FRUITS

Enzyme	*Molecular weight*[a]				
	Apple	*Pear*	*Peach*	*Tomato*	*Avocado*
Exo-PG	58000	63000	68000		
Endo-PG		160000	41000	44000; 84000[b]	
β-Galactosidase	46000			63000	
Endo-β-1,4-glucanase					49000
Pectinesterase	27000			23700–35500[c]	

[a] Data for enzymes of apple, Bartley (1978) and unpublished and Miyairi *et al.* (1975); pear, Pressey & Avants (1976) and Pressey (personal communication); peach, Pressey & Avants (1973*a*); tomato, Pressey & Avants (1972. 1973*b*) and Wallner & Walker (1975); avocado, Awad & Lewis (1980).

[b] Major endo-PG has molecular weight 44000.

[c] Four pectinesterases characterised, molecular weights 23700, 24300, 27000 and 35500.

pH optimum is usually 7·0–8·5 (Rexova-Benkova & Markovic, 1976). Pectinesterase is also activated by Ca^{2+}. For example, the pH optimum of the orange enzyme changes to 5·0–8·0 in the presence of 0·05M $CaCl_2$ (MacDonnell *et al.*, 1945).

Assay of endo-PG and exo-PG with galacturonan and methyl esterified galacturonan substrates (Pressey & Avants, 1973*a*; Bartley, 1978) indicates that the de-esterified substrate is more rapidly hydrolysed and suggests that pectinesterase may influence the rate of hydrolysis of cell wall polymers by the glycosidases. However, in apple, in contrast to pear, peach and tomato, the degree of esterification of the pectic polysaccharides does not decline during ripening but remains essentially unchanged, and this may question the postulated rôle of pectinesterase in this fruit (Doesburg, 1965; Knee, 1978*a*). The pH of the fruit cell walls is not known, although, from the pH optima of the glycosidases, it might be expected to be below 7·0. Partially purified PG's can release polymers of uronic acid from fruit cell wall preparations in *in vitro* incubations at pH 4·0–4·5 (Pressey & Avants, 1973*a*, 1976; Bartley, 1978).

It is notable that, of the enzymes associated with cell wall degradation in ripening fruits (see Table 1), only the endo-PG of pear has a molecular weight (MW) in excess of 100,000. A comparison of the hydrolysis of apple fruit cell wall preparations using a number of endo-PG's (MW 37-200,000) and also α-L- arabinofuranosidases (MW 40-350,000) suggested that enzymes with a molecular weight greater than 100,000 are excluded from the wall and cannot degrade it, irrespective of their activities with soluble substrates (Knee *et al.*, 1975). Carpita *et al.* (1979), working with living plant cells including cultured cells of sycamore maple, calculated that globular proteins having a molecular weight greater than 17,000 cannot diffuse freely through the cell wall.

WALL DEGRADATION DURING FRUIT RIPENING

Cellulose

Crystalline α-cellulose is very resistant to enzymic attack and can only be degraded by the combined operation of a number of glycosidases, including the endo-glucanases C_1 and C_x (Rees, 1977). The C_1 glucanase, which is thought to initiate degradation of the polymer, has not been detected in fruit and explains why degradation of cellulose has not been observed during fruit ripening.

Hemicelluloses

The small quantities of monomers characteristic of the hemicelluloses, xylose, glucose and mannose, do not decline during the ripening of apples (Bartley, 1976), strawberries (Neal, 1965), tomatoes (Gross & Wallner, 1979) and pears (Ahmed & Labavitch, 1980). Endo-β-1,4-glucanase is present in a number of fruits including apple (Bartley, unpublished data), pear (Yamaki & Matsuda, 1977), tomato (Pharr

& Dickinson, 1973), peach (Hinton & Pressey, 1974) and avocado (Awad & Lewis, 1980). The activity of the enzyme is normal in the non-ripening *rin* mutant of tomato (Poovaiah & Nukaya, 1979) and this observation, together with the analytical data, suggests that the enzyme does not have a primary rôle in fruit softening.

Loss of galactose residues

The major change in monomeric composition of the cell walls of ripening apples is the loss of about 70% of their galactosyl residue content (Table 2; Knee, 1973*b*).

TABLE 2
ANHYDRO SUGAR COMPOSITION OF POLYSACCHARIDES IN CELL WALL AND SOLUBLE FRACTIONS FROM APPLE AND STRAWBERRY FRUITS

	Apple[a]				*Strawberry*[a]			
	Unripe		*Ripe*		*Unripe*		*Ripe*	
	Cell wall	*Soluble*	*Cell wall*	*Soluble*	*Cell wall*	*Soluble*	*Cell wall*	*Soluble*
Rhamnose	0·11	ND	0·10	ND	0·63	0	0·35	1·39
Arabinose	2·68	0·07	2·00	0·14	3·51	0·15	0·81	3·17
Xylose	0·79	0·01	0·73	0·01	1·03	0·04	1·79	0·30
Mannose	0·32	ND	0·32	ND	0·47	0·02	0·94	0
Galactose	3·78	0·11	0·75	0·14	4·34	0·08	1·56	2·57
Glucose[b]	29·3	0·06	4·44	0·06	16·6	0·28	13·5	3·69
Galacturonic acid	3·90	0·10	2·51	1·00	21·0	4·01	6·74	16·3

[a] Apple results are quoted in mg gram^{-1} fresh weight, strawberry results as mg per fruit (unripe fruit weight 2·41 g, ripe fruit weight 8·68 g).
[b] Results for cell wall glucose include starch.
ND: not determined.

The loss of monomer occurs in the branched pectic polysaccharide fraction (Knee *et al.*, 1975). Similarly in tomatoes (Gross & Wallner, 1979), loss of galactose is the dominant change, but in pears (Ahmed & Labavitch, 1980) and strawberries (Neal, 1965) the change is smaller. However, there is evidence of turnover of galactose residues during growth of strawberry fruits (Knee *et al.*, 1977) and this seems to be a feature of cell expansion in other tissues (Labavitch & Ray, 1974). Apples (Bartley, 1974), tomatoes (Wallner & Walker, 1975) and pears (Yamaki & Matsuda, 1977) all contain β-galactosidase activity. The apple enzyme was shown to be capable of degrading β-1,4-linked galactan (Bartley, 1974) and releasing galactose residues from apple cell wall preparations (Bartley, 1978). Enzyme activity was present at all stages of ripening in the various fruits examined; in the apple, activity associated with the cell wall was detectable at all stages (Bartley, 1977). This leads to the suggestion that hydrolysis of terminal galactosyl residues is a constant feature of some fruit cell walls and that galactose residues decline only when the synthesis of rhamnogalacturonan carrying branches of galactose units fails to make good the losses. The significance of the loss of galactose residues for softening is not clear for in *rin* tomatoes the loss takes place after harvest, when little softening occurs (Gross & Wallner, 1979).

Increase in soluble pectic polysaccharide

It has been known for at least 60 years that the proportion of soluble pectic polysaccharide increases in ripening apples (Carre, 1922). The process is matched by a decline in the insoluble pectic polysaccharide fraction (see Table 2) and it is natural to conclude that a change in part of the insoluble fraction converted it to a soluble form. The ensuing years have seen various interpretations of what that change might be. Joslyn (1962) has reveiwed the older literature but we shall confine ourselves to more recent developments.

The soluble pectic polysaccharide in ripe apples has a low proportion of neutral monomers (Knee, 1973*a*) and this is in keeping with its supposed origin in the middle lamella. Its physical properties, and hence its molecular weight, are comparable with those of the soluble pectic polysaccharide in the unripe fruit (Knee, 1978*a*). The apple is exceptional among fruits in that it appears to lack endo-PG. In other fruits, the enzyme is an obvious means of achieving solubilisation, and the soluble pectic polysaccharide isolated from peaches (Pressey *et al.*, 1971), strawberries (Woodward, 1972) and pears (Knee, 1973*a*) shows progressive depolymerisation with ripening. Endo-PG activity increases in tomatoes and pears during ripening, but activity is not measurable in unripe fruit (Hobson, 1964; Tucker *et al.*, 1980; Knee, Casimir & Bartley, unpublished data). Radioimmunoassay of extracts from green and ripe tomato fruits suggests that the increase in endo-PG activity observed derives from net synthesis of protein (Tucker *et al.*, 1980).

An interesting contrast is provided by different varieties of peach. Freestone varieties possess high activities of both endo-PG and exo-PG; they show extensive softening and a marked rise in soluble pectic polysaccharide on ripening. Clingstone varieties have low activities of endo-PG and show less softening and pectic polysaccharide solubilisation (Pressey & Avants, 1978).

Endo-PG has not been detected in apple, but an exo-PG is present (Bartley, 1978) and this must account for the loss of about 10% of total uronic acid residues during ripening. The enzyme preferentially hydrolyses non-esterified galacturonan, but is capable of degrading apple cell walls to release monomers and some polymeric material. The viscosity of the pectic polysaccharide extracted from the cell wall with sodium polyphosphate declines during ripening (Knee, 1978*a*). This fraction is thought to derive from the middle lamella region of the cell wall and suggests that this is the site of action of exo-PG.

A MODEL OF THE CELL SEPARATION PROCESS

From the data discussed, a model can be proposed for the softening of fruits observed during ripening. It is suggested that the middle lamella is stabilised by junction zones formed by calcium ions and free carboxyl groups in predominantly esterified pectic polysaccharide molecules. Softening is initiated by the transport of

PG to the middle lamella, possibly together with pectinesterase. The apple lacks endo-PG, and the exo-PG would cause a limited degradation of cell wall polymers. In other fruits, endo-PG degrades the soluble pectic polysaccharide, and cell separation and softening are more extensive. The glycosidases display optimal activity at acid pH values; acidification of the cell wall could be brought about by a proton pump located in the plasmalemma. Acidification could also lead to a weakening of cell wall polysaccharide bonding by displacement of calcium ions from junction zones (Soll & Böttger, 1981).

Changes occurring in the abscission zones of plant tissues show analogies with those observed in fruit ripening. Although exo-PG has been prepared from the abscission zones of citrus and bean, endo-PG has not been detected (Riov, 1974; Berger & Reid, 1979). A number of isoenzymes of endo-β-1,4-glucanase have also been assayed. The time course of appearance of the pI 9·5 enzyme and its distribution are consistent with its involvement in abscission (Sexton *et al.*, 1980). It is thought that this enzyme degrades non-cellulosic glucan components of the cell wall.

Secreted proteins are often glycoproteins which undergo post-translational modifications following synthesis of the polypeptide moiety in the endoplasmic reticulum (Chrispeels, 1976). Osborne & Sargent (1976) suggested that hydrolytic enzymes present within the dictyosome vesicles could be secreted to the wall either after fusion of the vesicles with the plasmalemma or by passage of intact vesicles via the desmotubules of the plasmodesmata. Once within the plasmodesmata, any hydrolytic enzymes would be liberated by rupture of the vesicles in the immediate vicinity of the middle lamella. Ben-Arie *et al.* (1979) noted that a characteristic of middle lamella dissolution in ripe apple and pear fruits was the frequent occurrence of vesicles in the vicinity of the plasmodesmata complex. They also noted the persistence of the cell wall–plasmodesmata complex through ripening.

SYNTHESIS OF CELL WALL POLYSACCHARIDES IN RIPENING FRUITS

Most studies of cell wall changes in ripening fruit have concentrated on degradative aspects and the possible rôle of synthetic processes has rarely been considered. A study of polysaccharide metabolism in developing strawberry fruits (Knee *et al.*, 1977) revealed that incorporation of ^{14}C-glucose into wall polymers ceased at the onset of ripening. Strawberry fruits were also labelled with $^{14}CO_2$, 2–6 days after petal fall. A loss of label from insoluble cell wall polymers was observed during ripening of the fruits with a corresponding increase of label in the soluble polymers. This is consistent with the predominance of degradative changes in ripening.

In recent work with apple and pear, tissue discs prepared from the fruits at various stages of ripening were incubated with methyl labelled ^{14}C-methionine and ^{3}H-inositol selected as precursors of cell wall polysaccharides (Knee, 1978*b*). Inositol

incorporation into galacturonan declined sharply during ripening whilst methionine incorporation into methyl ester groups of galacturonan was maintained. This suggested that cell separation might be brought about by methylation of the galacturonan. However, further experiments with pear tissue discs in which uronic acid content was measured together with incorporation of ^{14}C-methionine indicated that the discs synthesised galacturonan and methyl esterified polymer in *in vitro* incubations (Knee, unpublished data). The uronic acid content of whole pears declines continuously during ripening, and the results with discs suggest that polysaccharide synthesis assayed could be a wound injury response to cutting of the tissue (Van Steveninck, 1975).

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CHEMICAL CHANGES IN FOOD BY THE MAILLARD REACTION

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ABSTRACT

The Maillard reaction results from a reaction between reducing sugars and amino acids. Reactive intermediates are formed by a variety of pathways and these can yield both volatile flavour components and brown melanoidins of higher molecular weight. The formation of these compounds is desirable in the heating (cooking) of many food products (meat, coffee, bread) but their occurrence during storage is undesirable and leads to a reduction in quality. The mechanism of the Maillard reaction will be explained and the most important intermediates and reaction products will be pointed out and their properties described. Reaction conditions for the Maillard reaction and methods for its inhibition will be discussed together with a description of methods currently available for the early identification of the Maillard reaction in foods.

The storage of food obviously occurs under conditions which best deter chemical reactions in food. Implicit in this statement is the realisation that food ingredients are chemical compounds which, according to the reactivity of their functional groups, can react more or less quickly. For this reason, a freshness guarantee date, obviously relating to enzymatic reactions and to the influence of microorganisms, is printed on packed food in the Federal Republic of Germany.

A brown colouring of food during storage is often observed, but the browning of fruit and vegetables especially, has nothing to do with the Maillard reaction. Rather, it is enzymatically controlled by polyphenol oxidases which cause oxidation of polyphenol systems like, for example, catechins, tannins and hydroxycinnamic acids which thereby become transformed to *o*-quinones, and quickly react further to brown–red pigments. Involvement of the Maillard reaction is excluded by the observation that such browning, for example, on apples, pears, or bananas, can be

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prevented by immersion in solutions of ascorbic acid, which is well known to be highly reactive in the Maillard reaction.

By the Maillard reaction ('non-enzymatic browning'), one understands reactions of reducing sugars with compounds having free amino groups; that is, with amino acids, peptides and proteins. It appears that the incorporation of a nitrogen function into the sugar molecule vastly reduces its stability, so that the sugar molecule ultimately becomes broken down. The resulting decomposition products, however, also react with amino acids in the Strecker Degradation, so that a multiplicity of reactive components are ultimately produced. These can further react to form brown polymers whereby, depending on the temperature, characteristic aromatic substances also arise. Especially during storage, both aromatic substances and brown polymers are undesirable. The following figures serve to illustrate the mechanism of the Maillard reaction as currently understood.

At the beginning of the reaction, a condensation occurs between a reducing sugar and a compound having a free amino group, e.g. an aromatic or aliphatic amine or, in natural systems, an amino acid. In the case of aldoses, *N*-glycosides arise, whereas ketoses yield their corresponding ketosides.

Whereas ketosides, in the sense of a Heyns–Carson Rearrangement, become transformed to derivatives of their corresponding aldoses, the corresponding 1-deoxy-1-aminoketoses arise from aldose *N*-glycosides by means of an Amadori Rearrangement (Fig. 1). This is catalysed by small quantities of acid whereby, first, the hemiacetal ring on the sugar residue is opened to allow the carbonium (IIa) ion or the immonium (IIb) ion to develop. Because, as we found more than 10 years ago, a glycoside in the furanose form becomes rearranged ten times faster than the

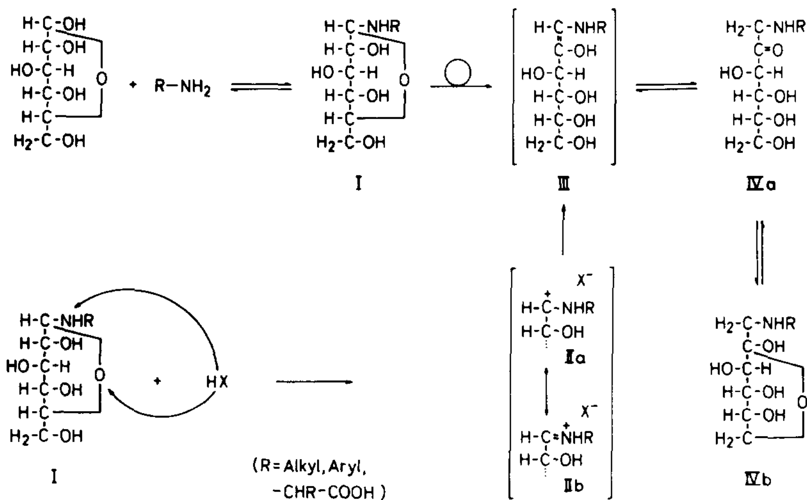


Fig. 1. The mechanism of Amadori Rearrangement.

corresponding pyranose, the ring opening appears to be rate limiting for the course of this reaction (Heyns *et al.*, 1970). Apparently, by means of a base catalysis of the corresponding anion, a proton from C-atom 2 of the sugar residue becomes detached to give the enaminol (III) form. The enaminol (III) stabilises as the rearranged compound in the open-chain form (IVa) and the corresponding hemiacetal form (IVb). At this stage the reactions are accompanied by intensive brown colouring. Moreover, because decomposition reactions run parallel to the rearrangement, the Amadori reaction products can often be isolated only in very small yields. Further, the stability of the hemiacetal ring, and, with it, the stability of the entire sugar molecule, are apparently important. The actual Maillard reaction, the nonenzymatic browning process, therefore takes place especially quickly when the Amadori reaction product (IVa) can develop a furanose system only instead of (IVb), or when, in any event, the Amadori reaction product (IVa) exists in the open-chain form. This is always the case when the C-6 position of aldohexoses is altered, as, for example, in glucuronic acid, or by aldopentoses which, in this sense, similarly transform themselves more rapidly.

One of the most marked characteristics of Amadori compounds appears to be their tendency to produce enediol forms which, similarly arise from ring opening (Fig. 2). Enol formation can occur between C-atoms 1 and 2 as well as between C-atoms 2 and 3 and in both cases, the characteristic decomposition sequences can be observed. In the first case, the allyl hydroxyl group on C_3 is removed, thereby creating a double bond between C_2 and C_3. This promotes a hydrolytic scission of the previously very tightly attached amine residue, thereby causing the formation of 3-desoxyhexosone (VI), a relatively stable compound already found in various

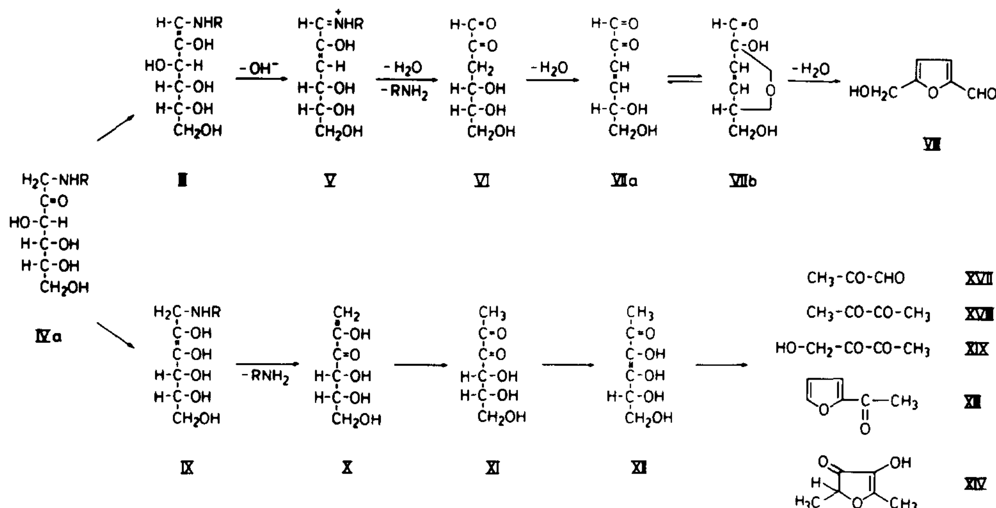


Fig. 2. The mechanism of the decomposition of Amadori Rearrangement products (Hodge, 1967).

browning products such as soy sauce and dried fruit. The reaction continues through further dehydration reactions and by translocation of the double bond until, finally, compound VIIb yields hydroxymethylfurfural (VIII).

In the second case, an enediol is first formed between C-atoms 2 and 3. This double bond facilitates the elimination of the allyl amine residue and this leads to the formation of 1-deoxyhexosone (XI). This decomposes further, yielding, ultimately, pyruvaldehyde, diacetyl, hydroxydiacetyl, acetyl furan (XIII) and predominantly, from 6-deoxy sugars, 2,5-dimethyl-3-2(H)-4-hydroxy-furanone (XIV).

A somewhat different decomposition route leading to a similar result was described 20 years ago by Anet (1959*a*). Browning reactions on freeze-dried apricot and peach purées, evidently accompanied by a reduction of the amino acid concentration, were observed (Anet & Reynolds, 1956). During the ion exchange column work-up procedure for fructose-amino acids, they also found difructose-amino acids (Anet, 1959*a*). Their formation is another indication that the Amadori product can react further with glucose.

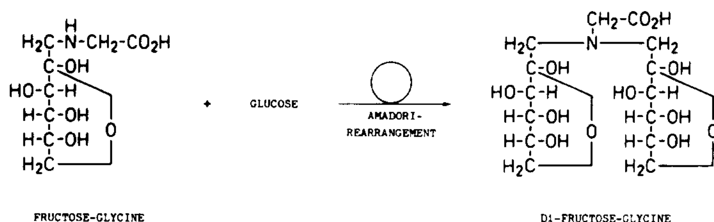
Di-D-fructose-glycine decomposes rather quickly (Anet, 1959*b*) (Table 1) and the proposed decomposition route is based on the assumption that a fructose residue is

TABLE 1
DECOMPOSITION OF DI-D-FRUCTOSE-GLYCINE TIMES FOR TWO-THIRDS DECOMPOSITION (REYNOLDS, 1963)

Temperature (°*C*)	*pH* 3·5	*pH* 5·5	*pH* 8
25	50 days	9 days	45 days
40	70 h	25 h	> 100 h
50	27 h	10 h	60 h
75	45 min	35 min	130 min
100	4 min	3 min	35 min

eliminated after transformation into 3-deoxyhexosone and the original Amadori compound is released (Fig. 3). The further reaction sequence runs identically to the description in Fig. 2, which goes back to a later hypothesis by Hodge (1967). Nevertheless, Anet (1962) partially and logically specifies hemiacetal structures. Basically, as before, the model relies upon a series of enolisation and dehydration reactions in the sugar residue which ultimately lead to the formation of furans and carbonyl compounds.

All of these highly reactive compounds react with amino acids very quickly to form melanoidins. For the variety of reactions possible, Hodge's noted schema are remembered. Moreover, a few of these compounds, namely α-β-dicarbonyl compounds, are the starting point of the Strecker Degradation in course of which amino acids become transformed into aldehydes and amino ketones (Fig. 4).



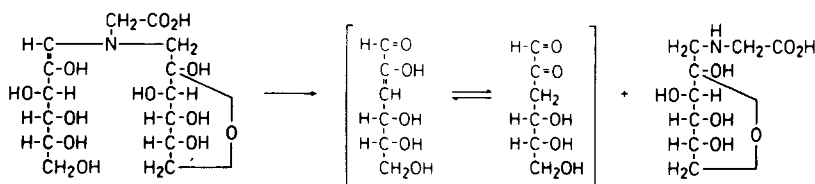


Fig. 3. The mechanism of fructose-glycine decomposition according to Anet (1959*a*).

Both, again, are themselves very reactive, and pyrazines, well known as aroma carriers of roasted products, evolve from the amino ketones. Besides leading to the formation of different heterocycles, condensation reactions of this kind occur for example in all heat-requiring aroma-formation in food. Baltes (1980) gives a summary of these compounds. Because their formation at lower temperatures seems to be less probable, these compounds ought to appear less frequently during food storage. Therefore, this undoubtedly very important Maillard reaction complex will be spared at this point. Nonetheless, mention must be made of studies by Tsuchida (1973), who produced pyrazines by the reaction of glucose with glycine under mild conditions. The pyrazines yielded by this reaction still possess unaltered sugar molecule residues in the side chain and, as such, the compounds are only very slightly volatile. Within the frame of a Strecker Degradation evidently they were formed after reaction of the amino acid with an osone. Moreover, compounds of this type could also be isolated from soy sauce.

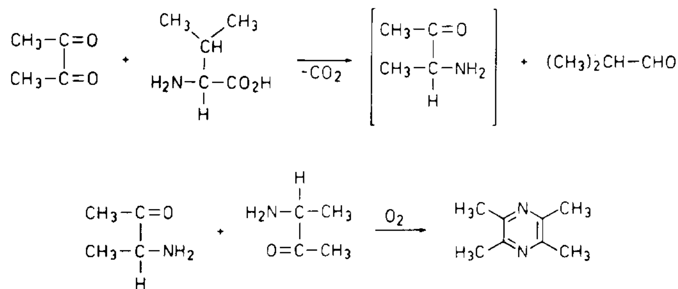


Fig. 4. The mechanism of Strecker Degradation and subsequent pyrazine formation.

As yet the structure of the melanoidins has not been verified. The reason lies in the variety of reactions stemming from the scission products formed chiefly from the sugar molecule. Consequently, one has to draw upon model reactions in order to devise an answer to the question of the structure of these compounds which, undoubtedly, we eat every day.

In recent years, we have carried out a few model reactions with an 'unnatural' system (Baltes & Franke, 1978; Otto & Baltes, 1980; Lessig & Baltes, 1981*a* and *b*). We chose the transformation of glucose with 4-chloroaniline which, like amino acids, initiates the Maillard reaction and which seemed to offer a few additional advantages. One advantage is that 4-chloroaniline is not subject to the Strecker decomposition.

Additionally, because its reaction with glucose occurs relatively slowly, it was hoped that the lower molecular weight products would provide clues concerning the structure of the melanoidins. Actually, we isolated and structurally classified about forty products from this reaction; among them were furans, pyrroles, indoles and predominantly, quinolines. It was often possible to identify the reacting carbonyl compounds in that these, through a Skraup synthesis, had spontaneously reacted with the amine to form the observed quinolines. Among other fractions, we also obtained a homogeneous reactive brown fraction, which we stabilised through permethylation to prevent its further alteration as it otherwise usually occurs with melanoidins. The brown fraction's molecular weight was about 1000; identical results were obtained by vapour pressure osmometry and by HPLC gel chromatography. Ultimately, the field desorption mass spectrometer, to which we obtained access just a short time ago, produced evidence of a large number of fragments suggesting compounds having a molecular weight of 825–1219 Daltons. Although the contrary is often supposed, the distribution of the peaks (chiefly representing molecular ions) supported the contention that no polymer homologous sequence was present. Through Curie point pyrolysis we obtained over 90 fragments which mostly represented structural elements of the brown fraction. This statement is based on our experience with lower molecular weight compounds of this kind whose structures were familiar to us.

According to one of our calculations, a hypothetical, average formula was $C_{45}H_{45}O_9N_3Cl_3(OCH_3)_3$, which corresponded to the combustion analysis values as well as to the methoxyl determination values. The number of double bond equivalents was also calculated. According to this, 4-chloroaniline residues would account for only nine of these equivalents. With one exception, catalytic hydrogenation showed that aromatic systems account for the other double bonds. Whereas the nitrogen functions seem to be saturated, the oxygen atoms could be incorporated in furan and oxazol systems as well as in acetyl residues. With an average composition (without three 4-chloroaniline residues) of about $C_{27}H_{36}O_9(OCH_3)_3$, these brown compounds give the impression of being considerably stable, aromatic systems. On the other hand, we were not able to detect

components, as described by Feretti *et al.* (1974) consisting of several condensed, furan rings which were linked by oxygen bridges or methylene groups.

Difficulty in obtaining NMR spectra was thought to result from the presence of paramagnetic, structural elements. However, by giving a clear signal by $g = 2004$ (13 gauss), the ESR spectra provided clear indications of the presence of free radicals. Because the signal position corresponded exactly to that of diphenylpicrylhydrazyl, we concluded the free electron must be on nitrogen.

To saturate these radicals the melanoidins were allowed to react with nitric oxide (NO). We succeeded in saturating only about 50 % of these apparently very stable radicals and, interestingly, the colour changed from dark brown to red-brown and became altogether much lighter. It is therefore reasonable to assume that the colour of the melanoidins is derived, at least partially, from these free radicals. In this context, similarities with hemiquinones should be borne in mind.

The occurrence of free radicals after heating or after irradiating food was described, among others, by Diaz-Santanilla *et al.* (1981) and Uchiyama & Uchiyama (1979, 1981).

Because of deviating signal positions, however, a structural similarity with the compound types we examined seems to be doubtful. However, Milic *et al.* (1978) observed free radicals of the same type during the reaction of glucose with aminobutyric acid. We have measured exactly the same signal position in melanoidins isolated from a reaction mixture consisting of aromatic amino acids and glucose. These correspond much more nearly to the natural systems in food than the reaction products of 4-chloroaniline, and we hope these systems simulate natural conditions of food in a better way. Similarities between Maillard reactions in foods and in suitable model systems were recognised earlier by, for example, Tatum *et al.* (1967) and Lee *et al.* (1979). Interestingly, Tatum and his co-workers described the formation of diacetone alcohol during storage of apricot purées; this is a precursor of mesityloxide, a sugar degradation product which was identified during our experiments.

Of course, conditions in both these reactions must be the same. It must be assumed that, after longer reaction times and at higher temperatures, melanoidins react to form larger units. For example, the melanoidins formed after the reaction of glucose with amino acids possessed molecular weights similar to those of the products which were isolated from the reaction with 4-chloroaniline (Lessig & Baltes, 1981*a*). On the other hand, from roasted coffee we found two melanoidin fractions with molecular weights of 9000 and 17000 Daltons.

From model systems it is known that Maillard compounds can react easily with nitrogen oxides to form nitrosoamines. In particular, pyrroles, pyrrolidines and thiazolidines (Sakaguchi *et al.*, 1978) can react to form nitrosoamines under suitable conditions. On the other hand, the currently often described, mutagenic characteristics of heated food (Commoner *et al.*, 1978) do not seem to be the result of Maillard-type transformations. It seems more likely that strong mutagenic products

of the kind which Kasai *et al.* (1980) and Sugimura & Nagao (1979) isolated from, for example, fried fish and meat or from heated soy protein relate to the pyrolysis of amino acids. Such compounds form only to a small degree except at excessive temperatures. There are only a few references to toxic effects of Maillard products. From a review by Adrian (1973), one can conclude that diminished digestibilities of proteins and reduced yeast growth in the presence of Maillard products is an effect of premelanoidins with lower molecular weight; melanoidins of higher molecular weights seem not to be absorbed by the body. Reduced protein efficiency rates (PER) of casein after Maillard reaction were recognised as being due to lysine deficiency (Mori *et al.*, 1977).

Melanoidins seem generally to have an anti-oxidative effect. Paik & Kim (1979) and Lee *et al.* (1975) ascertained that these effects were greatest at the beginning of the browning reaction. This leads to the conclusion that this characteristic originates from Maillard intermediates such as reductones. In this sense, similar characteristics of ascorbic acid are recalled. Products from the transformation of carbohydrates with basic amino acids (Lingnert & Eriksson, 1978) apparently gave the greatest effect.

Maillard reaction products also seem to possess bactericidal effects (Maeshige *et al.*, 1974). Reductones, osones, furan compounds and also Amadori reaction products were described as being effective components. Reaction products from the pyrolysis of carbohydrates also showed the same kind of effects (Radoev *et al.*, 1961). Because of these bactericidal effects, the addition of preservatives to bread totally surrounded by a crust will soon be banned in the Federal Republic of Germany, unless the bread is to be sold sliced.

It is well known that the Maillard reaction occurs faster as the temperature is raised, but this characteristic by no means excludes the reaction's occurrence at lower temperatures such as during food storage. In particular, Maillard reactions during storage can damage powdered foods containing carbohydrates and amino acids when their moisture contents are sufficiently high. The foods then become coloured yellow to brown and cause a bitter taste. From various examples (e.g. Heiss & Eichner, 1971), one can expect a Maillard reaction in such foods with an equilibrium relative humidity of 30–80% with the reaction's maximum lying between 60 and 80%. The equilibrium relative humidity is the same as the water activity and both are a function of the sorption-isotherm of water in the corresponding food.

In powdered foods, equilibrium relative humidities of 30–80%, correspond to actual water contents of 3–10%. Consequently, non-enzymatic browning reactions can already begin at lower moisture values than is the case, for example, in enzymatic reactions or in the growth of mould fungi, yeasts and bacteria. Only fat oxidation occurs at even lower moisture values.

A reaction of this kind is often observed in improperly stored milk powder. Reaction partners here are lactose and the lysine component of casein; this is

reactive because of the protruding ε-amino group. Because the lysine residue, through the Amadori rearrangement, becomes so tightly bound to the lactose molecules, lysine's nutritional availability is destroyed. In Table 2, the lysine losses after heating milk and milk powder are specified.

Similarly, the Maillard reaction may take place in egg powder. In addition to browning, the reaction of freeze-dried ovalbumin with glucose, caused the binding of lysine as observed through denaturation and conformational changes of the protein (Watanabe *et al.*, 1980).

TABLE 2
LYSINE LOSSES AFTER HEATING MILK AND MILK POWDER
(ACCORDING TO ROSS, 1959)

	Lysine Loss (%)
Fresh milk, shortly boiled	5
Condensed milk	20
Low-fat milk powder, shortly heated to 150 °C	40
Low-fat milk powder, heated 3 h to 150 °C	80

Apart from lysine the amino acid arginine suffered the biggest losses. Loncin *et al.* (1968) measured lysine loss and yellowing of milk powder samples stored 10 days at 40 °C at varying, relative humidities. The results show that the reaction apparently occurred optimally at a water content of 13 %. The Maillard reaction is observed to a lesser degree at higher, as well as at lower, water contents. These kinds of maxima are explained in that, on the one hand, at lower water content not enough substance is dissolved and, moreover, diffusion is restricted. On the other hand, at higher water contents, although enough substance is dissolved and diffusion has substantially increased, the concentration is then too low to give a high reaction rate. Lying in the middle, the optimal conditions provide relatively high substrate concentrations in the dissolved form for condensation reactions which, of course, eliminate water in the first step. Similarly, the reaction maxima in egg powder and dried meat lie in the area of respectively 9 and 11 % water content; in tomato powder about 12 % water value was measured (Eichner, 1980).

A preliminary thermal stress can be important for an occurrence of the Maillard reaction; this promotes reactions of precursors to form immediate, initial melanoidin products. As a result, obvious differences between products from tomato powders exposed to varying preliminary, thermal conditions could be observed (Ciner-Doruk and Eichner, 1979). Moreover, by a temperature increment during the air drying of carrot cubes, increased formation of Amadori products could be shown. Thereafter, the formation of the usual brown colouring was observed and this was accompanied at the same time by sensory changes in the product.

How can one now recognise that a Maillard reaction has already taken place in a food? First, the reaction is, of course, manifested by melanoidin formation. However, the liberation of aroma compounds as a manifestation of storage changes makes a chemical proof of spoilage unnecessary. It is normally too late, when the warehouse manager has to dispose of the food because the brown colouring is already conspicuous or when an increase in absorbance at 420 nm can be measured. The presence of hydroxymethylfurfural only serves to prove that a Maillard reaction has already occurred or that sugar-containing, acidic solutions have been heated (e.g. pasteurised fruit juices, artificial honey, jams).

By contrast, proof of the presence of the initial, colourless Amadori products provides a means of recognising the early stages of the Maillard reaction. The Moore and Stein amino acid analysis method can be used for this purpose (Fig. 5). It is particularly helpful in that a series of very characteristic products is eluted from the column just before aspartic acid; i.e. in a region in which interferences by other substances are not likely. Such products include primarily fructose–glutamic acid, fructose–asparagine, fructose–serine and fructose–threonine. These compounds, which arise by the reaction of glucose with the corresponding amino acids, are relatively stable and are therefore especially useful for the early recognition of incipient quality changes in food through the Maillard reaction (Ciner-Doruk & Eichner, 1979). The products from similar reactions of galacturonic acid, which is released through pectin degradation, are clearly more unstable, but, even so, they can also be found in the aminogram. Consequently, all of these compounds can be used successfully to identify storage changes before the first, sensory changes to the food become apparent.

On the other hand, it seems to be doubtful whether volatile products (e.g. from the Strecker degradation of amino acids such as, by tomato powders, the degradation of isovaleraldehyde from leucine) can be detected by gas chromatography before organoleptic changes are apparent. Light damage to milk powder, moreover, makes it important for the producers of milk-based infant food to check the milk powder's available lysine content, i.e. the lysine which, through a Maillard reaction, is not yet bound to milk sugar. A first indication of damage is given by finding furosine or pyridosine, in the Moore and Stein amino acid chromatogram of a hydrochloric acid hydrolysate of the milk powder.

Under these conditions, both compounds are formed by cyclisation and dehydration from the 2–3-osone derived from the initial Amadori product (Fig. 6). As Erbersdobler *et al.* (1979) recently showed, one can identify and quantitatively determine both compounds really well. Both furosine and pyridosine appear in areas of the aminogram where interference by other substances is unlikely. Both values are thoroughly suitable for judging incipient losses in milk powder quality and can be important in avoiding a lysine-deficient infant diet. The possibility of preventing Maillard-type reactions is an important topic for consideration. There are a series of varying, product-specific starting points for this. For example, the

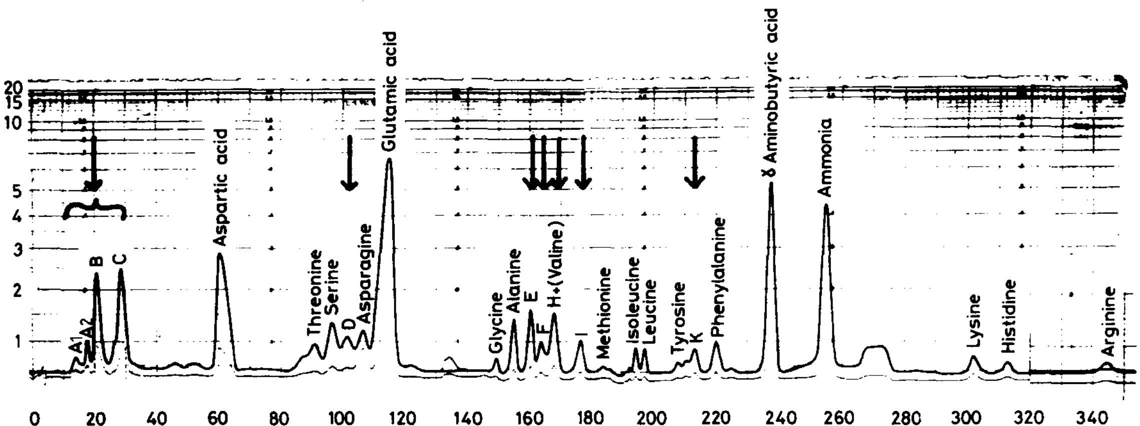


Fig. 5. Amino acid chromatogram, a tomato powder sample heated at 55 °C for 8 h after adjustment to an equilibrium moisture value of 33 %—Peak: Reaction product from: A_1 = galacturonic acid + aspartic acid. A_2 = glucose + aspartic acid; B = galacturonic acid + glutamic acid, asparagine, serine, threonine; C = glucose + glutamic acid, asparagine, serine, threonine; D = galacturonic acid + γ-aminobutyric acid; E, F = galacturonic acid + NH_3; H = glucose + γ-aminobutyric acid; I = glucose + NH_3; K = glucose + lysine (from Ciner-Doruk & Eichner, 1979).

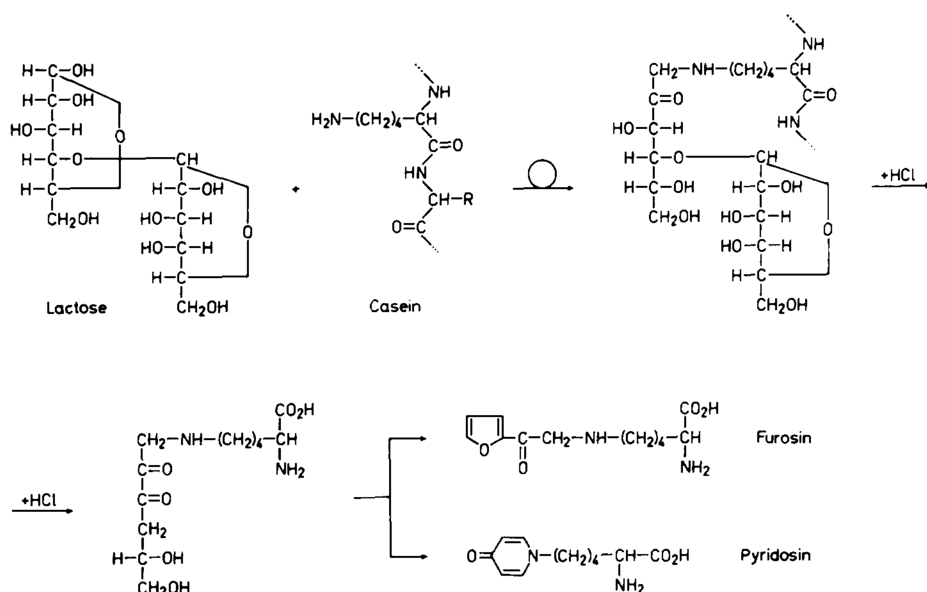


Fig. 6. Furosin and pyridosin formation from a lactose-casein condensation reaction.

glucose contained in liquid egg can be removed by the fermentation resulting from addition of yeast preparations. Another possible solution is the addition of the enzyme, glucose oxidase, whereby glucose becomes transformed to the unreactive gluconic acid. A thoroughly desirable side effect of this reaction is the removal of oxygen from the sealed container. Both methods have one thing in common, namely, the removal of one of the reaction partners for the Maillard reaction. When reactive sugars other than glucose are present, they must be removed first, because glucose, according to current knowledge, is the sugar having the most stable conformation. It therefore exhibits the lowest reaction rate in this reaction, and the removal of the other, faster reacting sugars should be given priority.

Related to this method of removing single reaction compounds are jam-production processes. By using small batches, the cooking process can be limited to 15–20 min and this ensures a smaller release of glucose by sucrose inversion in the acidic medium. In this way, for example, the production of deep red strawberry preserves is possible, whereas earlier, hour-long cooking processes, using the same type of fruit, led to brown-coloured products having strong, caramel aromas.

There are certain generally applicable technical steps for inhibiting the Maillard reaction during the drying of food which is intended to be stored in dried form.

Drying experiments showed that the Maillard reaction rate reaches a minimum at a water content of about 2%. Because the reaction's temperature coefficient is relatively high, the drying of such foods through the critical, relative humidity

condition must be achieved as quickly as possible. Additionally, the goods should not be left too long at higher temperatures. To attain this, the dry goods should be turned over as often as possible, or thin-layer drying should be implemented. In milk powder production, the water, for example, should be evaporated within one roller's passage; in spray-drying care should be taken that the dry goods do not become heated over 60 °C. Another generally applicable method insofar as the product allows, is the addition of a reaction inhibitor, i.e. sulphurous acid, which is usually assumed to operate through an addition reaction mechanism (Fig. 7). Accordingly, an acid residue is added to the 3, 4 position of the unsaturated osone as well as on to the aldehyde group as a bisulphite-addition compound (Anet & Ingles, 1964).

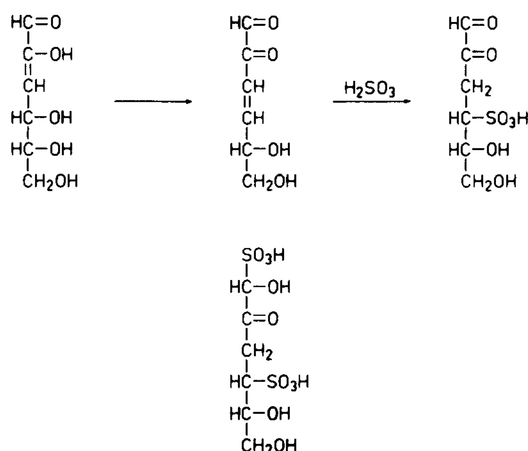


Fig. 7. Possible mechanism of the reaction of SO_2 with Maillard reaction intermediates.

Whether this is the only effect, must be doubted, however. In model attempts we found an inhibiting effect as long as free SO_2 was still demonstrable in the reaction's medium. After a while, all the SO_2 was oxidised to sulphate, and the browning reaction continued as in untreated samples. Accordingly, the reducing effect of SO_2 may be important in browning inhibition. Furthermore, ascorbic acid and cysteine can also inhibit the Maillard reaction to a limited degree (Arnold, 1969 and Shtal'berg & Radaeva, 1966). Our own results also indicate that reducing agents should inhibit the reaction, in as much as we have proved the involvement of intermolecular oxido-reductions during the reaction (Otto & Baltes, 1980). The addition of SO_2 is often used during food processing; e.g. in storage of fruit pulp intended for later processing to jam, in dried-fruit production, in wine preparation and in potato processing. The effect is twofold: not only is the non-enzymatic, Maillard reaction browning checked but also the polyphenoloxidase controlled,

enzymatic browning is hindered. When one now considers the possible storage periods for milk powder (9–15 months), jams and tomato pastes (up to 27 months), it means that foods properly stored probably become damaged to a lesser degree by Maillard-type reactions than, for example, through fat oxidation. Exceptions probably arise only when the product is not suitably prepared or when the preliminary steps of the reaction can occur during processing.

The Maillard reaction can be thought of as being a process especially characterised by the thermal formation of aromas in food; considerable numbers of volatile compounds are formed in this way and many of these are now well known. By contrast, the structure of the melanoidins remain unknown; hopefully, one day, this will be elucidated and we shall know what we actually eat.

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CHANGES IN THE COLOUR AND OPACITY OF MEAT

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ABSTRACT

On exposure to oxygen the purple ferrous haem pigment, myoglobin, forms the bright red covalent complex, oxymyoglobin. In fresh meat oxidation of oxymyoglobin to metmyoglobin, the unattractive brown pigment, is affected principally by the reducing capacity of the muscle, oxygen availability and temperature. In frozen meat, light is the major factor affecting the oxidation rate. Oxidation of nitric oxide myoglobin, the ferrous pigment in cured meats, is prevented by excluding oxygen but proceeds rapidly in light, if oxygen is present.

Stress, pre-slaughter handling of the animal and post-slaughter treatment of the carcass affect the light scattering properties of the meat. Glycogen depletion in the live animal results in translucent, dark firm and dry (DFD) meat with high pH and high oxygen uptake. Rapid post-mortem glycolysis causes structural changes in muscle which becomes opaque, pale soft and exudative (PSE). Electrical stimulation of the carcass and the rate of chilling also affect light scattering of meat. On storage, increase in light scatter is accompanied by a decrease in colour stability.

INTRODUCTION

The colour of meat is influenced by a number of independent but interacting variables. Animal husbandry practice, for example, the decision to rear entire male or castrated animals and the feeding regime employed, can markedly influence the initial colour of the meat, as can events prior to slaughter which might induce stress in the animal at, or on the way to, lairage. The process of converting muscle to meat for distribution and sale, that is, the chilling rate, the temperature and duration of chilled storage for ageing the carcass or primal joints for optimum tenderness, and possible use of frozen storage, all affect the stability of meat colour at retail. The

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major variables at retail are temperature of display and control of the atmosphere in which the meat is packed.

COLOUR

Colour is a subjective experience; the sensations of colour cannot exist without an observer to perceive them. Colour can be described in the psychological concepts of lightness, hue and saturation (Wyszecki & Stiles, 1967). Its perception by an observer with normal vision is trichromatic; that is, the colour sensation can be matched by an appropriate mixture of stimuli from three primary colours. This means that, although colour is psychological, it can be measured in physical quantities, provided the primaries and the illuminant are defined relative to a standard observer's vision. This psychophysical technique of expressing colour in terms of tristimulus values, established by the Commission Internationale de l'Eclairage (CIE), in 1931, has been much improved by converting the tristimulus values into visually uniform colour space. One transformation, commonly used in food colour measurement, is that of Hunter (1958). The space is defined in terms of lightness (L) and the chromaticness co-ordinates (a_L), green to red, and (b_L), blue to yellow, from which the hue angle (H°) and the saturation (S) can be calculated. Colour measurements reported in this paper were obtained on a Hunter D25 colour difference meter which gives values of L, a_L, b_L directly, or on Optica CF4DR or Pye Unicam SP8-100 recording reflectance spectrophotometers, and the colour terms calculated from the spectra.

The reflectance of an object depends on the amount of light absorbed and scattered. The Kubelka & Munk (1931) absorption and scatter coefficients (K) and (S) are related to reflectivity (R_∞) by the equation $K/S = (1 - R_\infty)^2/2R_\infty$ where R_∞ is the reflectance of an infinitely thick layer. The use of the Kubelka and Munk analysis to determine K and S and its application in colour formulation, pigment blending and opacity measurement is fully illustrated by Judd & Wyszecki (1975). Its application in food colorimetry, for example, as a method of relating pigment concentration to appearance, has been discussed by Francis & Clydesdale (1975) and its use in quantifying the effect of scatter on meat appearance by MacDougall (1970, 1971, 1977).

PIGMENTS

The pigment responsible for the colour of meat is the haem protein myoglobin along with residual quantities of haemoglobin, whose colour properties are similar to those of myoglobin. Light absorption and colour is a consequence of resonance of the prosthetic haem and reaction with ligands. Haem pigments are characterised by an intense absorption band in the blue region of the spectrum between 410 and 430 nm (the Soret band). The iron in the haem is co-ordinated with four pyrrole

nitrogens of the porphyrin, and the disc shaped haem is held in a cleft in the globin by van der Waals contacts and the fifth co-ordinate bond of the iron. This link, covalent with the proximal histidine imidazole of the F helix of the globin, is axial to the porphyrin. The sixth co-ordinate bond of the iron is available for reaction with ligands.

The chemistry of myoglobin and its reaction with ligands has been reviewed by Antonini & Brunori (1971), and, more recently, with particular emphasis on meat colour by Giddings (1977) and Livingston & Brown (1981). Oxymyoglobin, nitric oxide myoglobin and carboxymyoglobin are examples of sixth ligand ferrous 'covalent' complexes exhibiting two (α and β) absorption bands between 535 and 545 nm and 575 and 588 nm. They are red, but not identically red. The structural features of the haem–oxygen complex are well established. The symmetry is essentially octahedral and the geometry of the complex is that of a bent end-on configuration with a bond angle of 135° and either the outer dioxygen atom is H bonded or the inner oxygen atom is electrophilically attracted to a distal histidine imidazole. The bonding of oxygen is not strictly covalent. The resonance of the haem iron–oxygen bond (Caughey *et al.*, 1975) is predominantly 'dioxygen iron'; that is, the iron–oxygen complex is intermediate between covalent and ionic 'ferric superoxide'. Nitric oxide myoglobin has a bond angle of 110° with bonding between the haem iron and the proximal imidazole (Chien, 1969) and the complex is very stable in the absence of oxygen. In the case of myoglobin (deoxyferrous) the symmetry is square pyramidal because there is no sixth ligand. The α and β absorption bands are absent and replaced by one diffuse band with an absorption maximum at 555 nm. At concentrations found in meat, the colour is purple. The sixth ligand position in metmyoglobin, the unattractive ferric brown pigment, is occupied by a water molecule and cannot bind oxygen. The absorption bands have maxima at 505 and 630 nm which result in transmission (or reflectance) of more green and less red energy than either myoglobin or oxymyoglobin. Ferric pigments, which are red, are metmyoglobin nitrite and cyanmetmyoglobin. The ferrous pigment sulphmyoglobin, formed by reaction with H_2S, is green. Carboxymyoglobin, formed by the tight binding of CO, is red with a spectrum similar to oxy- or nitric oxide myoglobin. The pigments of cooked meat are complexes of haematin and denatured proteins. The ferrous pigments are pink and are readily oxidisable to the more common ferric which are brown. Ledward (1974) postulated that the ferric pigments were haematin di-imidazole complexes but Giddings (1977) doubts that two denatured protein-bound imidazoles can occupy the two haem axial co-ordination sites.

LIGHT SCATTER

Muscle cut immediately after an animal has been slaughtered is translucent and dark in appearance. Provided the animal was not subjected to exhausting stress

prior to slaughter and normal reserves of glycogen were present in the muscle, the pH falls from 7·0 to approximately 5·5 as glycogen is converted anaerobically to lactic acid (Bendall, 1973). During post-mortem glycolysis, beef muscle changes from deep dark purple to paler lighter purple which will rapidly redden on exposure to air. The increase in lightness is caused by changes in the muscle proteins; slight paleness from developing light scatter is evident at pH 5·9 and increases to 5·5 as the pH approaches the isoelectric point of the proteins and glycolysis ceases. Translucent meat with pH > 6·0 has a scatter coefficient $S\,\mathrm{mm}^{-1} < 0{\cdot}1$ and meat with normal pH has $S\,\mathrm{mm}^{-1}$ of 0·15 to 0·25, depending on both the chilling regime and the dimensions of the muscles in the carcass (MacDougall, 1977). If glycogen is depleted prior to slaughter, for example, by stress from transportation to the abattoir and mixing with strangers (Duchesne, 1978), or by shivering from sudden reduction in temperature and overnight holding in lairage, insufficient lactic acid may be produced for the pH to fall below 6·0, and the transition to semi-opaque from the dark jelly-like appearance does not occur. This condition is called dark cutting (DC) in beef and dark, firm and dry (DFD) in pork. The severity of the condition is directly related to the ultimate pH (MacDougall & Rhodes, 1972).

The inter-relationship of muscle pigment concentration and light scatter on the colour of oxygenated bovine muscle of normal pH is shown in Fig. 1. This figure was constructed using the Kubelka–Munk analysis of the reflectance spectra of 100 veal and beef samples of *M. longissimus dorsi* and *M. semimembranosus* spaced equally over the range of 1 to 6 mg g^{-1} myoglobin (MacDougall & Jones, unpublished data). A change in $S\,\mathrm{mm}^{-1}$ from 0·13 to 0·2 at 5 mg g^{-1} produces a difference in visual lightness, L, of approximately 5 units. A ΔL of 5 is a very large difference in colour which is easily distinguished and remembered. This range in L at constant pigmentation is typical of that found within single large muscles in beef hindquarter chilled by traditional methods (MacDougall, 1977; Taylor *et al.*, 1980–81). The variability in lightness is attributable to changes in protein structure brought about by the differences in cooling rate throughout the muscle. Portions cooled quickly have values of $S\,\mathrm{mm}^{-1}$ of $<0{\cdot}15$ but those cooled slowly, for example, the inner 10 cm of the *M. semimembranosus* next to the femur, normally have a value of $S\,\mathrm{mm}^{-1}$ in the region of 0·2 to 0·25, and extreme values $>0{\cdot}4$ have been observed (MacDougall, unpublished data). The latter have an appearance similar to that of pale, soft, exudative pork. Ageing for periods of up to 3 weeks, during which time considerable changes occur in the structure of the contractile proteins (Penny, 1980) accompanied by increased drip (Taylor *et al.*, 1980–81), causes a less severe but still important increase in $S\,\mathrm{mm}^{-1}$ equivalent to an increase in L of 2 units. One effect of electrically stimulating beef carcasses is the earlier development in light scatter during chilling because of the induced drop in pH. The lighter, more youthful appearance of the cut *M. longissimus dorsi* is sufficient to improve the quality grade in the USA because carcasses are graded on the day after slaughter (Savell *et al.*, 1978). The variability that small, as well as large, changes in scatter

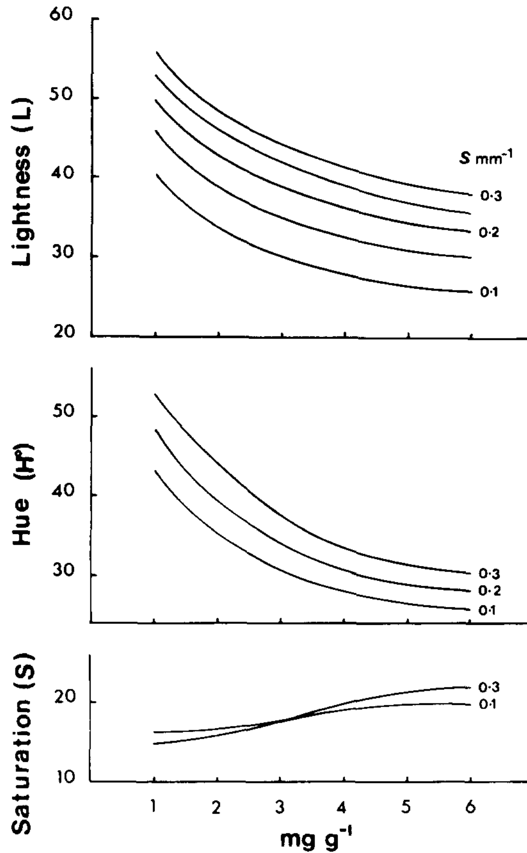

Fig. 1. Relationship of lightness (L), hue angle (H°) and saturation (S) to oxymyoglobin concentration (mg g^{-1}) and the Kubelka–Munk scatter coefficient (S mm^{-1}) for bovine muscle of normal ultimate pH (5·5–5·7).

have on reflectance illustrates the impossibility of estimating pigment concentration directly by reflectance photometry.

The relationship of lightness to pigment concentration at constant scatter is non-linear. A change of 0·5 mg g^{-1} between 1·0 and 2·0 mg g^{-1} produces a difference in L of 4 units whereas the same change in L requires >2 mg g^{-1} myoglobin at concentrations between 4·0 and 6·0 mg g^{-1}. Thus, in veal, small increases in myoglobin can produce changes in colour that have considerable consequences commercially (MacDougall *et al.*, 1973), whereas similar or greater increases in pigment concentration in meat from mature beef might not be noticed. Increase in L, either by decrease in pigment concentration or increase in scatter, is accompanied by an increase in hue towards yellow, increasing markedly at values of <2 mg g^{-1},

but the decrease in saturation is only half the increase in lightness. The change in hue and saturation is due to the dichroism of the oxymyoglobin spectrum (Wright, 1969); the moderately absorbing green region of the spectrum contributes proportionally less to reflectance with increase in pigment concentration than does the more transmitting red region. Thus, at 1 mg g^{-1}, the concentration found in veal or pork, the hue is reddish orange, but at 6 mg g^{-1}, the concentration in mature beef, the hue is purple red.

The pale, soft, exudative (PSE) condition in pork from stress-susceptible pigs, for example, Pietrain and, to a lesser extent, Landrace breeds, is characterised by an extremely rapid fall in muscle pH (Bendall & Wismer-Pedersen, 1962). Ultimate pH of 5·4 or lower is attained while the carcass is still hot, often within 1 h of slaughter. The trigger for rapid glycolysis and lactate production is directly related to the rate of Ca^{2+} anaerobically liberated from the mitochondria (Cheah & Cheah, 1976), the initial rate of Ca^{2+} efflux for stress-susceptible pigs being more than twice that of stress-resistant pigs. Development of light scatter occurs earlier in the PSE condition and by the time the carcass is chilled the meat is opaque. The prolongation of high temperature at low pH causes muscle proteins to denature; for example, the precipitation of sarcoplasmic proteins is associated with a decrease in myofibrillar solubility (Scopes, 1964), water is liberated and the wet meat reflects more light. Slow glycolysing pork has values of $S\,mm^{-1}$ similar to that of normally chilled beef and PSE pork has values of $S\,mm^{-1}$ of 0·3 to 0·4 which is comparable with that of slowly chilled beef.

The lower limit of $S\,mm^{-1}$ of 0·1 in Fig. 1 is the demarcation of normal beef from dark cutting (DC). It is not possible to show the effect of DC in the figure because the dark colour results from more than the effect of diminished light scatter. Typical values of L, H° and S for DC beef with $S\,mm^{-1}$ of 0·05 are approximately 24, 20 and 12 (MacDougall & Jones, 1981). The difference in saturation between dark cutting and normal meat can be greater than the difference in lightness. The less saturated purple colour is attributable to two factors. The higher oxygen consumption rate (Hall *et al.*, 1944; Bendall & Taylor, 1972) and the lower rate of inward diffusion of oxygen (Lawrie, 1958) considerably increase the time for formation of a sufficiently thick layer of oxymyoglobin on the surface to appear red. Because of the greater translucence, light penetrates further into the meat, more is absorbed by the pigment, which makes it appear darker, and that which is reflected has the absorption characteristics of myoglobin.

COLOUR CHANGES IN FRESH MEAT

Meat, freshly cut and exposed to air, rapidly changes in colour from the purple of myoglobin to the bright cherry red of oxymyoglobin, but the colour is unstable and fades to brown metmyoglobin. Autoxidation to metmyoglobin is non-enzymic and

involves free oxygen, but the reaction cannot be considered as a simple one-electron-transfer from the iron to the bound oxygen (Livingston & Brown, 1981). The reaction is highly dependent on oxygen tension (Brooks, 1935) and is most rapid at low values of partial pressure of oxygen which give a 1:1 ratio of oxy- to deoxymyoglobin (George & Strattmann, 1952). Oxidation is first order with respect to unoxidised myoglobin and the rate increases to 1–1·4 mm partial pressure of oxygen, and then decreases to a constant value above 30 mm. Thus, in oxygenated fresh meat, metmyoglobin forms first at the interface in the oxygenated layer adjacent to the myoglobin layer at the limit of oxygen penetration. The autoxidation of oxymyoglobin utilises 0·25 and liberates 0·75 mole of oxygen (Brown & Mebine, 1969), and since the most likely mechanism requires two electrons (Castro, 1971) it is postulated (Giddings, 1977) that reduction of the oxygen of oxymyoglobin to the peroxide oxidation state is an intermediate step in the reaction proceeding in such a way that the oxygenated haem iron contributes one electron and the deoxyferrous myoglobin the other. The autoxidation rate is increased by the presence of heavy metal ions (Snyder & Skrdlant, 1966).

The most important factors which affect fresh meat colour stability are temperature, the gaseous environment in the package, oxygen consumption and the reducing capacity of the meat. Individual muscles exhibit different rates of metmyoglobin formation (Hood, 1971, 1980; Ledward, 1971; MacDougall & Taylor, 1975); the *M. longissimus dorsi* has been cited as having a stable colour, and the *M. psoas major* as a muscle which discolours rapidly. A 3 to 5 °C increase in the temperature of the refrigerated display cabinet will double the rate of discoloration (MacDougall & Taylor, 1975), which is also affected by fluorescent and ultra-violet light (Hood, 1980). Oxygen diffusion into meat is greater at low temperatures (Urbin & Wilson, 1958) but the ultimate depth depends on the rate of oxygen uptake which decreases with ageing (Bendall & Taylor, 1972). Aged meat can have a brighter colour immediately after 'blooming' because of increased light scatter and deeper penetration (MacDougall, 1972), but it has poorer colour stability with loss of metmyoglobin reducing activity. Stewart *et al.* (1965) and Watts *et al.* (1966) showed that metmyoglobin reduction is mediated through nicotinamide adenine dinucleotide (NAD) under anoxic conditions. Giddings (1974, 1977) and Livingston & Brown (1981) have reviewed the literature on metmyoglobin reduction in post-rigor meat and conclude that it is primarily enzymic in nature involving the mitochondria which serve as a source of reducing equivalents for extramitochondrial pyridine nucleotide reduction. The gradual loss of activity on storage could occur from loss of co-factors and disintegration of mitochondrial particles. Until identification of a reduced NAD dependent metmyoglobin reductase from bovine heart muscle (Hagler *et al.*, 1979) no specific metmyoglobin reductase in muscle had been demonstrated convincingly. One technique of avoiding or delaying discoloration from rapid metmyoglobin formation is by controlled atmosphere packaging. Oxygen concentration of >60% and carbon dioxide

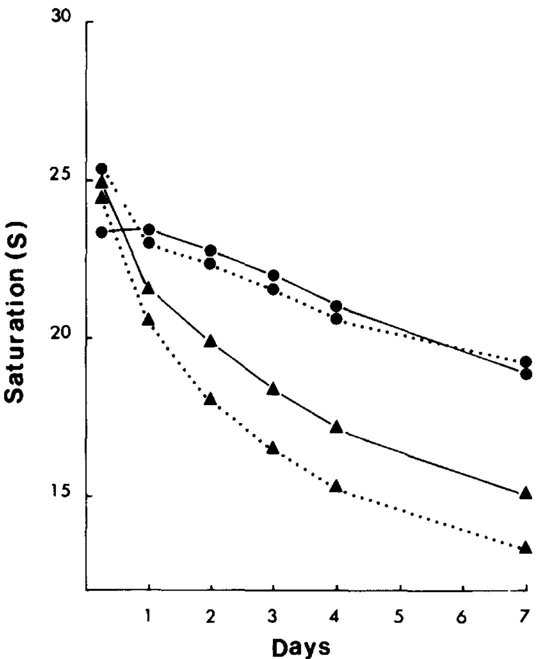

Fig. 2. Change in colour saturation (S) for *M. longissimus dorsi* and *M. psoas major* exposed to air at 5 °C and 1000 lux fluorescent illumination after anaerobic storage at 1 °C for 5 or 21 days. ● *M. longissimus dorsi*, ▲ *M. psoas major*, —— 5 days aged, . . . 21 days aged.

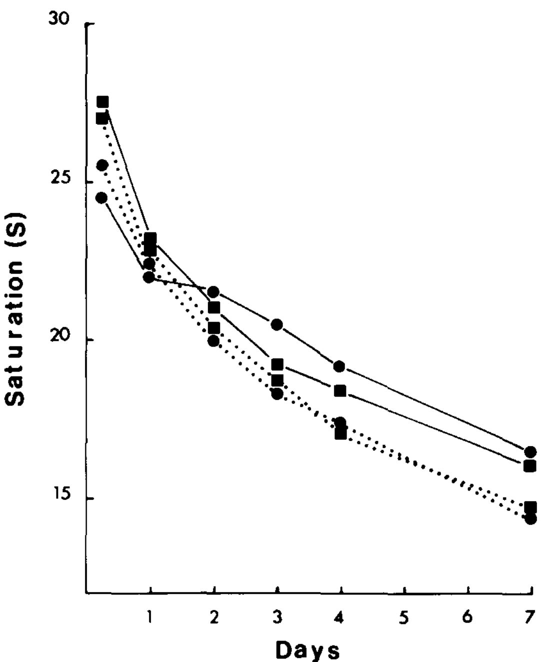

Fig. 3. Change in colour saturation (S) for *M. semimembranosus* exposed to air at 5 °C and 1000 lux fluorescent illumination after anaerobic storage at 1 °C for 5 or 21 days. ● 3 cm in from subcutaneous fat. ■ 3 cm from femur, —— 5 days aged, . . . 21 days aged.

(>20%) to retard bacterial spoilage submerges the metmyoglobin band to at least 1 cm below the surface (Taylor & MacDougall, 1973). Meat cuts <2 cm thick, thus packed, do not have a core at low partial pressure of oxygen and rapid formation of metmyoglobin does not occur.

The interaction of muscle type and degree of ageing on colour stability is shown in Figs 2 and 3. *M. longissimus dorsi* (LD), *M. psoas major* (PM) and *M. semimembranosus* (SM) primal joints were removed 48 h after slaughter from eight steer carcasses which were chilled for 7 h at 15 °C, followed by 0 to 1 °C with an air velocity of 0·5 m/s (Taylor *et al.*, 1980–81). The joints were vacuum packed and held for 5 or 21 days at 1 °C and then opened. Slices were overwrapped in oxygen-permeable film in polystyrene trays and the packages exposed to 1000 lux fluorescent light (Thorn 'Natural') at 5 °C. Colour changes are reported in S; values >20 are bright red, 18 is dull (20% metmyoglobin), 14 is distinctly brown (40% metmyoglobin) and < 12 is brown to grey–greenish brown (> 60% metmyoglobin). The LD was more stable than the PM, with the SM intermediate. The effect of ageing on the colour stability was shown by the more rapid decrease in S; at 2 days exposure the 21 days aged LD was < 1 unit less than the 5 days aged, and the PM 2 units less with the SM intermediate. A 2 unit loss in saturation can be remembered and is the level equivalent to the 20% metmyoglobin required for consumer discrimination for rejection (Hood & Riordan, 1973). The inner portion of the SM was slowly chilled and demonstrated the effect of increased light scatter. L for the outer portion was approximately 29 and for the inner was 32. There was little difference in the rate of saturation loss between the outer and inner portions which would appear to indicate similar reducing capacity. However, the lighter more denatured inner portion has been shown to have less reducing capacity. In a supplementary experiment 46 samples of aged SM were exposed to air and then packed under vacuum in oxygen-impermeable laminate pouches and held overnight at 1 °C. Of the 17 which had values of $S\,mm^{-1} > 0{\cdot}2$, 14 formed metmyoglobin on the surface which did not subsequently reduce, whereas 27 of the 29 which had values of $S\,mm^{-1} < 0{\cdot}2$ did reduce (MacDougall, unpublished data).

COLOUR CHANGES IN FROZEN MEAT

The colour of frozen meat is controlled by the freezing rate, the storage temperature, the intensity of light during display and method of packaging. Slowly frozen meat is excessively dark, whilst that frozen in liquid nitrogen is unnaturally pale (Jakobsson & Bengtsson, 1973). Freezing temperatures of −30 °C to −40 °C give an acceptable product (Taylor, 1979). The large variation in lightness is a result of differences in rate of ice crystal growth (Voyle, 1974). Small crystals, formed by fast freezing, scatter more light than large crystals formed by slow freezing, and hence fast frozen meat is opaque and pale, and slow frozen is translucent and dark. Meat packed

loosely in plastic bags rapidly develops freezer burn as moisture from the desiccating surface is deposited as snow on the inside of the film. Freezer burn is eliminated if the frozen cuts are packed in skin-tight wrapping. The major colour problem on storage is the photo-oxidation of the pigment. Thus, frozen meat under direct illumination oxidises from the surface inwards as compared with fresh, which oxidises from the subsurface outwards. Oxidation of frozen oxymyoglobin is temperature dependent (Zachariah & Satterlee, 1973); the rate increases from −5 °C to −12 °C and then decreases to a minimum at −20 °C. The rate is affected by illumination level and muscle type (Fig. 4) (MacDougall and Down, unpublished data). Aged beef *M.*

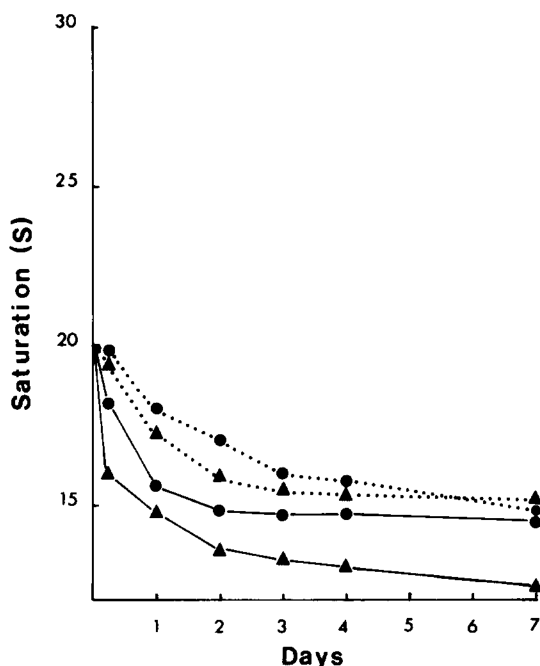

Fig. 4. Change in colour saturation (S) for frozen *M. longissimus dorsi* and *M. gluteus medius* wrapped in 'Surlyn' and displayed at −18 °C under 500 and 1000 lux fluorescent illumination after storage in the dark for 2 months. ● *M. longissimus dorsi*, ▲ *M. gluteus medius*, ... 500 lux, —— 1000 lux.

longissimus dorsi and *M. gluteus medius* slices, bloomed in air for 30 min and then blast frozen at −35 °C for 15 min, to freeze the exterior, were skin-packed in 'Surlyn' (Taylor, 1979). Freezing was completed at −35 °C and the steaks were held in the dark for 2 months before display. They were exposed to two levels in fluorescent illumination, 500 and 1000 lux (Thorn 'Natural'), in an open display case at −18 °C; 1000 lux is typical of the illumination level used for fresh meat display. The colour of

the meat at the start of display was attractive bright red, with a fresh meat appearance because of optical contact of the film with the meat surface. The saturation loss from time of packing to start of display was approximately 1–2 units of S. Fading for both types of meat was complete after 3 days at 1000 lux, although the LD was the slower to fade. The rate was halved at the lower 500 lux level and the extent of final saturation loss was less. These results are similar to those of Lentz (1971) who showed that frozen beef colour remains attractive for 3 months in the dark but only 3 days in the light.

CURED MEAT COLOUR

The reactions of nitrite in meat have been reviewed by Möhler (1973), MacDougall *et al.* (1975) and Cassens *et al.* (1979). The pigment responsible for the colour of uncooked cured meat is nitric oxide myoglobin and that of cooked cured meat is nitric oxide myochrome. In the former, nitric oxide is liganded at the sixth co-ordinated position of the iron, but, in the latter, heating may detach the haem from the denatured globin and an additional nitric oxide occupy the co-ordinating position of the iron formerly occupied by globin (Tarladgis, 1962; Lee & Cassens, 1976). The initial reaction of nitrite with myoglobin is to produce both nitric oxide myoglobin and metmyoglobin (Brooks, 1937). In the presence of reducing systems only nitric oxide myoglobin is formed. Several schemes have been proposed for metmyoglobin reduction in meat in the presence of nitrite, either chemical or enzymic. Koizumi & Brown (1971) showed that metmyoglobin is reduced anaerobically by NADH plus FMN, and Fox & Thomson (1963) showed that metmyoglobin forms the ionic complex metmyoglobin nitrite which can then react with a semistable nitric oxide reductant intermediate to give nitric oxide metmyoglobin which reduces to nitric oxide myoglobin (Fox & Ackerman, 1968). The enzymic scheme of Walters *et al.* (1967), is that ferrocytochrome c forms nitric oxide ferricytochrome c via NAD dehydrogenase, and the subsequently formed nitric oxide metmyoglobin is reduced to nitric oxide myoglobin by the dehydrogenase. Cheah (1976) has shown that lactic dehydrogenase can generate NADH for metmyoglobin reduction. Nitric oxide myoglobin, once formed, is stable in the absence of oxygen. The stability is limited by the rate of nitric oxide dissociation, and any nitric oxide dissociated will react with oxygen, if present. Dissociation is slow in the dark but, since nitric oxide myoglobin is photolabile, the reaction occurs rapidly in the light. Bacon that has been oxidised will recover if packed anaerobically, indicating that the reducing system is still active in the Wiltshire product (Cheah, 1976). Vacuum packed Wiltshire bacon will remain stable in colour for weeks, even under fluorescent illumination, although there is a gradual increase in the opacity of the product (MacDougall *et al.*, 1975).

DISCUSSION

Undoubtedly the most important colour change in meat is the loss of redness caused by the formation of metmyoglobin, with the exception of green sulphmyoglobin caused by bacterial spoilage, for example, in vacuum packed primal joints with high pH and slight oxygen permeability of the packaging material (Taylor & Shaw, 1977). Although opacity changes are important in our understanding of colour variability, they are of less consequence in the market place. Attractiveness of the product, usually considered as redness, may have little relationship to the final assessment of the product as eaten, but it is usually the only criterion, other than cost, which influences the consumer in making a decision to purchase. Discrimination is in favour of redness when meat is presented with that which is slightly brown, although the slightly brown might be purchased as sufficiently red if the red were absent. Since attractiveness is considered so important by the retail trade, fluorescent tubes with increased red energy are often employed. The colour difference produced by changing low colour rendering lamps for those which maintain redness can be as much as the equivalent of 25 to 50 % of the difference in meat colour and pigment oxidation which would cause rejection (MacDougall, 1981).

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REACTIONS OF SOME FOOD ADDITIVES DURING STORAGE

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ABSTRACT

The effectiveness of preservatives and antioxidants relies upon their chemical reactivity but these additives and those which are used for their physical effects (e.g. colours, flavours, vitamins) may also participate in reactions unrelated to their rôle as food additives. Examples of the situations in which changes can be encountered during storage are used to illustrate the factors involved and the three major experimental approaches which may be adopted in studying them.

INTRODUCTION

Provision of adequate amounts of a varied diet for an urban population demands the use of food additives. Many are selected for their chemical reactivity (e.g. antioxidants and preservatives) and this may manifest itself during storage and distribution; others may exert a physical effect (e.g. colours) but may incidentally participate in chemical changes. The range of additives which can participate in reactions is very wide and the present paper will not attempt a comprehensive coverage; examples of changes will be cited in relation to preservatives, flavours, colours and vitamins and will be used to exemplify the range of situations and effects which can be encountered during storage of food. The term 'storage' will be interpreted to include all periods of time when no effects are deliberately being promoted. It will therefore exclude changes which are particularly associated with processing conditions, but will include those which can be encountered during storage, either at low, ambient or elevated temperatures, and also during distribution and at the point of sale.

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SULPHITES

Sulphur dioxide and the sulphites are used very widely in the food industry and for a wide range of purposes. They are used as preservatives, as antioxidants, to control enzymic or non-enzymic browning or to modify protein rheology. In these applications they are used to inhibit, or—in other cases—to promote, some specific change in a particular constituent of the food product. In the course of exerting this effect, SO_2 will itself undergo a chemical change. The extent of these changes can be exemplified by some work conducted many years ago by Hearne & Tapsfield (1956) in studies on the non-enzymic browning of dehydrated potatoes during storage. These workers showed that 50–80% of the SO_2 measurable in food immediately after production could quite soon cease to be measurable if the product quality (particularly the moisture content) was poor, or if the storage conditions were hot. These observations were made in commodities which, in some cases, were heavily sulphited, i.e. 2000 ppm SO_2 or more. The test conditions usually precluded physical loss, so the amount of SO_2 being converted to other chemical products was between 1000 and 1600 ppm. Elevated temperatures are by no means necessary for extensive reaction of sulphite, and some work on the toxicology of sulphite (Til *et al.*, 1972) showed that when sodium metabisulphite was incorporated into pig rations at ambient temperature, the amount of SO_2 measurable the next day was only 65% of the expected value.

It is sometimes assumed that losses of measurable SO_2, particularly in the presence of air, are due either to volatilisation or oxidation to some other oxyacid of sulphur. However, over 30 years ago, Mangan & Doak (1947) studied the loss of SO_2 in dehydrated vegetables, and showed that only 20% of added sulphite was converted to sulphate, and that other sulphur oxyacids were found in even smaller amounts. They concluded that the other products were not inorganic and came from mechanisms which did not involve oxygen. Broadly similar results were obtained more recently (Gilbert & McWeeny, 1976). Sulphites are used extensively for their inhibiting effect upon the development of non-enzymic browning in dried fruits and vegetables. The browning reaction is a complex one; it is very dependent on temperature and on water activity (McWeeny, 1973) and it results in the formation of dark-coloured products, off flavours and protein damage which may reduce the nutritive value and/or the saleability of the product. The mechanism involved has never been fully established, but the early stages of the sequence have now been fairly well elucidated. Typically, the major reactants are either a reducing sugar or ascorbic acid and an amino acid or an amino side (or terminal) group on a protein molecule. Sulphites can exert an effect by removing some of the reducing sugar from the reaction system by the reversible formation of sugar hydroxysulphonates; customarily, these are measured as the so-called 'bound SO_2'. In a typical model system of glucose and glycine with added sodium bisulphite, the amount of free SO_2 decreases steadily until a very low figure is reached and at that

point colour development becomes marked, Fig. 1 (McWeeny *et al.*, 1969). Total measurable SO_2 also decreases, although rather more slowly, and, by difference, this provides a measure of the amount of SO_2 converted irreversibly to other compounds. The nature of these compounds has been investigated extensively and it is now generally agreed that one mechanism involved is the formation of a sulphonated deoxyosulose by the reaction of bisulphite ion with an osulose derived from the reaction of the reducing sugar and amino acid. In the case of glucose and

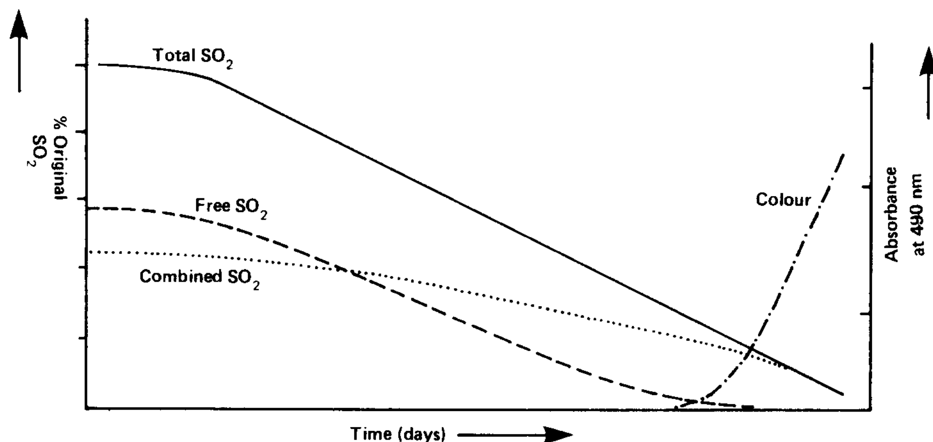


Fig. 1. Colour development and measurable SO_2 in glucose/glycine/bisulphite solution (after McWeeny *et al.*, 1969).

glycine the deoxyhexosulose was first isolated as an intermediate arising from the Maillard reaction by Anet (1960). Ingles (1962) postulated the reaction of this deoxyosulose with bisulphite in the control of the Maillard reaction in food, and isolated the predicted sulphonated deoxyosulose product from heated solutions of glucose and bisulphite. Subsequently, the product was identified in simple sulphited Maillard-type systems (Knowles, 1971). The pentosulose analogue was isolated from a sulphited ascorbic acid/glycine system by Wedzicha & McWeeny (1974*a*) and these sulphonates were isolated from stored dehydrated cabbage (Wedzicha & McWeeny, 1974*b*). Isolation of these two compounds provided the first concrete evidence about the mechanism of SO_2 in controlling the non-enzymic browning reactions in food during storage. One of these was probably identical with the unidentified sulphonate isolated from dried apricots by Stadtman *et al.* (1946).

Subsequently, the presence of sulphonated deoxyosuloses in amounts equivalent to 2500 ppm of SO_2 was demonstrated in some dehydrated cabbage after storage for 5 years (Wedzicha & McWeeny, 1975). Formation of the 4-sulphonated deoxyosulose may take place through direct substitution of the hydroxyl group on C_4 of the deoxyosulose, but, alternatively (Fig. 2), the deoxyosulose can dehydrate at

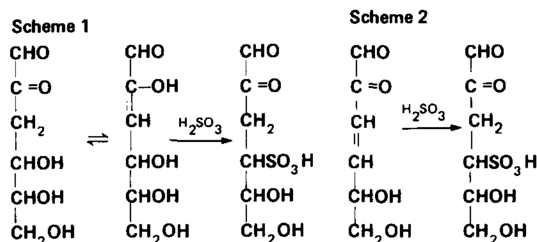
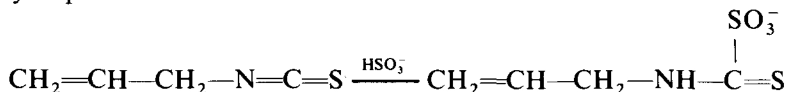


Fig. 2. Formation of 3,4-dideoxy 4 sulpho hexosulose (after Ingles, 1962).

carbons 3 and 4 to an unsaturated osulose which undergoes an addition reaction with the bisulphite ion (Ingles, 1962), i.e. the well-established mechanism by which the bisulphite ion undergoes an addition reaction with $\alpha\beta$-unsaturated aldehydes. This was regarded by McWeeny & Burton (1963) as being important in the sulphite inhibition of a wide range of non-enzymic browning reactions.

This reaction of bisulphite with unsaturated compounds is a general one and can manifest itself in various ways. A situation in which the flavour of foodstuffs can be affected by an analogous reaction involving an isothiòcyanate was reported recently by Frijters *et al.* (1981) in relation to the flavour characteristics of mustard. Allyl isothiocyanate is predominantly responsible for the flavour of mustard, and in studies on mustard pastes, with and without added bisulphite, measurements were made on (i) the abundance of the flavour compound, (ii) the amount of added bisulphite which was still measurable after storage, (iii) the abundance of the product derived from their interaction and (iv) the sensory quality of the mustard flavour. In sulphited pastes, the loss of flavour correlates well with the loss of bisulphite and of allylisothiocyanate and with the formation of allylaminothiocarbonylsulphonate.



$$CH_2{=}CH{-}CH_2{-}N{=}C{=}S \xrightarrow{HSO_3^-} CH_2{=}CH{-}CH_2{-}NH{-}\underset{}{\overset{SO_3^-}{\overset{|}{C}}}{=}S$$

There may also be reaction between additives and other additives or with the materials with which the food is in contact. Reactions of this sort may be exemplified by some studies conducted by Saxby (1977, 1978); solutions of various food colours containing added sulphur dioxide were stored in glass bottles close to 20 fluorescent tubes under conditions which might be experienced on, for instance, a supermarket display shelf. Over a period of weeks there were extensive changes and in the case of Sunset Yellow two new compounds were formed. One of these was sulphanilic acid, formed by scission of the —N=N— bond; the other was an unidentified compound which retained the chromophoric group, but showed an absorption maximum at a longer wavelength than the parent compound. In a similar experiment, an extensive change was encountered with amaranth over a period of weeks; no scission product was observed, but a new coloured compound was again observed. As with Sunset

Yellow, the absorption maximum of the new compound was at a longer wavelength than that of the parent compound, but its identity has not been established.

More dramatic changes, in terms of the timescale, can be observed if the food colour/SO_2 solution is placed in contact with a piece of tin plate on which the tin surface has been scratched to expose the underlying steel. Within a matter of hours, the colour was lost. Amaranth degraded to naphthionic acid; tartrazine and Sunset Yellow degraded to sulphanilic acid. In both cases there has been simple reductive cleavage of the diazo group. The conditions used for these tests were very mild and could be thought of as simulating those existing in a can in which the tin plate surface has some minor imperfections or when there has been chemical attack upon the tin plate by the can contents during storage or after opening.

NITRITES

The use of sodium nitrite in processed meat products is important in terms of its effects on the colour, flavour and microbiological stability of these commodities. Specifically, it has a major rôle to play in ensuring control of the botulinum hazard in cured meats.

A considerable proportion of the added nitrite survives the processing operation, and its continued reactivity in the meat is manifested in the progressive loss of measurable nitrite in the product. The chemical nature of the changes which take place during storage are not all well understood. Whilst there is much to be said for trying to produce a 'balance sheet' showing the nitrite consumed and the products formed, the limited usefulness of this approach must be appreciated, e.g. in cured meats the levels of volatile nitrosamines are in the 0–10 μg/kg range as compared with the 100 mg/kg nitrite which may have ceased to be measurable. Even a 99·9 % successful 'balance sheet' on nitrite reaction products could completely overlook any possible nitrosamine hazard. An alternative approach involves a systematic consideration of the potential reactions and a number of reaction mechanisms involving nitrite in food have been identified. The work has concentrated on the formation and properties of *C*- and *S*-nitroso compounds and the ways in which they may influence *N*-nitrosamine formation. It has been established that:

(a) Reactions of phenolic compounds (e.g. smoke phenols) can lead to the formation of nitroso-phenols. These are reactive compounds which can oxidise to the corresponding nitro-phenol (Knowles *et al.*, 1975).
(b) Neither nitroso- nor nitro-phenols are nitrosating agents capable of producing *N*-nitrosamines (Davies & McWeeny, 1977).
(c) Nitroso-phenols can act as catalysts promoting *N*-nitrosamine formation from the reaction between nitrite and secondary amines under moderately acid conditions (Davies *et al.*, 1978*a*, 1980).

(d) Reaction of nitrite with thiols can lead to the formation of *S*-nitroso compounds under mildly acid conditions (Davies *et al.*, 1978*b*).
(e) *S*-nitroso compounds can react with secondary amines and form nitrosamines under mildly acid conditions and at neutral or alkaline pH (Dennis *et al.*, 1979).
(f) Nitrogen oxides are important intermediates in some nitrosations and are quite soluble in the lipid phase; the presence of fat or a hydrocarbon phase can cause a marked increase in the formation of nitrosamines from any amines which partition into the lipid phase to a significant effect (Massey *et al.*, 1979).

The demonstration of the rôle of (i) nitrosophenols as catalysts, (ii) *S*-nitroso compounds as *trans*-nitrosating agents and (iii) a lipid phase in promoting nitrosation can provide a basis for a qualitative understanding (Fig. 3) of some of the confused and apparently conflicting results which are found in the literature relating to nitrosamine formation in cured meats. The pH can play a major part in determining which of the number of possible chemical mechanisms will dominate a particular situation. Physical factors—in this case solubility and partition coefficient—can determine the rate of chemical reaction, and an intermediate which shows a catalytic effect can have an importance much greater than its low abundance would normally imply.

The existence of these changes during storage can also be important in another

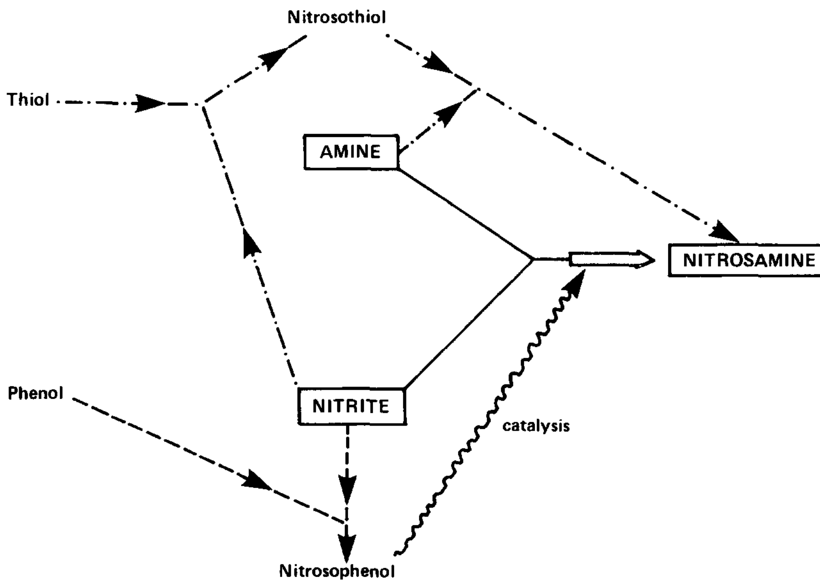


Fig. 3. Some reaction pathways involved in nitrosamine formation in food.

context. Measurement of nitrite is a routine quality control procedure in the cured meat industry and there are a number of widely used methods for this analysis (see review by Usher & Telling, 1975). The methods customarily employed do not specifically measure sodium nitrite. It is now possible to distinguish between the 'free' and 'bound' nitrite (Olsman & van Leeuwen, 1977) and although the chemical nature of the 'bound' nitrite is not fully understood, it is normally considered to include at least some of the *S*-nitroso compound present in meat. Not all *S*-nitroso compounds are equally potent as nitrosating agents, e.g. Dennis *et al.* (1980) have compared the nitrosating capability of *S*-nitroso cysteine, *S*-nitroso glutathione and of oligomers of the latter compound when linked to a macromolecule. Major differences in the rates of nitrosation of added secondary amines can be demonstrated with these three compounds. The differences between these various compounds as nitrosating agents and in their response to the analytical methods customarily employed for nitrite serves to underline the fact that our appreciation of the level of nitrite required to maintain botulinum control in cured meat is at best semi-quantitative.

PHENOLS IN SMOKED FISH

The products to which the fish is exposed during smoking include a group of phenolic compounds which contribute to the characteristic flavour of the smoked product and also to its enhanced microbiological stability. A systematic examination of the behaviour of some of these compounds after they have been deposited upon fish has recently been concluded by Moini (1980). This work included a study of the influence of time at sub-zero temperatures on the changes in the total amount of steam-volatile ether-soluble phenols which were isolated and identified in smoked cod.

The total steam-volatile ether-soluble phenols recoverable from each block of smoked cod after various time intervals is expressed in Fig. 4 as a percentage of the phenolic content of similar samples immediately after smoking and freezing. At each of the four storage temperatures the initial rapid loss of measurable phenols slows down and becomes almost imperceptible. At 'equilibrium' the percentage loss in total measurable phenols is twice as great at $-4\,°C$ (52 %) as at $-30\,°C$ (27 %). The conditions of measurement were such as to destroy any labile-binding of phenols and the observation of an apparent equilibrium condition was rather unexpected. However, these figures refer to total phenols and not to individual compounds, and further investigation showed clear differences existing between the amount and speed of loss of individual phenols in smoked cod; some were unmeasurable after only a short period of storage, while the amounts of others changed little.

A typical example (Table 1) of the data obtained by Moini (1980) shows the

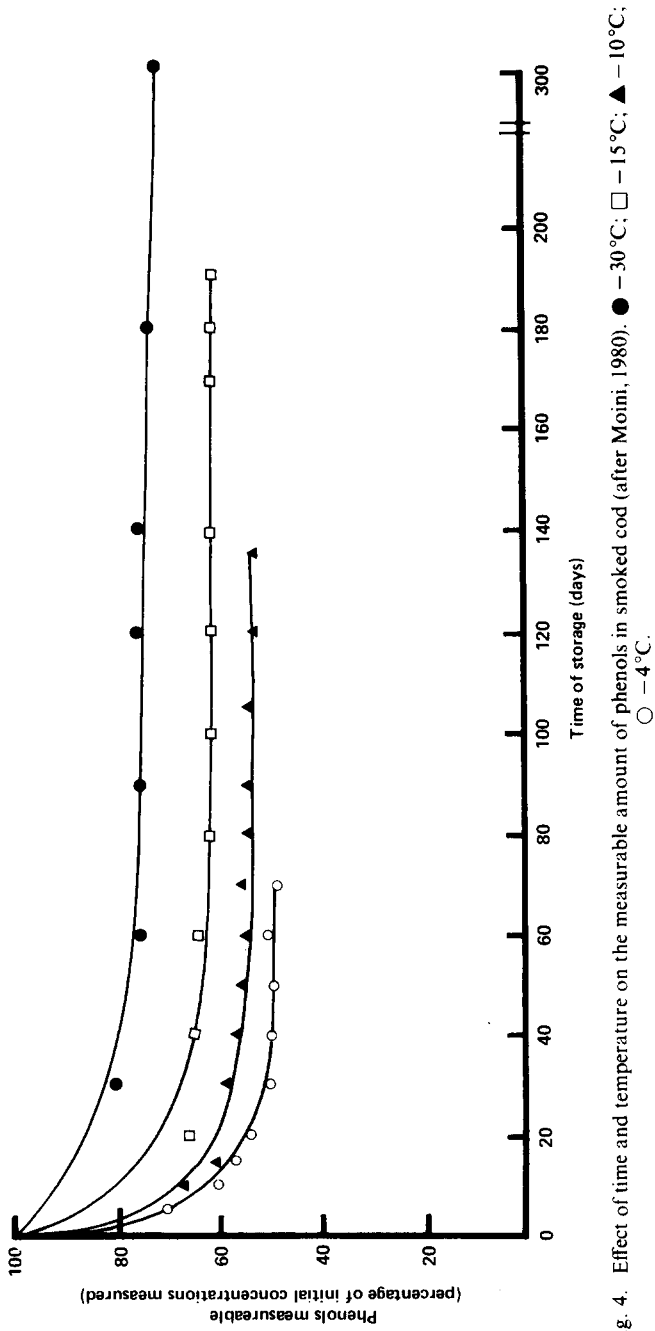


Fig. 4. Effect of time and temperature on the measurable amount of phenols in smoked cod (after Moini, 1980). ● −30°C; □ −15°C; ▲ −10°C; ○ −4°C.

TABLE 1
MEASURABLE AMOUNT OF PHENOLS IN SMOKED COD AFTER 40 DAYS' STORAGE AT −4 °C (AFTER MOINI, 1980)

Phenol	*Amount measurable* (%)
m/*p*-Cresol	69
Phenol	67
2,6-Dimethoxyphenol	63
Guaiacol	55
3,5-Dimethylphenol	54
Iso-Eugenol	51
4-Ethylphenol	44
4-Methylguaiacol	34
3,4-Dimethylphenol	24
4-Ethylguaiacol	13
Vanillin	0*
2,3-Dimethylphenol	0†
Eugenol	0†

* Some measurable after 10 days of storage.
† None measurable after 10 days of storage.

relative losses of each of fourteen phenols after storage for 40 days at −4 °C, beyond which time the smoked cod was unacceptable to the taste panel. The phenols can be roughly divided into three groups, the first group are measurable in quantities of at least 50 % of the original amount, the second group are found in quantities from 10–50 % of that originally present, and those in the third group fall to less than 10 % of their initial value. The loss of individual phenols is not related to volatility, i.e. the vapour pressure of cresol is forty times greater than that of vanillin at −4 °C, and yet cresol is retained much better than vanillin. Also, the activation energy of volatilisation of a range of phenols representative of those in smoked cod is within the range of 55–65 kJ/mole, whereas the observed activation energy associated with loss of phenols is about 44 kJ/mole. Covalent binding may be involved, particularly as a result of oxidation of phenols to quinones. It is thought (Mason, 1955; Pierpoint, 1977) that reactions of this sort can involve covalent bonding of quinones to proteins through protein terminal amino groups, the ε-amino group of lysine residues, the imino group of proline residues and through sulphydryl groups. The structure of the quinones may also be important, e.g. those with a second reactive ring position (or substituent) may form a cross-link by reacting with a second protein chain.

DEGRADATION OF β-CAROTENE

An unusual form of instability in a hydrogenated fat was encountered some years ago (McWeeny, 1968*a*,*b*,*c*) in relation to the formation of a green/blue discoloration

in a series of hydrogenated coconut or palm kernel oils. This reaction was notable insofar as the discoloration developed more rapidly in refrigerated store than it did at ambient conditions. Saponification and thin layer chromatography allowed separation of a carotenoid which was yellow in organic solvents, but which turned bright blue when exposed to even mildly acidic conditions. It chromatographed upon silicic acid as a blue spot which turned yellow when exposed to ammonia fumes. Chemical tests suggested that this compound was a 5,8-epoxide derivative of β-carotene. It was very similar to, but not identical with, mutatochrome. The compound was not fully identified, but was thought to be a specific geometrical isomer of mutatochrome.

Epoxides of this sort are normally formed by the action of a per-acid on β-carotene. Per-acids have not previously been reported in hydrogenated fats, but it is conceivable that they are formed in trace amounts by the action of atmospheric oxygen upon the aldehydo-glycerides formed by thermal breakdown of hydroperoxides during the deodorisation stage of fat refining; certainly, the reaction was dependent upon the presence of oxygen. A feature of the properties of per-acids is their thermolabile nature; they are normally used in the laboratory at temperatures in the 0–5 °C range. This may provide some explanation of the apparently negative temperature coefficient of the colour development. The reactions involved are depicted in Fig. 5. At ambient temperatures the per-acid decomposes thermally, rather than reacting with β-carotene, but, at refrigerator temperature, it may accumulate to the point where reaction with β-carotene becomes important. A review (McWeeny, 1968*d*) of reactions in food which show a negative temperature dependence lists a wide variety of situations in which the

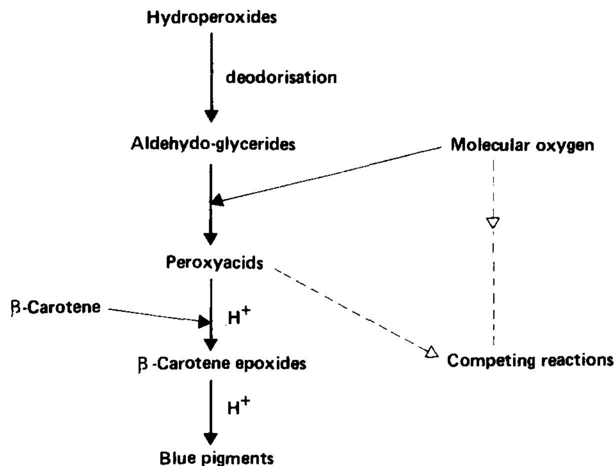


Fig. 5. Possible reaction mechanism involved in formation of green–blue discoloration in carotenised fat (after McWeeny, 1968).

common feature is that competing reactions with differing energies of activation are involved.

ASCORBIC ACID LOSS FROM COOKED POTATO

Storage is normally considered in terms of time spent at, or below, ambient temperature, but, in specific instances, it is proper to consider also the effects of storage at elevated temperatures, e.g. the warm-holding of cooked foods in catering establishments. Potato can be an important source of ascorbic acid, and potato products are frequently fortified with it. In a recent paper Jonsson (1981) studied vitamin retention in steamed potato during warm-holding for up to 4 h; the results exhibited the predictable destruction of ascorbic acid during the warm-holding process, but also served to illustrate a further factor in the situation. Two different batches of potato were prepared and, after cooking, each was held at 75 °C in air for up to 4 h. The ultimate loss of ascorbic acid was quite similar, but, during the first hour, the loss was widely different. In one case the loss was about 40 % of the original amount; in the other the loss was in excess of 80 %. The differences between the two batches was that in one case the tubers had disintegrated to a great extent during cooking and it was in this batch that the ascorbic acid loss was great. The distintegrated tubers have, of course, a much greater tendency to allow penetration of oxygen into their tissues and thereby promote the oxidation of ascorbic acid. These observations emphasise the need to bear in mind the importance of physical, as well as chemical factors, e.g. the results quoted suggest that during quite short periods of storage at elevated temperatures, loss of ascorbic acid from mashed potato could be much greater than from boiled potato.

CONCLUSION

It should be expected that food as consumed will contain substances deriving from, and additional to, the additives which are incorporated during production. In seeking to establish the nature and abundance of these products there are three basic methods of approach.

First, attempts can be made to isolate the reaction products from a food in which an additive has been employed; this is probably a difficult task because of the complexity of the food in chemical terms, and the low amount of additive employed.

Secondly, model systems can be used to examine the possible reactions of the additive with various food constituents, to determine the nature of the products and to devise analytical methods for their estimation prior to using the analytical methods in estimations of the anticipated products in actual treated foods. The drawback of this approach, of course, is that it relies on successfully predicting all the relevant model systems and evaluating them successfully.

A third approach involves the use of radioactive tracers. Here a labelled form of the additive is introduced to the food, subjected to appropriate simulation of processing, storage and cooking, and then compounds containing the labelled atom are isolated, identified and measured, and a balance sheet made up. The answers obtained will have three drawbacks, i.e. they relate only to the particular formulation and treatment which apply to that batch of food, the method follows only the labelled atom and minor products (e.g. nitrosamines) can be overlooked in producing what is apparently a very satisfactory balance sheet.

Each of these three types of approach has its own particular strengths and its own particular limitations, and, if used in isolation from the other two, may lead to inadequate, inaccurate and possibly harmful conclusions about what happens to additives in food during storage.

Most of the more reactive additives have been in use for a long time (e.g. the use of sulphur dioxide was practised by the ancient Greeks and Egyptians) and there is no evidence of significant harmful effects arising from reaction products from additives used in their traditional applications. However, advances in food technology involve additives in new combinations of conditions of pH, temperature, pressure and formulation. The possibility of novel and potentially harmful compounds being formed in this way, either during processing or on subsequent storage, is one to which the British Government and its expert advisers are alert and which receives their on-going attention; it is reassuring to find that so far such problems as have emerged have been amenable to relatively simple corrective actions.

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BIOCHEMISTRY OF UNDESIRABLE EFFECTS ATTRIBUTED TO MICROBIAL GROWTH ON PROTEINACEOUS FOODS STORED AT CHILL TEMPERATURES

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ABSTRACT

Red meats, poultry meat, fish and milk, the major foods considered in this paper contain, in microbiological terms, appreciable but variable amounts of carbohydrates, amino acids, nucleotides, essential minerals and lipids in addition to protein. Hence they provide an ideal environment for the growth of bacteria and it is the biochemical activities of these organisms which are normally held to be responsible for the changes in appearance, flavour, odour and sometimes texture, which occur during storage and eventually render the food unacceptable to the consumer.

It is now evident that initial growth occurs at the expense of energy derived from carbohydrate metabolism with the consequent formation of organic acids, alcohols, carbon dioxide, etc. Subsequently, lactic acid, amino acids and possibly nucleotides are metabolised with the formation of similar end products, and, in addition, ammonia, hydrogen sulphide, mercaptans etc. Breakdown of protein and lipid occurs only at a very late stage of the storage.

Details of the chemistry and the rôle of particular organisms in it are now becoming clearer, although it sometimes proves difficult to differentiate between microbiological and autolytic processes.

INTRODUCTION

Storage of red meats, poultry, fish and milk, the four proteinaceous foods considered in this review, inevitably leads to undesirable changes in one or more of the properties used by the consumer to assess their acceptability, i.e. their colour, taste, odour or texture.

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Whilst the rate at which deterioration occurs can be slowed by storage under refrigeration it cannot be totally prevented, unless the product is sterilised. The food is eventually rejected and said to be 'spoiled'. Off-odours and/or off-flavours are two of the more frequent causes of spoilage, and their chemical nature and the biochemical pathways involved in their formation are the subject of this paper. Their development frequently coincides with the presence of large microbial numbers, and it is usually assumed that the two are related. Evidence collected over a period of time suggests that this is often true, but contributions from autolytic processes—perhaps even overriding ones—cannot be dismissed. The most common are the oxidative and hydrolytic changes which occur to the lipids in many foods as a result of chemical and indigenous enzyme activities, and which seem to be particularly important in minced flesh foods (Branen, 1978) and milk (Shipe *et al.*, 1978). The possible presence of end-products from these processes certainly complicates the interpretation of the chemical data on volatile end-products in terms of microbial metabolism.

COMPOSITION

The lipid content of the whole fish, chicken or red meat carcass can vary widely between and within species, but the composition of the musculature is quite similar, each containing 70–80 % water, 17–20 % protein, 1–4 % lipid and 1–5 % soluble small molecular weight compounds including mineral salts (Paul & Southgate, 1978). Soluble organic compounds common to all three include amino acids, lactic acid, glycogen, glucose, glucose-6-phosphate, creatine and nucleotides and their degradation products. An important point to note is that the soluble carbohydrate, a preferred substrate for microbial growth, rarely exceeds 0·2 % and is frequently less than 0·1 %. The major difference is the presence of trimethylamine oxide (TMAO) only in fish. The pH of fresh, good quality beef is normally in the range 5·5–5·8 while pH values greater than 6·0 are more common in fish and poultry. Milk is somewhat different, containing 87 % water, 3 % protein, 4 % lipid and 5 % lactose and having a pH of around 7·0. Low concentrations of amino acids and monosaccharides are undoubtedly present, but their levels are not well defined.

MICROBIOLOGY

Foods so rich in nutrients and with such favourable pH values provide ideal cultural conditions for microbes. It is therefore no surprise that, during chill storage, those organisms which inevitably gain access to the product despite the implementation of stringent hygiene standards, and which are capable of growing at low temperatures, grow to large numbers. In minced flesh foods growth occurs throughout the mass of

the food, but in unminced samples, growth is normally restricted to the exposed surfaces, at least until spoilage becomes obvious from off-odours and/or the presence of visible slime. By this time, under aerobic conditions, bacterial numbers are *ca.* $10^8/cm^2$ and the flora is dominated by Gram negative rods belonging predominantly to the genera *Pseudomonas*, *Moraxella-Acinetobacter* and *Alteromonas* (Shewan, 1974). *Pseudomonas* spp. are also a major element of the flora of bulk, raw milk and occur in pasteurised milk as post-process contaminants (Law, 1979). For each food, time to spoilage can be anything up to 10–12 days depending upon the extent of initial contamination and the temperature of storage. However, in the case of red meats in particular, the shelf-life can be extended to several weeks by packing under vacuum in materials relatively impermeable to atmospheric gases. This leads to low oxygen tensions and the build up of carbon dioxide, with the result that the flora is now dominated by Gram positive bacteria, particularly lactic acid bacteria, and at spoilage their numbers are in the region of $10^7/cm^2$ (Gill & Newton, 1978).

GROWTH SUBSTRATES

Studies with single pure bacterial cultures inoculated on to meat showed that initial growth on aerobically stored meat occurred at the expense of glucose, and, in certain cases, glucose-6-phosphate, and that by the time bacterial numbers reached $10^8/cm^2$ and spoilage was becoming evident, glucose was no longer detectable at the surface (Gill & Newton, 1978). Subsequently, ammonia concentrations increased at the surface, whilst the concentrations of certain amino acids and lactic acid decreased and there were accompanying increases in pH. Glucose diffusing to the surface continued to be metabolised while the concentrations of other amino acids increased. This was the first indication that proteolysis was occurring in the samples, an observation consistent with many earlier ones which had failed to detect protein breakdown prior to overt spoilage (Dainty *et al.*, 1975). This order of substrate utilisation confirms and extends earlier, but less detailed findings for fish (Beatty & Collins, 1939; Shewan & Jones, 1957) and minced beef (Gardner, 1965). In addition, in fish, TMAO is reduced in substrate concentrations to trimethylamine while acting as an electron acceptor for microbial oxidation of other substrates (Shewan & Jones, 1957). The data for chicken are less complete, only amino acids and, less definitely, glucose, having been shown to be utilised (Lea *et al.*, 1969*a*,*b*). Under laboratory conditions Gram negative bacteria are able to metabolise many of the other soluble components in flesh foods, but there is no evidence that they do so under storage conditions. Further work is clearly needed on this point.

During storage of red meat in vacuum packs, only two substrates are utilised; glucose, followed by L-arginine (Gill & Newton, 1978). This is consistent with the known metabolic capabilities of the dominant lactic acid bacteria and, together with

the fermentative type of metabolism which must predominate under conditions of low oxygen tension, explains the lower cell numbers found at spoilage than under aerobic conditions. It also explains the different types of volatile end-products associated with the two storage conditions.

Thus, protein breakdown does not appear to play a quantitatively important part in the *development* of spoilage in flesh foods, nor is there any evidence of significant lipid metabolism. However, at advanced stages of spoilage, degradation of structural (myofibrillar and connective tissue) proteins, as well as the soluble sarcoplasmic proteins, can occur and the food may even disintegrate.

Evidence for substrate utilisation during microbial growth in stored milk is very limited. But, since the types of bacteria are very similar to those in flesh foods, it seems likely that carbohydrates and amino acids are again the initial substrates for growth. Some evidence supporting this is provided by growth of streptococci at the expense of lactose, in milk subjected to poor temperature control (Shipe *et al.*, 1978). In apparent contrast to the situation in flesh foods, stored milk may contain substantial amounts of microbially produced lipases and proteinases (Law, 1979) and lipid and protein breakdown may therefore be of greater importance for microbial growth in this case. The proteinases are particularly active against the various types of casein and, as a result, the processing properties of the milk, e.g. its heat stability, may be adversely affected. In addition, both the lipases and the proteinases are sufficiently heat stable to survive pasteurisation and may produce quality defects in milk products, e.g. off-odours in cheese.

VOLATILE END-PRODUCTS

A somewhat abbreviated list of compounds which have been extracted from, or detected in the headspaces of proteinaceous foods after various periods of storage from freshness to overt spoilage is given in Table 1. Of course not all of them are ever likely to be found in one commodity at one time, but experience has shown that as many as fifty may be present (e.g. Stutz, 1978). Many of the compounds are present in trace amounts even in fresh foods and can be recovered, but not necessarily in constant proportions, throughout storage. At these concentrations they do not detract from the quality of the food and may even be essential constituents of its desirable properties (Kinsella, 1969) or perhaps precursors of cooked flavour. It is only when the concentrations of particular groups of compounds begin to rise significantly above their flavour thresholds that off-odours begin to appear, and it is to this process that bacteria are known to contribute. Hence, quantitative comparisons between fresh and stored, bacterially contaminated samples are essential and, ideally, stored sterile samples should also be included. Although such analyses have not always been done, we are now able, with a fair degree of

TABLE 1

VOLATILES RECOVERED FROM MEAT, FISH, POULTRY AND MILK AT VARIOUS STAGES OF SPOILAGE

	Meat[a]	Fish[b]	Chicken[c]	Milk[d]
Pentane	*		*	
Heptane	*		*	
Octane	*		*	
1-Heptene			*	
Heptadiene	*		*	
Benzene	*	*		
Toluene	*	*		
Ethanal	*	*	*	*
Propanal	*	*	*	*
Butanal	*	*		
Pentanal	*	*	*	*
Hexanal	*		*	
Heptanal	*		*	
2-Methylpropanal		*		*
3-Methylbutanal		*		*
2-Methylbutanal		*		*
Benzaldehyde	*			
Acetone	*	*	*	*
Diacetyl	*	*	*	*
Acetoin	*	*		
2-Butanone	*	*	*	*
2-Pentanone	*	*	*	*
2-Hexanone				*
2-Heptanone	*	*	*	*
Ethyl acetate	*	*	*	*
Ethyl propionate	*		*	
Ethyl butyrate	*	*		*
Ethyl caproate		*		*
Methyl acetate	*		*	

	Meat[a]	Fish[b]	Chicken[c]	Milk[d]
Methanol	*	*	*	*
Ethanol	*	*	*	*
Propanol	*	*	*	*
Butanol	*		*	*
Pentanol	*		*	
Hexanol	*		*	
2-Butanol	*			
3-Methylbutanol	*	*		
Hydrogen sulphide	*	*	*	*
Methyl mercaptan	*	*	*	*
Dimethyl sulphide	*	*	*	*
Dimethyl disulphide	*	*	*	*
Dimethyl trisulphide		*		
Formic acid		*		*
Acetic acid	*	*		*
Propionic acid	*	*		*
Butyric acid	*	*		*
Isobutyric acid	*	*		
Valeric acid	*	*		*
Isovaleric acid	*	*		
Caproic acid	*	*		*
Methylamine	*	*		
Dimethylamine	*	*		
Trimethylamine	*	*	*	
Putrescine	*	*		
Cadaverine	*	*		
Histamine	*	*		
Ammonia	*	*	*	

* Compound detected.
[a] Data compiled from Stutz (1978); Patterson & Bolton (1981); Dainty *et al.* (1979); Lakritz *et al.* (1975).
[b] Data compiled from Angelini *et al.* (1975); Wong *et al.* (1967); Miller *et al.* (1972; 1973*a*,*b*,*c*).
[c] Data compiled from Grey & Lea (1969); Freemen *et al.* (1976).
[d] Data compiled from Kulshrestha & Marth (1970); Kinsella (1969).

confidence, to associate particular odours with the production of specific chemicals, and in many cases bacteria have been shown to be involved.

Sulphur odours

Combinations of hydrogen sulphide, dimethylsulphide (DMS) and methyl mercaptan have been shown to be responsible for the sour, sulphidy, cabbage-water smells which develop on stored fish (Herbert *et al.*, 1975). Their formation was clearly associated with microbial growth since none of the compounds was present in stored, sterile samples and many of the *Pseudomonas* spp. isolated from the fish were shown to produce them (Herbert & Shewan, 1976). The same compounds plus dimethyldisulphide (DMDS) have also been shown to be major components of the putrid odours of minced beef (Stutz, 1978) and of chicken (Freeman *et al.*, 1976). They rarely reach high concentrations in milk.

The source of the compounds was shown to be cysteine and methionine (Herbert *et al.*, 1975; Herbert & Shewan, 1976) and not the glutathione or the taurine present in the tissues. Cysteine is probably the major source of hydrogen sulphide, while methyl mercaptan can be formed from methionine by oxidative deamination, followed by demethiolation. DMS was also shown to be formed from methionine, but no mechanism was suggested whilst DMDS is readily formed from methyl mercaptan by oxidation. A further oxidation product, dimethyltrisulphide, was detected in fish inoculated with pure bacterial cultures, but has not been reported in natural spoilage situations (Miller *et al.*, 1972, 1973*a*,*b*).

Fruity odours

The development of fruity odours in spoiled milk (Shipe *et al.*, 1978), fish (Miller *et al.*, 1973*c*) and minced beef (Stutz, 1978) has been traced to the presence of short chain fatty acid esters and shown to be associated particularly with the growth of *Pseudomonas fragi*. In all cases ethyl esters predominate, with ethyl butanoate and hexanoate being the two main components in milk; the same two plus ethyl acetate were present in fish. Minced beef contained methyl acetate in addition to ethyl acetate, and both have also been reported to be important components of the off-odours of chicken, but there was no indication whether they imparted a fruity tone to the odour in this case (Freeman *et al.*, 1976).

The esters are produced by reactions between the appropriate alcohol and acid components, presumably, but not necessarily, catalysed by bacterial esterases (Hosono *et al.*, 1974). In natural, mixed population situations there are indications of concerted action by more than one organism to produce esters (Stutz, 1978). Since many organisms found in stored milk produce lipases, milk lipids which contain appreciable amounts of butanoic and hexanoic acids seem a likely source of these ester components, while ethanol could be formed from a number of sources including glucose or amino acids via acetaldehyde (Lees & Jago, 1978). In the other foods, hexanoate is probably still derived from lipid, but the butanoate, acetate and

ethanol are more likely to be formed in carbohydrate and/or amino acid metabolism.

Amine/ammoniacal odours

Amine/ammoniacal odours are associated with greater than normal amounts of amines and ammonia, the latter normally making a greater contribution, except perhaps in fish. In this case because of the presence of substantial amounts of TMAO and its reduction to trimethylamine (TMA) by bacteria (Beatty & Collins, 1939), TMA tends to be the major amine contributing to the off-odours. In fatty fish (e.g. mackerel and herring), other bacterially produced amines, including putrescine, cadaverine and histamine (Ritchie & Mackie, 1980) may be found. However, unless there has been poor temperature control, the levels are not usually very significant until the flesh becomes grossly spoiled. Similarly, amine production in meat only normally becomes significant during post-spoilage processes (Patterson & Edwards, 1975; Lakritz *et al.*, 1975) the major ones found being methylamine, dimethylamine, trimethylamine, histamine, cadaverine and putrescine. A recent publication suggests, however, that high concentrations of putrescine and cadaverine produced by bacteria may be present in pork before spoilage (Slemr, 1981) and therefore contribute to the odours.

Histamine, cadaverine and putrescine arise by decarboxylation of histidine, lysine and ornithine/arginine, respectively, whilst decarboxylation of glycine could account for methylamine formation. In foods other than fish, trimethylamine could be formed from the action of choline deaminase on choline released from phospholipid, whilst dimethylamine could arise by decarboxylation of sarcosine derived from creatine. An alternative route of formation of methylamine and the longer chain alkylamines sometimes detected (Patterson & Edwards, 1975) is via the transamination of aldehydes arising from amino acid, carbohydrate or lipid metabolism (Maga, 1978).

Acid odours

The appearance of acetic and propionic acids in raw milk subjected to temperature abuse leads to a sour/acid off-condition during the predominantly lactic fermentation of lactose by lactic acid bacteria (Shipe *et al.*, 1978). A similar condition is described in vacuum-packaged meat stored for long periods, both straight (C_2–C_6) and branched chain (C_4 and C_5) fatty acids having been detected (Sutherland *et al.*, 1976; Dainty *et al.*, 1979). The two branched chain acids and acetic acid have also been found in aerobically stored samples of meat (Gibbs *et al.*, 1979; Patterson & Bolton, 1981; Dainty & Hibbard, 1980) and elevated levels of fatty acids may occur in stored fish (Van Spreekens, 1977). All of the acids may be formed during either carbohydrate or amino acid metabolism, but it seems likely that straight chain acids are mainly derived from carbohydrate, and the branched chain ones from the relevant amino acids.

Malty odours

This off-condition is predominantly associated with milk (Shipe *et al.*, 1978) but the same term has been used to describe spoilage of meat under certain conditions (Patterson & Gibbs, 1977). The compound predominantly responsible for the odour in milk has been identified as 3-methylbutanal, but smaller amounts of 2-methylbutanal, 2-methylpropanal and 3-methylthiopropanal may also be present (Morgan, 1970; Shipe *et al.*, 1978).

The mechanism of formation in each case involves transamination of the appropriate amino acid, leucine, isoleucine, valine or methionine, to its keto acid followed by decarboxylation to the aldehyde. With the exception of 3-methylthiopropanol, the aldehydes have also been detected in fish, while the corresponding alcohols, which could be formed by reduction, have been reported in fish and meat (Table 1).

Lipolytic/rancid odours

The release of medium chain C_6–C_{10} fatty acids from lipids produces this kind of deterioration in milk and milk products (Law, 1979). Although milk lipase is probably a major contributor to this condition at normal microbial populations, higher microbial numbers may also make a significant contribution.

Many of the hydrocarbons, alcohols, aldehydes and ketones listed in Table 1 are typical end-products of the oxidative breakdown of fats (Eriksson, 1974) and may contribute to the development of rancid odours in flesh foods. They are particularly common in minced flesh foods and while non-microbial processes are probably a major source, a contribution from microbes should not be discounted because many organisms are able to catalyse such reactions (Alford *et al.*, 1971).

Miscellaneous compounds

Experiments with pure cultures of some bacteria normally only present as relatively minor components of the spoilage flora of proteinaceous foods have revealed the production of various characteristic compounds. Examples are (a) 2-methoxy-3-isopropyl-pyrazine, produced by *Pseudomonas perolens* in fish and milk (Miller *et al.*, 1973*b*) and responsible for a musty/potato odour, (b) phenethyl-alcohol produced by growth of an *Achromobacter* (*Moraxella*) sp. on fish (Chen *et al.*, 1974) and (c) isobutyronitrile and isobutyraldoxime *O*-methyl ether, produced in meat by various Gram negative species (Gibbs *et al.*, 1979; Harper & Gibbs, 1979). According to Harper and Gibbs the ether may be the unidentified component found by Freeman *et al.* (1976) in chicken. The formation of each of these substances appears to involve amino acid metabolism; the pyrazine from threonine, the alcohol from phenylalanine and the nitrile and oxime from valine.

In many of the major studies quoted above, 2-butanone and acetone have been detected among the volatiles but their contribution to the odours have not been assessed (Freeman *et al.*, 1976; Stutz, 1978). Stutz has claimed that, together with

dimethylsulphide and dimethyldisulphide, they could be useful indicators of the microbial quality of meat since they were the only compounds produced (a) by most of the isolates tested, (b) in each sample of meat examined, (c) in increasing quantities throughout the storage period. A possible pathway for 2-butanone formation from acetoin (and hence carbohydrate), involving reduction to 2,3-butanediol followed by dehydration and subsequent oxidation to the ketone, has been proposed. Acetone can clearly arise from either carbohydrate or lipid via decarboxylation of acetoacetate.

The dominance of a particular spoilage situation by any of the individual compounds or groups of compounds described above does not preclude the presence of concentrations of other components significantly above the levels in fresh or stored sterile samples. The results of Stutz (1978) clearly demonstrate that it is the relative proportions of the various classes of substance, above a certain minimum concentration of total volatiles required for any off-odour perception, which determines the nature of the smell. Thus, he found that, whilst high levels of sulphur compounds often produce variations on an overall putrid odour, the same levels in the presence of elevated levels of esters and aromatic compounds give rise to an estery, rotten-vegetable smell. It is obviously possible to get a progression of smells as the proportions vary, and this clearly happens in many natural spoilage situations.

In summary, the spoilage of all three flesh foods considered shows many similarities in terms of microbiology, growth substrates and volatile end-products. That of milk also has much in common, its tendency to develop greater proteolytic and lipolytic activities at an earlier stage of spoilage being the major difference. The data on volatile compounds indicate that while many of the important spoilage odour compounds probably arise from amino acid metabolism, as suggested from the substrate studies, others may be derived from carbohydrate and/or lipid and be important indicators of the microbiological quality of the food.

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THE CHEMISTRY OF FLAVOUR AND TEXTURE GENERATION IN CHEESE

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ABSTRACT

Cheese texture and flavour are obtained through a series of chemical changes which occur in the curd during the early stages of ripening. The lowering of pH and Eh, a result of lactic bacteria metabolism, greatly influences texture through water and mineral contents, but has also further repercussions on some chemical changes. Lipid hydrolysis leads to free fatty acids which serve as a substrate for further reactions. Proteolysis influences texture, but mainly flavour, as it results in the formation of peptides and amino acids which, for flavour, leads to aroma compounds through enzymatic and, perhaps, purely chemical reactions.

INTRODUCTION

Milk has unique nutritious properties but, as it is highly perishable, unless properly heat-treated and refrigerated, it has a very short shelf-life. For centuries cheese making has been the only means of preserving the most valuable constituents of milk, and among the great variety of cheese types some can be considered as products with real long-term storage possibilities.

Starting from a liquid which, although it is not flavourless, has normally a very bland aroma, the cheese maker can, using different technologies, create a series of new products varying even within the same type of cheese over a large range of texture and flavour properties. Differences can also be encountered between cheeses

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from the same batch; this is particularly true for soft types, and it is not unusual to find detectable sensorial differences, even between the two sides of the same cheese. These texture and flavour properties are not obtained until after a ripening period, the length of which varies with the type of cheese, and cannot be maintained at their best for an indefinite period of time. This means that what we have to observe is not constant with time. As a consequence of the heterogeneous nature of the product, and of the complexity of its constitution, the chemical basis of cheese flavour and aroma has not yet been elucidated, despite a large number of publications, most of which have placed emphasis on the volatile aroma, permitting, in some cases, one to obtain an insight into the broad mechanism, but, regrettably, still leaving many questions unanswered. The texture of cheese, even though it is recognised as important for consumer preferences, has not, on the whole, been very extensively studied. This is why it is difficult to discuss the chemical basis of texture and flavour in cheese, the more so as no new results have been published since the subject was excellently reviewed by others (Behnke, 1980; Law, 1981).

Protection of the valuable milk constituents against spoilage is achieved by raising the dry matter content through clotting of milk protein, with subsequent elimination of whey, lowering the pH by lactic acid starters, and adding a certain amount of salt to the curd (salting or brining).

The fresh curd thus obtained is still rather bland in flavour and has a texture which differs considerably from the well-ripened products. These properties will be obtained after a series of enzymatic or non-enzymatic reactions. Water-soluble substances, fat and protein will follow an evolution which can be more or less controlled by varying the parameters such as moisture content, pH and Eh of the curd.

INFLUENCE OF pH AND PHYSICO-CHEMICAL CONDITIONS

The basic reaction in cheese making is the production of lactic acid by starters. Carbohydrates are fermented via the well known hexose diphosphate pathway to pyruvic acid. Lactic acid is then formed from pyruvic acid, which acts here as a hydrogen acceptor, so that the reduced NAD can be reoxidised for a further oxidation of glucose.

The lactic acid production makes the pH drop to a certain value which determines the future formation of the cheese. In Camembert cheese, for example, the pH drops to about 4·6, and sometimes lower, as a consequence of the large amount of lactose which remains in the curd still rich in water at the end of draining. The acidity of the curd leads to an important, almost total, solubilisation of the phosphates and calcium, linked to the protein micelles, and temporarily lowers the activity of the lipolytic and proteolytic enzymes (Mocquot, 1971). The calcium level is an important factor, as this element acts as a cement in the cheese body. The difference

in cohesion between the body of an Emmental (0·9 to 1·0 %Ca) and that of a soft cheese (0·2 to 0·3 % Ca) is obvious. This, together with the water content, limits the size of each type of cheese and, consequently, the possible ripening time. Mineral equilibrium also plays a rôle in the texture modification during ripening. Thus, as Camembert ripens, there is an important Ca-transport which, initially, is more or less uniformly present in the curd, towards the outside of the cheese (Metche & Fanni, 1978). The core, with a low Ca content, keeps acidic and firm with a low proteolysis. Lactic acid later serves as a substrate for surface flora, allowing the pH to rise to a level where enzymes become more active, leading to a highly flavoured product. Moreover, the increase of pH itself may also contribute to the softening of cheeses such as Camembert (Noomen, 1977).

The technique of washing the curd, which is sometimes used in soft cheese technology, results in less acidity and more minerals with, as a consequence, a different body. The more neutral flavour is a consequence of the lower activity of the *Penicillium* and metabolism is limited by the low level of available lactic acid. This may serve as an example of the late influence of lactic acid fermentation on the cheese's development.

Another consequence of the development of a lactic acid flora is the lowering of the Eh (Galestoot & Kooy, 1960) to a potential of about −130 mV or lower. As we shall see later, the existence of a negative potential will help to explain why some conversions occur and others are made impossible. At least, in Cheddar, reducing conditions achieved artificially have been shown to be essential for the production of key aroma compounds (Manning, 1979).

As well as metabolising lactose, starters can also use citrate as a substrate. This results in the production of pyruvic acid from oxaloacetic acid with acetic acid and CO_2 as by-products. This pyruvate, as it is not needed for reoxidising the reduced NAD, is used to produce diacetyl, according to a mechanism which is pH- and oxygen-dependent (Collins, 1972; Dwivedi, 1973).

Pyruvate is first decarboxylated to acetaldehyde TPP complex, which reacts with a molecule of acetyl CoA to form, directly, diacetyl. In many instances, diacetyl is reduced enzymatically to acetoin, which is subsequently reduced to 2,3-butylene glycol.

LIPIDS AND THEIR DEGRADATION

Fat plays a very important rôle in the development of a good texture, and it is well known that a higher fat content leads to a less firm and elastic body. During recent years there has been an increased interest in cheeses (Cheddar and Swiss) with lower fat content (20 to 30 % only) and the consumer has been able to notice the excessive firmness and lack of smoothness of such cheeses. These differences can be explained by the presence of more protein matrix in the cheese (Emmons *et al.*, 1980). From a

strictly practical point of view, it is necessary to raise the water content of the non-fat matter in order to obtain a texture more identical to that of normal cheese.

The reduction of the size of fat globules does not produce a distinct difference in the texture of Cheddar and the slight decrease in firmness and elasticity noticed in cheeses made from homogenised milk could result from a small increase in the water content (Emmons *et al.*, 1980).

Fat composition can also have an influence on the texture. A relationship has indeed been observed in Emmental between firmness and iodine value (IV) (Steffen, 1975). A higher IV (i.e. a more unsaturated fat) resulted in a softer body. Along the same lines, it appears that Gruyère cheese, made from the milk of grazing cows, or cows fed on green fodder supplemented with coprah oil, had a more open texture than cheeses made with the milk from cows not receiving any supplement (Mocquot, 1979).

During the ripening period, the amount of free fatty acids differs according to the type of cheese. In Camembert (Kuzdzal-Savoie & Kuzdzal, 1966) it can be up to 10% of total fatty acids. Although the influence of lipolysis on texture has not been really investigated, it is generally considered that it has no great influence on the rheological properties of the cheese.

The rôle of fat is also important for the perception and formation of flavour. It is commonly observed that cheese made from skimmed milk does not develop a full aroma (Ohren & Tuckey, 1969). If the fat content is increased above a certain limit, the flavour is not improved, and there may even be more frequent off-flavours. Substituting vegetable or even mineral oil for milk fat seems to favour a certain aroma-development—at least in Cheddar (Foda *et al.*, 1974). This seems to prove that one important action of fat is to dissolve and hold the flavour components. The fact that milk fat has to be used in order to obtain real cheese flavour emphasises the influence of the composition of milk fat on flavour genesis, whilst experiments reincorporating milk fat into skim milk, with or without the use of an emulsifying agent, seem to suggest that the fat–water interface has an important influence on flavour development, although it is not yet fully understood.

As in every type of food with a high fat content, lipolysis and oxidation are likely to occur. Lipolysis is known to be an enzymatic reaction. Milk lipases have been shown to be more active than starter lipases in Cheddar (Reiter & Sharpe, 1971). They seem to hydrolyse the fat selectively and to be able to attack triglycerides, whilst lactic streptococci lipases seem to be active mainly on mono- and diglycerides (Stadhouders & Veringa, 1973). The free fatty acids pattern of cheese shows, on the whole, a certain specificity towards the liberation of long chain fatty acids (Kuzdzal & Kuzdzal-Savoie, 1966; Umemoto & Sato, 1975). Free fatty acids will partition between water and lipid phase and be present as soaps. The liberated fatty acids are involved in several types of reaction which vary in importance according to the type of cheese considered.

In cheeses where mould growth occurs, production of methylketones is very important. This production follows a two-step scheme: the fatty acids are first oxidised to β-ketoacids, which are then decarboxylated to the corresponding methylketones with one carbon atom less (Hawke, 1966). Both resting spores (Lawrence, 1966) and mycelium (Lawrence & Hawke, 1968) seem equally efficient in the conversion. Besides this main mechanism, it seems that certain short chain carbonyl compounds could also result to a limited extent from the metabolic activity of the mould on the β-ketoacids (Dartey & Kinsella, 1971). The latter are normally present in small quantities in milk fat, i.e. in the ketoglycerides which represent about 1% of milk fat. This second mechanism, which is based on the constitutive β-ketoacids, is the main pathway in cheeses where mould growth is not involved in the ripening.

The amount of ketones produced during the curing does not depend directly on the amount of available fatty acids precursor (Anderson & Day, 1966) as 2-heptanone always predominates in Blue cheese (ewe or cow—despite considerable variations between samples) while 2-nonanone is the more abundant ketone in the soft type. Many factors affect the rate of formation of individual ketones: temperature (Dolezalek & Hoza, 1969), pH (Jolly & Kosikowski, 1975; Lawrence & Hawke, 1968), physiological stage of the mould (Fan *et al.*, 1976) and the ratio of concentration of fatty acid to dry weight of spores (Fan *et al.*, 1976). It appears that free fatty acids do not accumulate in the mixture during methylketone formation, which means that the lipolysis rate does not normally exceed the oxidation rate of the liberated fatty acid, thus avoiding the toxic effect of the fatty acids, which is more noticeable on the mycellium than on the resting spore (Fan *et al.*,1976). This toxic effect removes the efficiency of the technique of initiating lipolysis by homogenisation of milk fat compared with the use of microbial lipase for enhancing the rate of flavour development.

Data on the concentration of individual methylketones during blue cheese ripening show large fluctuations, which suggest interconversion mechanisms. Indeed, methylketones are further metabolised by *P. roqueforti* into the corresponding secondary alcohol, the reaction being reversible under aerobic conditions. The rate of ketone disappearance again depends on the influence of the physiological stage of the mould and the concentration of ketones (Fan *et al.*, 1976). The presence of nonenone in Blue cheese and Camembert, together with that of undecenone and tridecenone in Camembert made from milk heavily contaminated with *Pseudomonas* (Dumont *et al.*, 1977), raises the question whether these ketones are formed from monounsaturated fatty acids normally present in milk fat or whether another mechanism is to be postulated. The existence of a pathway from monounsaturated fatty acids to unsaturated ketones would mean a preferential lipase activity, as the medium chain length monounsaturated fatty acids are present in milk fat in much smaller quantities than the corresponding saturated acids.

Another possible reaction, in which polyunsaturated and, perhaps, monounsaturated, fatty acids can be involved, is oxidation. The amount of oxidation in cheese is, however, rather limited, as milk fat would normally be very susceptible to oxidation in the conditions (pH, and copper content when copper vats are still in use) which prevail in cheese. The existence of a low redox potential, together with the presence of natural antioxidants, could prevent the initiation of oxidation mechanisms, or create conditions in which the primary oxidation products are further reduced. The second hypothesis would explain the existence of 1-alkanols (including methanol) in cheeses: these alkanols would arise from hydroperoxides, and, by similarity to what has been demonstrated for butter fat oxidation (Stark & Forss, 1965), octanol, for example, can be formed from the 10-hydroperoxide of oleate (Stark & Forss, 1966). This is supported by the finding of positive PV in cheese fat (Pradel). Oxidation also explains the occurrence of oct-1-en-3-ol in Camembert, where it is thought to be a metabolite of *P. caseicolum*. We do not know the exact mechanism of its formation, but it is likely that it could be formed from linoleate, as demonstrated in oxidised butter (Stark & Forss, 1964). In a similar manner, the occurrence of aromatic hydrocarbons in cheeses (toluene, xylene, methylnapthalene, etc.), could be explained by the existence of an oxidative process.

Aliphatic and aromatic esters play an important part in the flavour, and sometimes the off-flavour, of cheese. They can be of enzymic origin, as different micro-organisms have been shown to produce esterases (Hosono *et al.*, 1974; Morgan, 1976) but can also easily result from a purely chemical reaction, at least in cheeses which are ripened for a long period of time. Amides have been identified in cheese (Wirotama & Ney, 1973), but no mechanism has ever been proposed for their formation. However, it should be remembered that, according to pure chemistry, amides are easily formed by the action of ammonia on ethyl esters.

γ- and δ-lactones have been identified in cheeses, particularly Cheddar cheese, where they have been considered as important for the flavour (Wong *et al.*, 1973). The accepted mechanism of formation in cheese supposes a hydrolysis of hydroxy fatty acids known to be a normal constituent of milk fat, followed by a lactonisation which is chemically favoured in an acid-aqueous medium. The mild conditions prevailing in cheese are far from those known to lead to lactone formation, namely, when heating milk fat in the presence of water. The study of the formation rate of lactones in Cheddar cheese suggests that lactone formation is more complex than simple hydrolysis (Wong *et al.*, 1975). Another source of lactone, at least in rancid cheese, where lactone levels are higher than in normal cheese, could be an enzymatic reduction in the cheese medium of oxy fatty acids known to exist in milk fat. Unfortunately, the suggested mechanism has not been demonstrated in cheese. It should also be remembered that other mechanisms are known to occur in nature: δ-oxidation of saturated fatty acids in the rumen (Dimick *et al.*, 1969) and free radical, as well as oxidative mechanisms (Vajdi *et al.*, 1979), but to suggest that they exist in cheese would be mere speculation.

PROTEINS AND THEIR DEGRADATION

Proteins play an important rôle in the texture of cheese as they represent the only continuous solid phase of the cheese. Electron microscopy studies have shown that proteins constitute a network in which the fat is entangled. Any modification of the nature or the amount of the protein present in the cheese will modify its texture.

The level of milk protein in itself could have a certain effect: Gruyère cheeses made during September–November, a period during which the protein level of milk is higher, are recognised to have a better texture than those made during the rest of the year. This observation is confirmed by the improvement obtained in the cheese body quality when the protein level is raised through membrane ultrafiltration (concentration factor, 1·2 to 1·7) (Rousseaux *et al.*, 1978). The firmness itself is related to the protein content of the cheese, the increase of which leads to a harder texture (Steffen, 1975). The relationship between the amount of protein in the non-fat matter of the cheese and the firmness has been characterised (De Jong, 1978). By applying multiple linear regression analysis to a sample of eleven cheeses of different types it has been shown (Chen *et al.*, 1979) that the protein level was more significant than water, NaCl, fat content and pH in explaining the observed differences in firmness.

During past years, several techniques have been developed to increase the cheese yield by incorporating whey protein. The elevation of the pasteurisation temperature of milk has been shown to lead to a slight firmness increase in Gouda (Van den Berg, 1979). A relatively low addition (1 to 2 g/litre) of heat denatured serum protein, obtained by the Alfa Laval Centriway process, gives a cheese of lower quality which presents a soft and greasy texture (Van den Berg, 1979). The texture of Camembert, made from ultrafiltrated milk, is somewhat different from that of traditional cheese, the former being more granular, at least at the beginning of the ripening period.

As the ripening proceeds, proteolysis modifies the textural and flavour properties of the curd. The mechanism of protein breakdown during ripening is well documented (Desmazeaud & Gripon, 1977). It begins with the action of rennet, which cleaves the Phe_{105}—Met_{106} bond of κ-casein, thus inducing clotting. In addition, rennet cleaves the Phe_{23}–Phe_{24} and Phe_{24}–Val_{25} bonds of α_{s1}-casein during the early stage of ripening, with subsequent appearance of an α_{s1}I fraction. The action of the rennet goes on during the whole ripening period, inducing, mainly, the release of large molecular weight peptides, but no free amino acids are produced by the enzyme. β-casein shows little modification, and serum protein remains unmodified, acting as a bulk (de Koning *et al.*, 1981). The extent of the action of starters, endopeptidases and exopeptidases (mainly aminopeptidases) results in a selective amino acid production. In the Gruyère type, proteolysis leads to a softer and less elastic body (Steffen, 1975). In Meshanger cheese, a type of soft cheese on which the surface flora has not an important rôle, rennet alone seems to be

responsible for the softening, while other proteolytic enzymes have no influence (Noomen, 1977). Increasing the amount of rennet results in a more pronounced hydrolysis of α_{s1}-casein and a more rapid softening. Conversely, in cheese in which rennet has been inactivated, there is neither α_{s1}-casein degradation nor softening of the body (De Jong, 1977). The influence of rennet could also be very important in other types of cheese, such as Gouda and Edam (De Jong, 1977; Noomen, 1977). In some types of soft cheese, the texture modifications are pronounced, the cheese body turning softer and, in some cases, even fluid. In Camembert cheese, this has been attributed to the action of the *P. caseicolum* proteases which migrate right through the curd, bringing casein degradation (Knoop & Peters, 1971; Seeler, 1968).

Even if differences are observed in the texture, as proteolysis proceeds, only a few studies have dealt with the relationship between the intensity of protein degradation and texture modification. With the same nitrogen solubilisation, very different textures are obtained according to the type of cheese. Thus, in Gruyère cheese, soluble N may reach 30%—a figure comparable to that found in Camembert (Lenoir, 1963).

The water content of the product is a determining factor for texture, and also limits the storage aptitude. As observed for Gruyère and Emmental (Mocquot *et al.*, 1947; Mocquot, 1979) it is interesting to note that small variations in the water content will greatly influence the firmness. A modification of the dry matter content has a greater influence on the firmness of cheeses with a high dry matter level than on those with low dry matter level (De Jong, 1978). The water content mostly depends on the cheese-making conditions, but a loss of water occurring by evaporation during ripening can affect the cheese consistency (De Vries, 1979), and even as demonstrated for Emmental, explain the formation of a split-defect (Reiner *et al.*, 1949).

Proteolysis also produces substances which are either important, in themselves, for flavour and aroma, or which act as aroma precursors (Mulder, 1952). Furthermore, as suggested (Biede, 1977; McGugan *et al.*, 1979) another important consequence of proteolysis could be the release of aroma components which were previously bound to the protein.

Many attempts have been made to elucidate the rôle of protein in the development of flavour. A slurry process has been used (Kristoffersen, 1973) assuming that the fermentation in the slurry is similar to that which occurs in the cheese itself. This experiment has led to the conclusion that there exists a relationship between flavour development and β-casein degradation (which has previously been said to be rather limited during cheese ripening), the whole system being under the influence of an equilibrium between the different protein fractions. Recent experiments (Green *et al.*, 1981) have shown that protein hydrolysis decreases when cheese milk is concentrated by ultrafiltration. These results can perhaps be explained by the disruption of the protein equilibrium. Addition of

proteolytic enzymes has been shown to accelerate the development of cheese flavour, but our own experiments on soft-type cheeses, with *micrococcus* protease added to the milk, do not confirm their views.

The recognised importance of non-volatile water extractable fractions (McGugan *et al.*, 1979) has been interpreted as a direct effect of the proteolysis products. As an increase of the free amino acid level has been shown, elsewhere, to make no difference in the intensity of flavour (Law & Sharpe, 1977) it seems that peptides could be responsible for the observed beneficial effect. The study of peptide formation has been directed more towards the formation of the bitter peptides, as, beyond certain limits, bitterness becomes a flavour defect.

Bitterness in cheese results from the presence of low molecular weight peptides which are not further degraded to non-bitter peptides and amino acids by starters when the strains which are used are peptidase deficient. A difference is thus made between bitter and non-bitter strains. This is also the case for *P. roqueforti* (Tchebotarev *et al.*, 1975) and *P. caseicolum*, where certain strains are responsible for the development of bitterness in Camembert cheese. Studies have shown that bitterness is more dependent on the nature of the protein than on the proteolytic enzyme used, which indicates the importance of the amino acid sequence. Among proteins, casein produces more bitterness than others. Among the different caseins, α_{s1}-casein always produces more bitterness than β-casein. This may explain why ewe or goat milk cheeses are usually less bitter than cheeses made from cow's milk, as there is relatively little α_{s1}-casein in ewe's or goat's milk (Pelissier, 1973; Pelissier & Manchon, 1976). Bitter peptides have been isolated from cheese (Hodges *et al.*, 1972) or from model experiments on casein (Pelissier, 1973) and their structure has been elucidated, thus giving a better understanding of the specific action of rennet on α_{s1}-casein, with a preferential attack on the carbonyl of phenylalanine and leucine residues. Bitter peptides obtained from casein follow the well known hydrophobicity rule (Ney, 1971), and, from their structure, it is possible to conclude that bitter peptides contain more phenylalanine and leucine than others.

Many other substances present in cheese can also add to bitterness: amino acids, amines, amides, substituted amides, long chain ketones, some monoglycerides (Ney, 1979) and probably others, too.

Peptides also seem to contribute to other flavours. Large peptides have been found to be important for the brothy flavour in Swiss cheese, whilst it was suggested that the typical sweet flavour results from an interaction of calcium and magnesium with small peptides (Biede, 1977).

The amino acid pattern of cheese is not simple, as the one we observe is in a dynamic state. This pattern results from the enzymatic degradation of peptides by various microorganisms and also from amino acid interconversion, excretion and degradation. However, each type of cheese has its own characteristic pattern. In Camembert, for example, the percentage of free tyrosine and lysine is lower than could be expected from casein hydrolysis, while free alanine, leucine and

phenylalanine are present in higher proportions (Do Ngoc *et al.*, 1971). Amino acid interconversion has been demonstrated by mean of radiotracers (Cecchi *et al.*, 1979) in Taleggio, an Italian cheese, giving rise to various metabolites such as α-ketoglutaric and pyruvic acid, which can participate in interconversion reactions. This discussion, however, is outside the scope of this paper. Far more interesting for the aroma are the products which result from a series of reactions of desamination, transamination and decarboxylation.

Volatile, as well as non-volatile, amines have been identified in all types of cheese, and the ability of various strains to produce them has been demonstrated (Golovnya *et al.*, 1969; Tokita & Hosono, 1968). For most identified amines, a simple decarboxylation of the usual free amino acids can explain their formation. For others, such as secondary or tertiary amines, or even n-butylamine, there is no readily available explanation of their origin (Golovnya *et al.*, 1969).

Comparing the relative amount of some amines with those of the parent amino acids, one readily notices that distortion occurs. Histidine, for example, is usually more abundant in cheese than tyrosine, while tyramine is often more abundant than histamine (Smith, 1981). This may be the result of differences in the rate of decarboxylation, or in the rate of deamination, the reaction by which amines are converted to neutral or acidic compounds, as demonstrated in cheese for tyramine (Raibaud *et al.*, 1959). Amino acids can also undergo deamination and such a reaction, followed by hydroxylation, would explain the formation of hydroxyphenylacetic acid in Camembert (Simonart & Mayaudon, 1956).

Another reaction in which amines could be involved is acetylation. It would explain the formation of *N*-isobutylacetamide regularly identified in Camembert (Dumont & Adda, 1978), even if the mechanism was not demonstrated in cheese, but in wine, where *Saccharomyces cerevisiae* has been shown to have the ability to transform primary amines in anaerobic model fermentation (Schreier *et al.*, 1975). This explanation seems more likely than the one which results from the observation that *N*-isobutylacetamide developed formula is similar to that of the dipeptide Val–Gly after it has been decarboxylated and desaminated. One reason for this is that the sequence Val–Gly is not found in casein. Gly–Val is found instead. So the formation of Val–Gly would involve a reaction of transamination, possible if catalysed by proteolytic enzyme, the occurrence of which has never been demonstrated in cheese.

Oxidative desamination can lead to volatile fatty acids: glycine, alanine and serine lead to acetate, threonine to propionate, valine to isobutyrate, whilst isoleucine leads to isovalerate (Nakoe & Elliot, 1965). Amino acids will also serve as substrates for the formation of aldehydes, either by an enzymatically catalysed transamination which gives an intermediary imide, followed by decarboxylation or by a pure chemical process known as the Strecker degradation (Keeney & Day, 1957). The first type of reaction explains the formation of branched aldehydes such as isobutanal, 3-methylbutanal, 3-methylthiopropanal and phenylacetaldehyde

from isoleucine, leucine, valine, methionine and phenylalanine by streptococci (McLeod & Morgan, 1958; Morgan, 1976).

Whatever may be the mechanism by which these aldehydes are formed, they seem to be reduced as they are produced, for they are not normally found in cheese, yet the corresponding alcohols are usually present in large amounts. This is particularly the case for phenylethanol, 3-methylthiopropanol and 3-methylbutanol.

Methanethiol is produced from methionine (Grill *et al.*, 1967). It may be produced in cheese by two different mechanisms. It has been shown (Law & Sharpe, 1977) that coryneform bacteria and some Gram negative rods were able to produce methanethiol from methionine by demethiolase activity. The enzyme isolated from one of the rods, although probably not active in the conditions prevailing in cheese, is only active on free methionine, indicating that the rate of CH_3SH production depends on the rate of proteolysis. This specificity might exist for coryneform bacteria, as we have observed that addition of free methionine to the curd greatly enhances the CH_3SH production in surface-ripened cheese, and shortens the ripening time. The enzymatic reaction does not seem to occur in Cheddar, as coryneform bacteria are normally absent, and a reaction of a purely chemical nature, which could explain the formation of methanethiol from methionine or methionine residues, has been postulated. The reaction would be initiated by the action of a reducing agent, which, in the proposed scheme (Manning, 1979) would produce H_2S from cystine/cysteine. H_2S then reacts with methionine to produce methanethiol. This proposed mechanism calls to mind an earlier hypothesis (Kristoffersen, 1973), according to which the state of oxidation of the protein sulphur was supposed to be the determinant for the development of the right flavour in Cheddar cheese, so that an active S—H group would be able to release H_2S later when hydrogen becomes available to reduce the previously oxidised S—S group.

Whatever may be its mechanism of formation, methanethiol appears to be a key compound: it can very easily lead to DMDS and to the corresponding trisulphide. Methanethiol can also be esterified by short chain fatty acids by an unknown mechanism which, however, could be enzymatic, as the ester formation has only been obtained in model systems when micrococci were present (Cuer *et al.*, 1979). The addition of methanethiol with formaldehyde could explain the formation of 2,4-dithiapentane (Sloot & Harkes, 1975). By analogy to what has been demonstrated in the model system (Schreier *et al.*, 1976) other interesting components, such as 2-methylthiophan-3-one, can be related to methionine, but, here again, by an unknown mechanism. The mechanism by which dimethylsulphide, an important component of Swiss cheese (Langler, 1966) and known to be a metabolite of propionibacteria (Keenan & Bills, 1968), is formed, has not been demonstrated.

Besides sulphur amino acids, aromatic amino acids are the source of interesting aroma components such as phenol, cresol, acetophenone, indole, but, even if the existence of enzymatic reactions is to be suspected because their presence is related to that of certain microorganisms, no mechanism has been demonstrated. This also

applies to pyrazines about which there is still great doubt as to how they are formed in cheese (Morgan, 1976).

CONCLUSIONS

In conclusion, we must admit that we still understand very little about the mechanisms which lead to a good quality cheese. The scattered knowledge, which has been obtained up to now, is, most of the time, not sufficient to give the control of flavour and texture. The hypothesis that the flavour development in Cheddar is the result of non enzymatic reactions and is under the influence of redox potential is an interesting one. Further work is necessary to know if it is also valid for other types of cheese. If so, it would help to understand why some contaminating micro-organisms—known to be able, even if present only in small numbers, greatly to lower the redox potential—can sometimes have a positive effect on cheese quality.

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THE STABILITY OF FLAVOUR CONSTITUENTS IN ALCOHOLIC BEVERAGES

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ABSTRACT

Some changes in flavour that take place when beer, wine and cider are stored are reviewed. The nature and mode of formation of the compounds responsible for stale flavours in these beverages is also considered.

INTRODUCTION

The flavours of beer, cider and wine all change on storage and the development of stale flavour notes is usually accelerated by storage at elevated temperatures. The development of instrumental techniques, such as gas chromatography, mass spectrometry and high performance liquid chromatography, has facilitated the study of many of the chemical changes that occur when these beverages are stored. It is convenient to consider separately the stability of flavour constituents present in beer, cider and wine.

STORAGE OF BEER

The raw materials used by the brewing industry have been described by Hough *et al.* (1971) who have also discussed the various stages of the brewing process. Over 600 compounds have been identified in beer and these include amino acids, aldehydes, amines, carbohydrates, esters, ketones, heterocyclic compounds, nucleotides, organic acids, proteins, polyphenols, sulphur compounds and vitamins. These compounds are either derived from the raw materials used in brewing or are metabolic products from yeast fermentation. The flavour of beer soon starts to alter on storage, although not all the constituents or groups of constituents change in concentration. There are a number of comprehensive reviews (Dalgliesh, 1977;

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Hashimoto, 1981; Hudson, 1981) on beer staling and only the more important changes that take place when beer is stored will be discussed. Consideration will also be given to the main pathways for the formation of compounds which are thought to be responsible for these changes in flavour.

During storage, many beers darken in colour (Hashimoto & Koike, 1971) and haze starts to form. There are two kinds of haze. 'Chill-haze' forms when beer is cooled to ~4 °C, but redissolves when the beer regains ambient temperature, whereas 'permanent-haze', once it develops, is always visible and increases in amount as the beer ages. The susceptibility of beer to form both kinds of haze—and thus the haze-life—depends on the methods of production and conditions of storage. Beers may be protected against haze-formation for 6 months or more, but flavour deterioration (staling) is usually noticeable before the development of chill-haze becomes significant.

The major sensory changes that take place when beer is stored have been described by Dalgliesh (1977) and are illustrated in Fig. 1. These changes are usually accelerated by storage at elevated temperatures and by the presence of oxygen in the beer or the headspace above the beer. All beers develop a sweet or toffee-like flavour on storage and there is an apparent decrease in the level of bitter taste (Pangborn *et al.*, 1977). Hence, as beer deteriorates on storage, there is a shift towards a sweeter

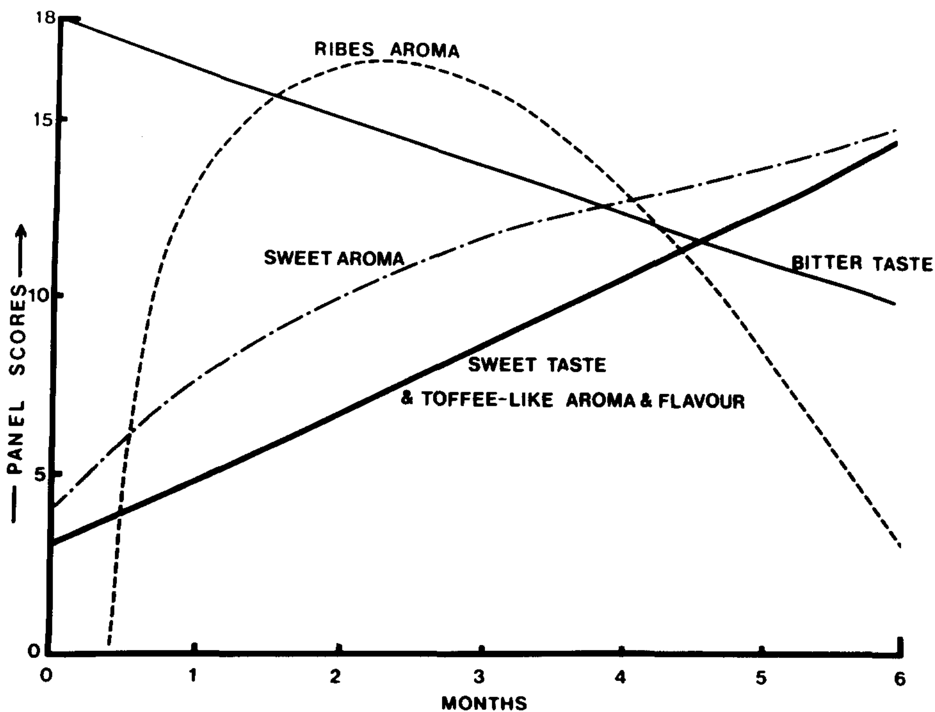

Fig. 1. Beer flavour: sensory changes during ageing.

flavour. Other prominent flavour notes that develop when beer is stored are often described as catty (ribes) and cardboard-like. The catty flavour notes occur in both ales and lager beers and develop as a result of oxidative deterioration. Such flavours, are often transient and may be very intense after 2 to 3 months' storage, but not noticeable in beer that has been kept for more than 6 months.The development of cardboard flavours is usually most noticeable in lager beers and is associated with the formation of certain carbonyl compounds.

Recent work at the Brewing Research Foundation has been concerned with describing the flavours of a range of ales and lagers which were all considered to exhibit stale flavours by the brewing companies who provided the samples. It soon became clear that, although tasters could readily distinguish the stale beer from the fresh control, the overall pattern of stale flavour notes was very different for the various brands of beers. Hence, not all stale beers exhibit all of the flavour notes shown in Fig. 1.

CHEMISTRY OF BEER STALING

Formation of carbonyl compounds

The formation of carbonyl compounds during the storage of beer has been the subject of extensive investigations during the last 10–15 years (Drost *et al.*, 1971; Palamand & Hardwick, 1969; Wang & Siebert, 1974). Wort contains a complex mixture of carbonyl compounds which contribute to its characteristic malty flavour. Thus, the pronounced malty aromas present in distillates obtained by steam distillation of wort at reduced pressure can be removed by the addition of 2,4-dinitrophenylhydrazine reagent. During fermentation the majority of carbonyl constituents present in wort are transformed into other compounds, e.g. alcohols, which are generally much less flavour active than are the corresponding aldehydes and ketones (Peppard & Halsey, 1981). Consequently, fresh beer usually contains very low levels of carbonyl compounds ($\sim 40\ \mu g$/litre) and, even after prolonged storage, the majority of aldehydes and ketones are present in beer at concentrations substantially below their flavour thresholds. Furfural, which is normally present in only trace quantities in fresh beer (Bernstein & Laufer, 1977), can increase markedly in concentration during storage, particularly if packages contain high levels of air in the headspace. Increase in the concentration of furfural is often used as an indicator of oxidised flavour (Brenner & Khan, 1976) although its concentration in stored beers is always below its flavour threshold.

Unsaturated aldehydes and certain ketones contribute to cardboard flavours in some lager beers and compounds such as 2-heptenal, 2-octenal and 2-*trans*-nonenal all have flavour thresholds of less than 1 μg/kg (Hashimoto, 1981). The increase in concentration of 2-*trans*-nonenal has been studied by numerous investigators, and this compound is present at concentrations above its flavour threshold in many stored beers which have a pronounced cardboard flavour (Jamieson & Van

Gheluwe, 1970; Meilgaard *et al.*, 1971; Wang & Siebert, 1974). However, the development of cardboard flavour notes in beer almost certainly results from the synergistic effect of a number of unsaturated carbonyl compounds and is not solely due to the presence of 2-*trans*-nonenal.

The mode of formation of carbonyl compounds in beer has interested brewing chemists in recent years. Four main pathways of formation have been distinguished. These are Strecker degradation of amino acids, oxidation of higher alcohols, autoxidation of fatty acids and the oxidative degradation of *iso*-α-acids (Hashimoto, 1981). The Strecker degradation of α-amino acids results in the formation of an aldehyde containing one less carbon atom than the amino acid (Chang *et al.*, 1970). Dicarbonyl compounds are formed as intermediates by the reaction of sugars and amino compounds, and these intermediates then react with α-amino acids to form aldehydes, e.g. *iso*-valeraldehyde is formed from leucine by this reaction. However, the amounts of simple aldehydes, e.g. formaldehyde, acetaldehyde, *iso*-butyraldehyde, etc., produced by such reactions are far too small for these compounds to contribute to beer staling.

Beer contains a spectrum of higher alcohols which are either metabolic products of fermentation or arise by chemical reduction of carbonyl compounds present in wort. Some of these higher alcohols are gradually transformed into aldehydes and ketones during the storage of beer. Molecular oxygen present in beer does not seem to oxidise higher alcohols, except in the presence of melanoidins formed from reducing sugars and amino compounds (Hashimoto, 1972), Polyphenols such as catechin and quercetin may also catalyse the oxidation of higher alcohols (Dadic *et al.*, 1974). However, the aldehydes and ketones formed by such reactions are not very flavour active and would not be responsible for cardboard flavour in beer.

Unsaturated carbonyl compounds are known to be formed in many foods (milk, butter, vegetable oils, etc.) by the autoxidation of lipids (Hashimoto, 1981). Worts and beers contain a number of unsaturated fatty acids which are mainly derived from the cereals used in brewing. Model systems have been used to demonstrate that a mixture of aldehydes, including 2-*trans*-nonenal, can be formed by the oxidation of the wort and beer constituent linoleic acid (Hashimoto & Kuroiwa, 1975). The concentrations of a number of unsaturated fatty acids in beer (MacPherson & Buckee, 1974) are well in excess of those required to form the corresponding unsaturated aldehydes in amounts in excess of their flavour thresholds. However, attempts to alter the flavour life of beer by selecting raw materials which contain differing levels of lipids have not been successful (Meilgaard *et al.*, 1971; Hashimoto, 1974). Processes for reducing levels of lipids in malt or cereal adjuncts are unlikely to be practical on the commercial scale.

Volatile compounds are known to be formed from hop resins and their derivatives, either when hops are stored or during wort boiling (De Mets & Verzele, 1968; Regan & Elvidge, 1969). It has now been shown that carbonyl compounds

such as 2-methyl-propanal can be formed in a model beer system by the oxidative degradation of *iso*-α-acids (Hashimoto & Eshima, 1979). Such compounds are formed by the oxidative cleavage of the various acyl and alkenyl side chains present in *iso*-humulone and its homologues. However, carbonyl compounds formed by this route are unlikely to be present in beer in sufficient concentration to contribute to stale flavours.

Clearly, most of the pathways proposed for the formation of carbonyl compounds as beer ages do not account for the levels of flavour active compounds required for the development of cardboard aroma. Hashimoto & Kuroiwa (1975) have shown that saturated aldehydes can undergo condensations, in a model beer system containing proline, to form a mixture of 2-alkenals including 2-*trans*-nonenal. Such a route might account for levels of unsaturated aldehydes if Aldol condensations occur in beer during storage.

Sunstruck flavour

When beer is exposed to sunlight a pronounced sulphury flavour can develop. This unpleasant flavour is usually termed 'sunstruck flavour' (Kuroiwa & Hashimoto, 1981) and is often a serious problem for beers packaged in clear glass bottles. However, such flavouring can also develop in the short time required to pour beer from a can into a glass, providing the transfer is carried out in the presence of sunlight.

Kuroiwa & Hashimoto (1961) showed that sunstruck flavour was reduced in intensity by the addition of reagents such as silver nitrate, 2,4-dinitrofluorobenzene and *p*-chloromercuribenzoate which indicated that the flavour was due to a sulphur-containing compound(s) which had a sulphydryl group (Kuroiwa & Hashimoto, 1961). It was also demonstrated that the bitter *iso*-α-acids were involved in the formation of sunstruck flavour and, as expected, the off-flavour did not develop in unhopped beers. The compound responsible for sunstruck flavour has been identified as 3-methyl-2-butene-1-thiol (Kuroiwa & Hashimoto, 1961; Gunst & Verzele, 1978) and is formed by the photodegradation of the *iso*-α-acids present in beer (see Fig. 2). The thiol donor was originally thought to be hydrogen sulphide but is more likely to be a sulphur-containing amino acid.

The development of sunstruck flavour can be prevented by using reduced *iso*-α-acids to bitter beer (Todd *et al.*, 1972). If *iso*-α-acids are treated with sodium borohydride, the carbonyl group in the *iso*-hexenoyl side chain is converted into a secondary alcohol and the oxidative cleavage shown in Fig. 2 cannot occur. Reduced *iso*-α-acids are available from commercial sources and are used to bitter beer by certain brewing companies in America. Thus, sunstruck flavour, unlike cardboard flavour, is due to the presence of a single compound in beer and its identification and prevention is one of the notable successes achieved by brewing chemists.

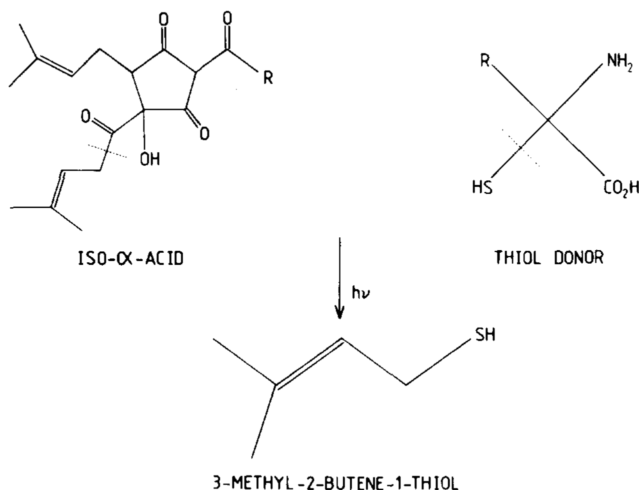

Fig. 2. Formation of 'sunstruck flavour' in beer.

Catty flavours

Catty flavour notes are usually noticeable in beer before the development of cardboard flavours (Dalgliesh, 1977) and hence can be regarded as evidence of oxidative damage.

The compounds responsible for the development of catty off-flavours when beer is stored have not been identified. However, in an unusual case, a catty flavour in some South African beers was shown to result from 4-methyl-4-mercaptopentan-2-one which was formed from the reaction of mesityl oxide with hydrogen sulphide (Cosser *et al.*, 1980). The mesityl oxide was traced to an impurity in a paint used in the maltings and the hydrogen sulphide was formed as a normal metabolic product of fermentation.

Certain varieties of hops, e.g. Bullion and Yakima Cluster (Cosser *et al.*, 1980) have a catty aroma, but this off-flavour does not usually persist into the finished beer because the majority of hop volatiles are lost with the steam when wort is boiled (Sharpe & Laws, 1981). Consequently, beers of sound flavour can be produced from Bullion and Cluster hops provided they are added to wort at the start of boiling. However, it is traditional practice when brewing lager beers to add a portion of hops towards the end of wort boiling, and if Bullion and Cluster hops are used in this way any off-flavours present in the hops may persist into the beer.

Compounds of general structure $R_1R_2C(SH)CH_2(C{=}O)R_3$ often have a pronounced catty flavour and it seems likely that compounds having this structure may be formed when beer is stored. 4-Methyl-4-mercaptopentan-2-one is very flavour active and has a flavour threshold of 1 mg per 100 kg of product (Cosser *et al.*, 1980). There are clearly considerable analytical difficulties in detecting sulphur

compounds at such low concentrations. Oxygen undoubtedly plays the most important rôle in the formation of catty taints and development of this off-flavour can be reduced by ensuring low levels of oxygen in packaged beer. However, it is unlikely that all the problems associated with catty taints in beer will be solved until after the compounds responsible have been chemically characterised.

STORAGE OF WINE

Wine, like beer, has a shelf-life which is affected by time and temperature during storage. However, the objective of the winemaker, i.e. to get the product to the consumer whilst it is in peak condition, is even more difficult to achieve than the similar objective facing the brewer. Beer is generally in prime condition on leaving the brewery to go into trade. On the other hand, Fig. 3 shows that many wines are considerably improved by moderate storage after packaging (Peterson, 1974).

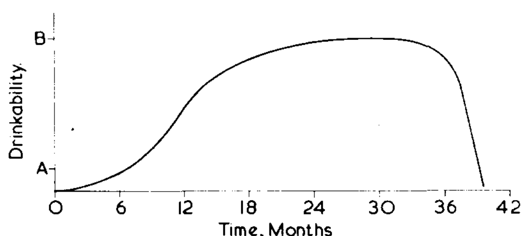

Fig. 3. Drinkability as a function of time after bottling for a white wine (from Peterson (1974)).

Thus, storage conditions, which can produce a fine wine from a less distinguished one, may subsequently yield a mediocre wine on further storage. Hence both beneficial and deleterious chemical changes take place when wine is stored. As has already been discussed in relation to beer, many of these changes, and particularly those considered to be deleterious, involve oxidation. Consequently, except in the manufacture of special products such as sherry and madeira, care is taken to avoid aeration during the production of wines. Oxidation defects in wine will generally be manifested in an alteration in colour (red wines becoming brown, white wines also becoming progressively darker), together with an increase in cooked and rancid flavour notes (Thoukis, 1974). Sulphur dioxide is used extensively in the wine industry to prevent browning and appears to be the most practical inhibitor of oxidation (Ivanov, 1967; White & Ough, 1973).

A large number of flavour compounds, both volatile and non-volatile, contribute to the aroma and taste of wine (Schreier, 1979; Singleton & Noble, 1976). The majority of these are derived from the grape itself, are products of fermentation or originate from oak wood casks when these are used in the first stage of the wine

maturation process (Schreier, 1979; Singleton, 1974; Singleton & Noble, 1976). However, the appearance of new flavour compounds, and alteration in the relative levels of those already present, mark the onset and progress of the maturation and ageing processes.

Schreier (1979) has listed well over one hundred esters which have been identified in various wines, and numerous workers have investigated the fate of some of these compounds during natural and forced ageing processes. From experiments involving storage of model solutions and wines, it appears that esters may be hydrolysed, may be formed through chemical esterification or may remain at equilibrium concentrations, depending upon amounts present immediately after fermentation (Ramsey & Ough, 1980). The formation of ethyl and acid ethyl esters of lactic, succinic, malic and tartaric acids is one of the principal chemical changes occurring during the ageing of wine, although the periods required to reach ester equilibrium may differ between acids as well as between wines and, of course, are dependent on the temperature of storage (Shinohara *et al.*, 1979). The concentrations of certain esters in wine have been used as indices of ageing (Amerine & Joslyn, 1970; Watanabe & Shimazu, 1978); for example, the concentrations of ethyl acetate and to a lesser extent, the ethyl esters of hexanoic, octanoic and decanoic acids, increase during ageing whilst esters of higher alcohols, such as *iso*-amyl, hexyl and 2-phenylethyl alcohols, decrease in concentration (Marais & Pool, 1980). Sherry generally contains about twice as much ethanol (*ca.* 17–20 % v/v) as does table wine, thus giving rise to faster rates of formation of ethyl esters; the characteristic flavour of sherry is supposed to be due in part to the high levels of these compounds (Shinohara *et al.*, 1979).

Approximately fifty aldehydes and ketones have been reported to be present in grapes, although the majority of these appear to be chemically reduced by yeast during fermentation of the must (Schreier, 1979). During storage of wine, the total level of aldehydes is found to increase, especially at higher temperatures (Marais & Pool, 1980; Sachde *et al.*, 1980). Thus, various authors have reported increases in levels of furfural, acetaldehyde, and different higher aldehydes from *iso*-C_4 up to C_7 (Bayer, 1966; Schreier, 1979; Simpson, 1979). It is very likely that the bulk of aldehydes in wine do not actually exist in the free form, but, instead, may be chemically bound to sulphur dioxide or to ethyl and higher alcohols as acetals (Borea *et al.*, 1980). Various investigators have recorded the presence in wine of acetals, such as 1,1-diethoxyethane (Fig. 4; (I)) and 2,4,5-trimethyl-1,3-dioxolane (II) (Brander *et al.*, 1980), and have noted that their levels increased during storage (Schreier, 1976, 1979; Schreier *et al.*, 1976). In the manufacture of sherry, which involves thermal and/or oxidative treatment of white wine containing *ca.* 17 % ethanol, aldehydes such as acetaldehyde and 5-hydroxymethylfurfural (III) are formed in relatively large amounts (Amerine, 1979). Consequently, acetals have also been identified in sherry (Galleto *et al.*, 1966; Müller *et al.*, 1978).

One of the principal components responsible for the character of lager beer,

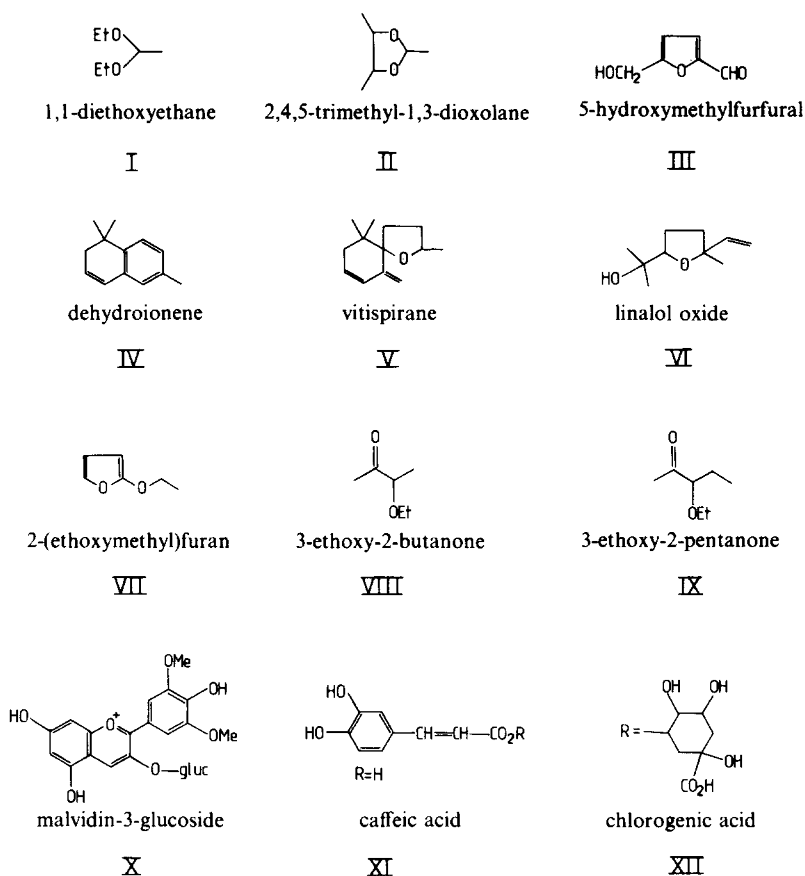

Fig. 4. Some flavour constituents of wine.

dimethyl sulphide, has been identified in wine and found to increase in concentration during storage, and evidence has been presented for its contribution to the bouquet of aged wine (Du Plessis & Loubser, 1974; Loubser & Du Plessis, 1976; Marais & Pool, 1980). Two further compounds which have been shown to be formed in ageing wine are dehydroionene (IV) and vitispirane (V) (Simpson, 1978, 1979; Bertuccioli & Viani, 1976). Both compounds were reported to be generally absent in new wines, but were formed in relatively large amounts in both naturally matured and force-aged wines. However, only dehydroionene appears to be formed in amounts sufficient to contribute to the bouquet of aged wine. Similarly, ethyl furoate and the oxides of both linalol (VI) and nerol have also been found at higher levels in older wines (Simpson, 1979). The detection of 2-(ethoxymethyl)furan (VII) in aged wine indicates that ageing processes involving ethanol can occur not only by

esterification of carboxylic acids but also by esterification of alcohols such as 2-furanmethanol (Bertuccioli & Viani, 1976). Furthermore, Schreier (1980) has recently demonstrated the occurrence of 3-ethoxy-2-butanone (VIII) and 3-ethoxy-2-pentanone (IX) in wine. It was suggested that these compounds are formed by acid-catalysed etherification of the appropriate α-hydroxyketones, both of which are known fermentation products.

All constituents of wine flavour mentioned so far are, to a greater or lesser extent, volatile. However, the group of substances which frequently has most impact on wine flavour is the non-volatile phenolic constituents (Singleton & Noble, 1976). These comprise two main groups, namely: (i) cinnamic and benzoic acid derivatives, which make up *ca.* 200 mg/litre of many white and red wines and (ii) flavonoids, which can vary from *ca.* 50 mg/litre in a typical white wine to *ca.* 1200 mg/litre in a typical red wine. The non-volatile phenolic constituents, particularly the flavonoids, are responsible for the majority of the bitter and astringent tastes. Furthermore, the colour of wine can also be attributed to the presence of flavonoids, red wine colour being mainly due to anthocyanins, such as malvidin-3-glucoside (X) and/or related polymeric substances (Ribereau-Gayon, 1974; Singleton & Noble, 1976).

In many wines, particularly red ones, phenolic compounds are the main pool of substances capable of autoxidation under usual wine-ageing conditions. Thus, the processing and ageing of wine can produce changes in the flavour of the product which result directly from modification of the structure of phenols, or indirectly through associated chemical reactions (Singleton & Noble, 1976; Wildenradt & Singleton, 1974).

The flavour effects of the hydroxybenzoates and hydroxycinnamates of wine are evidently rather mild, and most of the individual characterised compounds are present in wine at levels less than their flavour threshold (Singleton & Noble, 1976). Of this group of wine constituents, hydroxycinnamic acids, such as caffeic acid (XI) and its esters, e.g. caffeoyl tartaric acid and chlorogenic acid (XII), are most notable. However, the concentrations of caffeoyl and *p*-coumaroyl tartaric acids change only slightly during storage, indicating that such compounds are not directly responsible for the development of aged flavours (Nagel & Wulf, 1979). On the other hand, evidence has been presented showing that the oxidation of ethanol to acetaldehyde by dissolved oxygen occurs at an appreciable rate only in the presence of certain polyphenolic substances, such as caffeic acid and the flavan-3-ol, (+)-catechin (Wildenradt & Singleton, 1974).

In light white wines, most of the flavonoid content will usually be accounted for by a group of flavan-3-ols, the most prominent members of which are generally (+)-catechin and (−)-epicatechin. This is not true, however, for red wines and Table 1 lists the levels of flavonoid compounds present in a typical young red table wine.

It is well known that young red wines are often bitter, astringent and harsh, and that ageing is required before the product is considered palatable. The structural modifications which produce these sensory changes on ageing are exceedingly

complex and certainly have not yet been fully elucidated. However, it is clear that polymerisation is involved. Thus, the presence of anthocyanins is difficult to demonstrate in old wines (Ribereau-Gayon, 1974), and catechins, too, show a marked, though somewhat less dramatic, decrease in level during storage (Nagel & Wulf, 1979). Conversely, the molecular weight of polymeric tannin in very young wine is apparently of flavonoid dimer or trimer size, increasing to about 8–14 flavonoid units after a few years of ageing and eventually decreasing again in very old wine, presumably due to precipitation from solution (Ribereau-Gayon, 1974).

TABLE 1
FLAVONOID CONTENT OF A TYPICAL YOUNG RED TABLE WINE

Flavonoid	*Content (mg/litre, measured as gallic acid equivalent)*
Anthocyanins	120
Flavonols	50
Flavanones	5
Catechins	250
Anthocyanogenic tannins	750

(Source: Singleton & Noble (1976)).

Actually, tannins of molecular weights as high as 50,000 have been claimed to be present in some old wines (Somers, 1966). Several schemes have been put forward to account for these polymerisation reactions. The formation of dimeric and oligomeric procyanidins from catechins is now well known (Lea *et al.*, 1979). In addition, anthocyanins have been shown to undergo condensation reactions with both catechins and procyanidins, although the simultaneous involvement of various other compounds has been suggested (Jurd, 1967, 1969; Timberlake & Bridle, 1974; Haslam, 1980).

Several methods have been used to reduce the levels of polyphenols in wine in order to improve the flavour of the product and its keeping quality. These include ultrafiltration (Amano *et al.*, 1980), and the treatment of wine with synthetic poly(vinylpyrrolidone) or polyamide resins (Thoukis, 1974; Vojnovic *et al.*, 1977).

Whilst much effort has been made to elucidate the polymerisation reactions undergone by flavonoids during wine ageing, there is some evidence to suggest that certain low molecular weight phenols can be responsible for the development of aged flavours in wine. Thus, a substituted benzopyran was tentatively identified as one of the two major components in an extract prepared from wines which had been stored for over 2 years (Wulf & Nagel, 1980). Interestingly, the other component, which was not identified, was reported to have an aroma exactly like that of a sherry or an oxidised wine.

STORAGE OF CIDER

Compared with both beer and wine, cider is consumed to a much smaller extent over a more limited geographical area. Even so, quite a lot of information has been obtained regarding the flavour constituents of cider, particularly by workers at the Long Ashton Research Station. Thus, Williams *et al.* (1974, 1975, 1978) have characterised many of the volatile flavour constituents of cider, whilst Lea *et al.* (1974, 1978*a*,*b*, 1980) have concentrated on the nonvolatile flavour compounds.

Flavour taints in cider have received some attention, but, generally, researchers seem to have concentrated on taints arising from chemical contamination or microbiological defects (Tucknott, 1978). Flavour characteristics described as 'stale' are known for cider (Williams, 1975), but as yet very little work appears to have been directed towards chemically characterising the substances responsible.

Many of the volatile flavour constituents of cider are also present in beer and wine. Thus, whilst changes in the levels of these compounds during cider ageing seem not to have been specifically studied, it is quite possible that they would be found to resemble those occurring during the ageing of beer and/or wine. Aldehydes, ketones, acetals and certain esters probably contribute to stale flavour notes in cider.

Regarding the non-volatile flavour constituents of cider, the most important groups are sugars (often added post-fermentation), acids and phenolics (Williams, 1974). The first two groups provide cider with sweetness and acidity, respectively although, as with the volatile flavour components of cider, little is known about how they are affected by ageing. It is assumed that acids, for example, will be involved in esterification/hydrolysis equilibria, as is the case with beers and wines. The third group, the phenolics, provide cider with bitterness and astringency. Oxidation and condensation of phenolics throughout the process of cider making, and presumably storage, too, can affect both the colour and taste of the final product.

The phenolic compounds in cider can be classified into four groups, namely: (i) phenolic acids, (ii) phloretin derivatives, (iii) catechins and (iv) procyanidins (Lea, 1978*a*). However, it seems that only the procyanidins, which are reported to cover a range of molecular size from dimeric to heptameric, are present at levels sufficient to contribute significant bitterness and astringency to the product.

Taste panel work on procyanidin fractions of cider shows that bitterness is predominantly associated with oligomeric structures, whilst astringency is mostly connected with polymeric structures (Lea, 1978*a*; Lea & Arnold, 1978). It is known that the oxidation of procyanidins leads to an increase in polymeric material. It might therefore seem reasonable to expect cider, on being subjected to oxidative processes during ageing, to undergo a decrease in bitterness with a corresponding increase in astringency. However, ciders subjected to oxidation during the later stages of processing have been found to undergo a decrease in both bitterness and astringency (Lea & Timberlake, 1974; 1978). It has been suggested that the 'native' polymeric

procyanidins decrease in concentration through oxidation and subsequent precipitation, whilst those produced oxidatively increase, but at the same time the latter are less bitter and astringent than the former (Lea & Timberlake, 1978).

Beers, ciders and wines all alter in flavour on storage and the formation of carbonyl compounds contributes to stale flavours that develop in these beverages. The polyphenols of cider and wine are responsible for bitter and astringent tastes, but they are of less importance in beer because they are present at lower levels.

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EDITORIAL

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GORDON BIRCH

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CHANGES IN PHYTATE AND MINERALS DURING GERMINATION AND COOKING OF FENUGREEK SEEDS

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ABSTRACT

The effects of germination, cooking and roasting on the phytic acid content, total phosphorus, water soluble inorganic phosphorus and mineral content of fenugreek seeds have been estimated. After 96 h germination, the dry weight of fenugreek seeds decreased while total ash content increased. Phytase and phosphatase activity of the ungerminated and germinated seeds have been assayed. It is observed that during germination the phytic acid values diminish and the water soluble inorganic phosphorus values increase. Phytase activity which is absent in the ungerminated seeds originates after germination and the phosphatase activity is increased in the germinated seeds. Heat treatment practised in cooking and roasting fenugreek seeds has less effect on phytate elimination than does germination. Changes in calcium, magnesium, iron, manganese, copper and zinc are found to be dependent on the loss of dry weight which occurs during processing of fenugreek seeds.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) is a leguminous herb which is consumed in various ways. It is commonly added to corn flour for making bread in Egypt. The seeds also are roasted, ground and cooked with molasses and eaten as a sweet, or eaten raw after sprouting (known to Egyptians as 'hulba'). In many Arabic countries the seeds are used to brew a hot beverage after the addition of sugar or molasses (El Madfa & Kuhl, 1976). In India, the leafy part of the plant is consumed as a vegetable

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and the ground seeds are used in spice mixtures, predominantly curry powder (Rosengarten, 1969). In the United States, it is used in the manufacture of chutneys and various spice blends but its most important culinary use is as a source of Fenugreek extract, a principal flavouring ingredient of imitation maple syrup. It is used in recipes like vegetable bean soup and Fenugreek beef stew (Shankaracharya & Natarajan, 1972).

Fenugreek seeds are found to contain on average about 24·7 % protein (El Madfa & Kuhl, 1976), 37·5 % total sugars (Shankaracharya *et al.*, 1973) and high levels of minerals, e.g. 270 mg total phosphorus, 160 mg calcium, 530 mg potassium, 14·1 mg iron and 19 mg sodium in 100 g dry seeds (Winton & Winton, 1939). Although it finds extensive use in food, nothing is known about the phytate content of fenugreek seeds.

Phosphorus containing compounds found in leguminous seeds can be classified into phytates, phospholipids, nucleic acid, phosphoproteins and inorganic phosphorus (Reedy *et al.*, 1978).

Phytin is a water insoluble mixed salt of calcium and magnesium with myo-inositol hexaphosphoric acid (phytic acid). It is the principal storage form of phosphorus in many kinds of leguminous seeds. Mature beans (*Phaseolus vulgaris*) contain relatively large amounts of phytate phosphorus (Makower, 1969) and 99·6 % of the phytate phosphorus is present in water soluble form (Lolas & Markakis, 1975).

The phytate ion complexes with divalent and trivalent metallic ions (Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}) and in many cases it forms insoluble compounds and may lead to decreased absorption of these minerals in the alimentary tract (Sharpe *et al.*, 1950, Roberts and Yudkin, 1960; Oberleas *et al.*, 1966). Phytates also interact with proteins to form insoluble complexes (Smith & Rackis, 1957).

The aim of the present study was to determine the changes in phytate and some mineral constituents in fenugreek seeds during germination and cooking.

MATERIALS AND METHODS

Materials

Type of seeds: Geiza 2 variety of fenugreek seeds were purchased from a local Egyptian market at Alexandria.

Germination procedure: Cleaned seeds were surface sterilised by soaking in alcohol for 1 min, washed with distilled water, then soaked in five volumes of distilled water for 6 h at room temperature (30–35 °C). The water was then removed and germination started on a screen in the dark for 96 h. The seeds were washed twice every 12 h with distilled water. The germinated seeds were dried at 40 °C in a fan assisted oven for 12 h. Germination for enzyme activity was made by the same method using 0·5 g of fenugreek seeds placed in a Gooch crucible.

Cooking of seeds: Fenugreek seeds were placed in a beaker containing distilled

water (1:4 seeds to water). The beaker was covered with aluminium foil and boiled until the seeds became edibly soft (20 min). The cooking water was decanted for analysis and the seeds were dried at 40 °C in a fan assisted oven for 12 h.

Roasting of seeds: Roasting was carried out at 185 °C for 15 min in a manual coffee roaster.

Preparation of samples for analysis: Different samples were ground in a laboratory Wiley mill to pass through a 40 mesh screen. The ground samples were stored at 5 °C until analysed. The water used for cooking was made up to a constant volume and aliquots were taken for chemical determinations.

Methods

Samples were analysed for dry weight, moisture and total ash contents following the standard methods of the AOAC (1975).

Determination of minerals: Iron, calcium and magnesium were determined in the ashed samples according to the AOAC methods (1975). Iron was analysed colorimetrically using α, α-dipyridyl and the absorbance measured at 510 nm. Zinc, copper and manganese were estimated by atomic absorption spectrophotometry using a Shimadzu AAS 630 instrument. The ashed samples were prepared for mineral determinations according to Chen *et al.* (1975). The different forms of phosphorus compounds were extracted and assayed for their phosphorus content using the following methods.

Total phosphorus: Samples (1 g) were digested with 20 ml of a mixture of sulphuric acid and perchloric acid (10:1) (Belavady & Banerjee, 1953). The digests were diluted to 100 ml, a 5 ml aliquot of each neutralised, the volume made up to 25 ml and phosphorus determined in the filtrate.

Water soluble inorganic phosphorus: Water soluble inorganic phosphorus was analysed by the method described by Belavady & Banerjee (1953) after extraction with 5 % trichloro-acetic acid (TCA) and phosphorus was estimated in the filtrate.

The phosphorus content of the different phosphorus compounds was determined using the colorimetric method of Lowery & López (1946). The blue colour was measured absorptiometrically at 770 nm using a spectrocolorimeter (Spekol, Carl-Zeiss Jena, Germany).

Phytate phosphorus: Extraction and precipitation of phytate phosphorus was performed according to the method of Wheeler & Ferrel (1971). The phytate phosphorus estimation was carried out according to the method of Makower (1970). Finally, the iron content of ferric phytate was measured by the AOAC method (1975) using α, α-dipyridyl reagent. The ratio of iron to phosphorus in ferric phytate was assumed to be 4:6 in order to calculate phytate phosphorus content.

Phytase activity: The substrate for phytase activity was prepared as calcium phytate from wheat bran according to the method of Anderson (1915), then converted to sodium phytate and purified by the method of Chang (1967). The phytase activity was assayed according to Peers (1953) with minor modifications.

Samples (0·5 g) of ground seeds were homogenised with prewarmed (32 or 52 °C) 0·2 M acetate buffer (pH 5·61) containing 4 mmol magnesium sulphate. The homogenate was transferred to a 50 ml Erlenmeyer flask followed by the addition of an appropriate volume of sodium phytate solution to bring the total volume to 50 ml and the final substrate concentration to 150 μg (0·16 μmol) per ml. An aliquot (2 ml) of the suspension was removed immediately after the addition of substrate and the remainder was incubated (at 32 or 52 °C); further 2 ml samples were removed after appropriate time intervals. The withdrawn samples were added to 1 ml 10 % (w/v) TCA, filtered and diluted to 10·0 ml. Aliquots (0·5 ml) of the clear filtrates were used for the determination of inorganic phosphorus using the method of Lowery & López (1946). Blanks for enzyme without substrate and substrate without enzyme were deducted under the same conditions as the experiment. Phytase activity is expressed as mg P released per g of seeds under the described conditions.

Phosphatase activity: The phosphatase activity of the fenugreek seeds was assayed by the method of Gibbins & Morris (1963) with disodium phenylphosphate (10 mM) as substrate under the same conditions used for the phytase activity. Two incubation temperatures were used; 32 °C which is the optimum for phosphatase and 52 °C which is used in the assay of phytase activity. Phosphatase activity is expressed as mg P released per g of seeds under the above-mentioned conditions.

RESULTS AND DISCUSSION

Dry weight and ash as affected by germination

Figure 1 shows the changes in dry weight and ash contents of fenugreek seeds during 6 days germination preceded by 6 h soaking. At the end of the 6th day, the dry weight was reduced to about 75 % of its original. The total ash content increased gradually throughout the germination period. These results concur with the investigations of Mayer (1956), Hall & Hodges (1966) and Reedy *et al.* (1978) on legumes other than fenugreek. As stated by Mayer & Poljakoff-Mayber (1963), a net loss of dry weight occurs as a result of oxidative breakdown of stored compounds in the seeds.

Phosphorus compounds as affected by germination

The results shown in Table 1 are expressed on a dry weight basis. A slight increase in total phosphorus was observed after 96 h germination. This apparent increase is mainly due to the decrease in dry weight. The inositol hexaphosphate (phytic acid) content determined as phytate phosphorus in ungerminated seeds amounted to 42·6 % of total phosphorus. After 96 h germination, the value decreased to 10·5 % of total phosphorus content. The loss in phytate phosphorus after germination is accompanied by liberation of inorganic phosphorus which increased from 10·2 % to 69·4 % of the total phosphorus content. At the same time non-phytate phosphorus

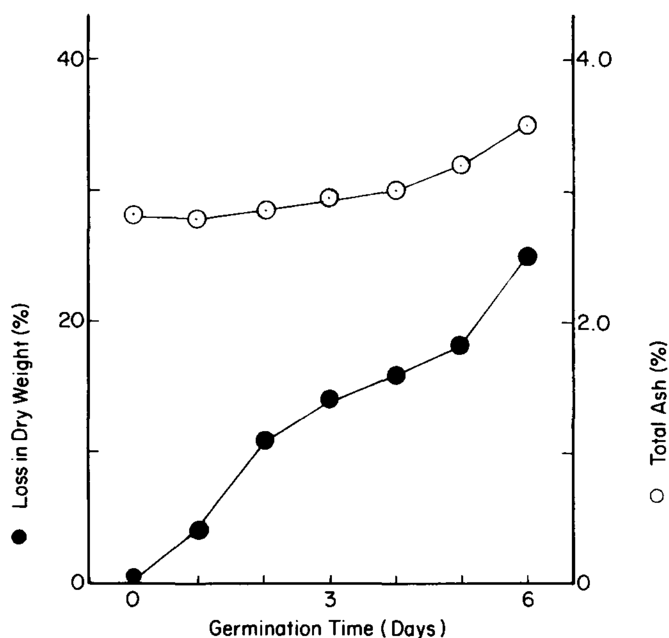

Fig. 1. The changes in dry weight and total ash in germinating fenugreek seeds.

TABLE 1

CHANGES IN PHOSPHORUS COMPOUNDS CAUSED BY GERMINATION, COOKING AND ROASTING (ON DRY WEIGHT BASIS)[a]

Phosphorus containing compounds	*Fenugreek*				
	Ungerminated	*Germinated*	*Cooked*	*Cooking water*	*Roasted*
Total phosphorus (TP)[b]	207	269	255	5·7	252
Phytic acid[b]	414	132	304	0·0	249
Phytate phosphorus[b]	88·4	28·2	64·9	0·0	53·1
Phytate phosphorus as a percentage of TP	42·6	10·5	25·5	0·0	21·1
Inorganic phosphorus[b]	21·2	187	30·3	4·3	73·9
Inorganic phosphorus as a percentage of TP	10·2	69·4	11·9	74·6	29·4
Non-phytate phosphorus[b]	119	240	190	5·7	199
Non-phytate phosphorus as a percentage of TP	57·4	89·5	74·6	100	78·9
Organic phosphorus other than phytate phosphorus[b]	97·8	53·9	160	1·5	125
Organic phosphorus other than phytate as a percentage of TP	47·2	20·1	62·7	25·4	49·6

[a] Mean of three determinations.
[b] Expressed as mg per 100 g.

TABLE 2

PHYTASE AND PHOSPHATASE ACTIVITY[a] OF UNGERMINATED AND GERMINATED FENUGREEK SEEDS

Enzyme	*Assay conditions*		*Enzyme activity*	*Fenugreek*			
	Temperature (°C)	*Incubation period (h)*		*Ungerminated*	*Soaked*[b]	*Germinated*[c]	*Germinated and dried*
Phytase	32	24	[d]	0·0	0·44	4·05	0·46
			[e]	0·0	10·9	100	11·4
	52	3	[d]	0·0	0·14	0·43	0·41
			[e]	0·0	32·6	100	95·0
Phosphatase	32	24	[d]	5·56	6·57	6·64	7·91
			[e]	83·7	98·9	100	119
	52	3	[d]	52·8	56·4	55·9	57·5
			[e]	94·4	101	100	103

[a] Mean of three determinations.
[b] Seeds were soaked for 6 h.
[c] Seeds were germinated for 96 h.
[d] Activity expressed as mg phosphorus released per g dry sample.
[e] Activity as a percentage of that found in germinated seeds.

increased from 57·4 to 89·5 % of the total phosphorus content. The increase in the inorganic phosphorus is much larger than can be accounted for from the decrease in phytate phosphorus values during germination. It seems, therefore, that during germination, phosphorus compounds in fenugreek break down to give rise to water soluble inorganic phosphorus. This observation was previously reported by Belavady & Banerjee (1953).

Although phytate phosphorus represented approximately 42·6 % of the total phosphorus, the combination of nucleic acid phosphorus, lipid phosphorus and protein phosphorus also makes up a sizeable portion of the total phosphorus amounting to 47·2 %. As studied by Hall & Hodges (1966), phytates are also rapidly mobilised in the endosperm and it would appear that there is nearly a direct conversion of these materials into nucleic acid phosphorus, lipid phosphorus and protein phosphorus in the developing roots and shoots. The source of phosphate for this additional synthesis could have been the inorganic phosphorus originating from phytate or perhaps, to some extent, from the breakdown of phospho-proteins. In the present study it appears that after 96 h germination the value of total organic phosphorus other than phytate phosphorus decreased from 47·2 to 20·1 % of total phosphorus. It seems that fenugreek seeds do not possess the capacity for net synthesis of nucleic acid during germination in the dark. As stated by Ingle & Hageman (1965), it is possible that some plant species, such as corn, possess the capacity for net synthesis of nucleic acid during germination and are able to immediately utilise the liberated inorganic phosphorus. On the other hand Hall & Hodges (1966) found that in the case of germinating oats, only in the presence of light would the inorganic phosphorus be used for synthesis of organic phosphate substances.

Phytase and phosphatase activities as affected by germination

The rapid decomposition of phytate after germination of fenugreek seeds was correlated with a steady rise of phytase and phosphatase activities and inorganic phosphorus. Sobolev (1963) reported that the enzymatic hydrolysis of phytate phosphorus during germination of seeds is accomplished by two phosphatases. One of these phosphatases, namely phytase, is responsible for the initial breakdown of phytate up to the stage of formation of inositol monophosphates, and the other enzyme accomplished a complete dephosphorylation of inositol phosphates into inositol and inorganic phosphates. As shown in Table 2, phytase activity seems to be absent from ungerminated fenugreek, whereas soaked and germinated seeds show phytase activity. On the other hand the results indicate that phosphatase activity is present in both germinated and ungerminated fenugreek. Soaked and germinated fenugreek, however, shows greater phosphatase activity.

Increased activity of phytase during the course of germination was inversely correlated with the disappearance of its substrate, phytate. Such a correlation was demonstrated in seven species of pulses, namely *Phaseolus mungo*, *Lens esculents*,

Cicer arietinum, Pisum sativa, Vigna sinensis, Cajanus indicum, Phaseolus radiatus (Belavady & Banerjee, 1953), in lettuce seeds (Mayer, 1956) and in *Pisum sativa* (Chen & Pan, 1977).

Changes in phosphorus compounds during cooking and roasting

Cooking of fenugreek seeds results in a loss of phytate phosphorus (Table 1). This loss is not attributed to the solubility of fenugreek phytate, as the analysis of the cooking water shows no phytate. Release of inorganic phosphorus was found to be small compared with the phytate loss, while the amount of inorganic phosphorus leached into the cooking water was small, amounting to 4·3 mg per 100 ml, in comparison with the original inorganic phosphorus in the raw fenugreek seeds. This may be attributed to the cooking method used which did not give the chance for leaching inorganic phosphorus. The loss of phytate and phytate phosphorus in roasted fenugreek (39 %) was not much greater than in cooked seeds (31 %), although a high temperature and relatively long time are used. It is clear that heat treatment has less effect on the elimination of phytate than has germination. The latter removes 68 % of the phytate content after 96 h of germination.

Changes in mineral composition caused by processing

Mineral compositions of ungerminated, germinated, cooked and roasted fenugreek seeds are shown in Table 3. Total phosphorus, calcium, magnesium, manganese, copper and zinc showed apparent increases after germination, while iron content decreased by 9 %. Taking into consideration the 25 % reduction in dry weight after 6 days of germination, the results clearly showed that all the determined minerals decreased in the following decreasing order as a result of leaching: iron, zinc, magnesium, manganese, copper, calcium and total phosphorus. Kumar *et al.* (1978) found that calcium, iron and zinc decreased during germination of black gram while magnesium increased after the ninth day of germination.

The values of all minerals except iron had increased after cooking of fenugreek

TABLE 3
EFFECT OF GERMINATION, COOKING AND ROASTING ON THE MINERAL CONTENTS OF FENUGREEK SEEDS[a]

Fenugreek	*Moisture* (%)	*Ash* (%)	*Mineral contents (mg per 100 g dry weight)*						
			TP	*Ca*	*Mg*	*Fe*	*Cu*	*Mn*	*Zn*
Ungerminated	10·9	3·36	207	173	163	10·9	0·7	1·1	2·6
Germinated	80·0	3·50	269	224	204	9·9	0·9	1·4	3·1
Cooked[b]	75·8	2·8	255	218	201	9·7	0·8	1·2	2·98
Cooking water	98·3	0·8	8·90[c]	18·0[c]	19·1[c]	1·1[c]	0·0	0·0	0·0
Roasted[d]	2·9	3·44	252	190	183	12·2	0·8	1·2	2·8

[a] Mean of three determinations.
[b] Loss in dry weight after cooking equals 18·8 %.
[c] Values are expressed as mg per 100 ml.
[d] Loss in dry weight after roasting equals 10·6 %.

seeds as a direct result of the reduction in dry weight amounting to 18·8 %; however, the increase was less than that observed in germinated seeds. Iron content decreased and the loss was found to be equal to the value recovered in drained cooking water.

Roasting of fenugreek seeds resulted in a decrease of moisture content and loss in dry weight amounting to 10·6 % of original dry matter. On the other hand, the observed increase in metal content is attributed to the loss in dry weight during roasting.

In conclusion, this study shows that the phytate, which is an antinutritive factor of fenugreek seeds, is diminished by germination and to a lesser extent by roasting and cooking.

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SOME CHANGES IN COTTONSEED OIL DURING FRYING OF FLAFEL AND EGGPLANT

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ABSTRACT

The changes in the characteristics, composition and technological properties of cottonseed oil during heating for 50 h at 200°C in the presence of air were studied. The effects of the heated oil in frying two types of Egyptian vegetable—flafel and eggplant—were also examined. The results showed that as the heating time of the oil increased: (a) the free fatty acids increased and both iodine value and peroxide number decreased; (b) the triglycerides hydrolysed and (c) the loss of oil weight, the tendency of oil to foam and the time necessary to fry the vegetable increased.

INTRODUCTION

The changes occurring in the nutritional, physiological and physico-chemical properties of frying fats and oils have been studied by Lang *et al.* (1973), Strausse & Billek (1974) and Vidyasagar *et al.* (1974). The free fatty acid (FFA) content, the absorption of light and the tendency of the oil to foam increased, while the iodine value decreased (Freeman, 1965; Schmidt *et al.*, 1969; Pokorny, 1973; Brooker, 1975). Basco *et al.* (1973) showed that the acid value and iodine number of rapeseed oil increased with the increasing frying time and the quantity of potatoes fried. Pokorny (1973) studied the changes in sunflower seed oil during deep fat frying of fish. He found that the peroxide value first decreased and later increased slightly while the thiobarbituric acid value increased proportionally with frying time. Stefanov (1974) showed that oxidation and hydrolysis of refined sunflower seed oil, refined corn oil and lard were accelerated at high temperatures up to 120°C during frying. Kishi *et al.* (1975) determined the acrolein, carbon dioxide, ethylene and other saturated hydrocarbons in heated frying oils by gas–liquid chromatography. They reported that acrolein caused marked inhibition of respiration, reduction of heart beat and a slight rise in blood pressure in rabbits.

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In Egypt, refined cottonseed oil is the major oil used for frying. The process for most Egyptian fried foods is usually carried out at temperatures ranging from 180 to 200 °C in the presence of air. After frying, the heated oil is usually left to cool and then stored in glass containers at room temperature for subsequent use. When its colour becomes dark due to repeated usage, the oil is either discarded or mixed with refined fresh oil. This investigation was initiated to explain the changes that occur during the heating of cottonseed oil and while frying vegetables in it. Flafel (bean paste) and eggplant were the two chosen vegetables for frying in the cottonseed oil since they are the most widely fried products on a commercial scale throughout Egypt.

MATERIALS AND METHODS

Materials

Refined cottonseed oil: The refined cottonseed oil was obtained from the Alexandria Company for Oil and Soap. This oil was heated at 200 °C in a stainless steel frying pan continuously for 10 h daily, then cooled and stored in a brown glass container in a dark place at room temperature. This process was repeated until the heating time of the oil reached 50 h. Samples of this heated oil were taken: (a) before heating; (b) when the oil temperature reached 200 °C and then after 10 h at 200 °C and (c) at 5 h and intervals thereafter until the total time of heating reached 50 h. The samples were kept in closed brown glass bottles and stored at 4 °C until analysis.

Eggplant (Solanum melongena) or Basengani: The mature fruits were obtained from the local market, peeled and cut into equal and similar finger-shaped pieces (2 g in weight), frying taking place immediately thereafter.

Broad bean paste (Flafel): This is a popular food in Egypt made from cracked dehulled broad beans (*Vicia faba*) and some green vegetables—e.g. onion, leek and pepper—and also coriander. The dried mixture and the directions for use of the broad bean paste prepared by the Nimo Company were utilised in this study. Surface frying at 200 °C was utilised to fry the two foods. Frying was carried out exactly as reported above. During the first 10 h the oil and food samples were taken every 2 h. The food samples were cooled and the different analyses were then carried out. The oil samples were stored at 4 °C to await analysis.

Methods

Technological methods: The weight of the oil and the fried food samples, the changes in surface areas and frying times were determined as described by Vidyasagar *et al.* (1974).

Chemical determinations: The moisture and oil contents of the two types of food and the characteristics of the oil before and after frying were determined according to the methods of the AOAC (1975). The methods described by Osman *et al.* (1977)

were used in: (a) fractionating the oil into classes and (b) the separation of the different triglycerides. These analyses were carried out on the oil samples obtained before and after frying.

RESULTS AND DISCUSSION

Oil characteristics

The results of the changes in the cottonseed oil characteristics during frying showed that: (a) the free fatty acid as oleic acid was increased from 0·15 % to 0·95 % while the iodine value decreased from 108·2 % to 62·2 % after 50 hours of frying; (b) the peroxide value increased from 6·4 to 11·2 Meq/kg in the first hour of oil heating, then subsequently decreased to about zero after 50 hours of frying; (c) slight changes occurred in both the specific gravity (from 0·912 to 0·925 g/cm^3) and saponification value from 191·6 to 145·2 with increase in time of oil heating and (d) both types of fried food had no effect on the changes in the oil characteristics during frying. Generally, these results agree with those found by Freeman (1965), Schmidt *et al.* (1969), Basco *et al.* (1973) and Brooker (1975). Pokorny (1973) showed that the peroxide value first decreased and later increased slightly with frying time.

Oil composition

Oil classes: Osman *et al.* (1977) found that the crude cottonseed oil could be separated into eight classes, which appeared on a TLC plate in the following sequence from the front to the baseline: hydrocarbons, triglycerides, unknown class (X_1) free fatty acids, unknown class (X_2), steroids, diglycerides and phospholipids, TLC showed that: (a) the refined cottonseed oil contained triglycerides, diglycerides and hydrocarbons as well as very small amounts of other components found in crude oil except monoglycerides which disappeared completely from the refined oil, (b) heating refined oil for more than 3 h at 200 °C in the presence of air led to a degradation of the oil into several products. These products appeared very close to each other owing to their similar molecular weights. The formation of these products increased with the increase in heating time, (c) less hydrolysis took place in the tri- and diglycerides during heating. Therefore, they could be considered more heat stable than the other oil classes and (d) the type of fried food had no effect on the hydrolysis. Hence, hydrolysis may be attributed mainly to heating.

Oil triglycerides: According to Osman *et al.* (1977) the different groups of triglycerides of cottonseed oil appeared in the following sequence from the front to the baseline of the TLC plate; triglycerides with one, two, three, four, five and six double bonds. TLC showed that these groups were found in fresh refined oil. After the oil was heated some of the previous classes disappeared and this was accompanied by the appearance of several other components. These components

TABLE 1

SOME CHARACTERISTICS OF THE FRIED FOODS AS AFFECTED BY THE REFINED COTTONSEED OIL HEATED AT 200 °C FOR DIFFERENT PERIODS

Food characteristics	*Moisture content*[a] (%)		*Oil absorbed*[b] *on a dry weight basis* (%)		*Weight loss* on a dry weight basis* *Observed loss* (%)		*Actual loss* (%)		*Diminution*[c] *in surface area* (%)		*Frying time* (*min*)	
Fried food	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*
Heating time												
0·0**	37·3	55·8	11·4	18·8	5·81	3·91	9·41	4·25	14·3	38·1	2:2	4
2	36·7	54·1	11·3	18·1	5·87	3·80	9·07	4·42	18·9	35·7	2:25	4
4	36·7	53·3	11·1	17·5	5·70	3·92	8·83	4·51	21·1	34·8	2:03	3:55
6	38·2	53·8	11·4	17·3	5·74	3·81	9·02	4·39	17·7	35·8	2:20	4
8	38·3	53·0	11·8	17·8	5·81	3·89	9·05	4·42	21·8	35·8	2:15	4
10	36·8	53·3	11·6	17·7	5·70	3·89	8·97	4·57	22·3	36·6	2:15	4

[a] The moisture contents of fresh flafel and eggplant were 49·4 and 0·8 %, respectively.
[b] The oil contents of fresh flafel and eggplant were 0·50 and 1 %, respectively, on a wet weight basis.
[c] The surface areas of fresh flafel and eggplant were 8·37 and 6·53 cm^2, respectively.
* Observed loss is the loss not taking into account the amount of oil absorbed. Actual loss is the loss on an oil-free basis.
** When the oil temperature reached 200 °C.

appeared very close to each other owing to their similar molecular weights. Generally, the triglyceride classes of refined cottonseed oil can be classified, according to their heat stability, into three groups: (a) heat labile groups that include the triglyceride classes numbers 3, 5 and 6. These classes disappeared completely after 5 hours of frying; (b) semi-heat labile groups that include the triglyceride classes numbers 2 and 4, and which are hydrolysed completely after 15 hours of frying and (c) a heat stable group represented by the triglyceride, number 1. The hydrolysis of the triglycerides was mainly due to the heating process and was not affected by type of fried food. Stefanov (1974) showed that oxidation and hydrolysis of refined oil was accelerated at high temperatures, reaching 120 °C during frying.

TABLE 2
THE EFFECT OF PROLONGED HEATING AT 200 °C DURING FRYING OF FLAFEL AND EGGPLANT ON THE TECHNOLOGICAL PROPERTIES OF REFINED COTTONSEED OIL

Property	*Percent oil weight loss*		*Frying time (min)*		*Tendency of oil to foam*	
heating time (h)	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*	*Flafel*	*Eggplant*
0·0*	—	—	2	4	No	No
5	—	—	2	4	No	No
10	13	18·8	2	4	No	No
15	—	—	2	4	Slight	Slight
20	22	32·3	2:30	6	Moderate	Moderate
25	—	—	2:30	6	Moderate	Moderate
30	36·3	44·3	2:30	6:30	Pronounced	Pronounced
35	—	—	3	6:30	Pronounced	Pronounced
40	43·1	52·3	3	6:30	Pronounced	Pronounced
45	—	—	3	6:30	Pronounced	Pronounced
50	52·1	65·3	3	6:30	Pronounced	Pronounced

* When the oil temperature reached 200 °C.

Fried foods

Table 1 lists the characteristics of the foods fried at 200 °C for different periods. The data show that: (a) the frying process lowered both the surface area and the moisture content of food, (b) the oil heated for 2, 4, 6, 8 and 10 h is considered suitable for food frying and (c) the eggplant needed more time for frying than flafel.

The results in Table 2 indicate that: (a) the loss of oil weight was increased as the heating time of the oil was increased, (b) the time needed for frying increased abruptly when oil heated for 20 and 30 h was used and (c) a slight tendency of the oil to foam was observable after 15 hours of frying. This tendency increased until it became quite pronounced after 30 hours of frying. Both flafel and eggplant disappeared beneath the foams during frying. They also browned in a shorter period. This change in colour was not concurrent with the end point of frying.

CONCLUSIONS

From the above results and discussion it can be concluded that: (a) after 15 hours of heating refined cottonseed oil in the presence of air at 200 °C, the oil showed loss of some of its frying properties and some foaming started with oil heated for 30 hours and increased with longer periods. The triglyceride classes numbers 2 and 4 (as shown by TLC) were hydrolysed. This may indicate that there is a relationship between the degradation of these types of triglyceride and the frying properties of the oil; (b) the lowering of the iodine values and the increases in free fatty acids during the oil heating paralleled the degradation of the oil and the triglyceride classes numbers 3, 5 and 6. In the case of oil heated up to 15 hours the hydrolysis of triglyceride classes numbers 1, 2 and 4 seems to be responsible for the changes occurring in both iodine values and free fatty acid content; (c) the increase in peroxide value during the first hour of oil heating is, in all probability, due to oxidation. With prolonged heating the oxidised substances (peroxides), decompose to carbonyl and acid components not detected by the peroxide test and hence lower peroxide values would be obtained; (d) refined cottonseed oil heated for not more than 10 hours could be considered suitable for food frying, as its properties indicate (Tables 1 and 2) and (e) the TLC technique can be applied to differentiate between fresh refined oil and heated oil. It is recommended that further work be carried out along these lines.

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DETERMINATION OF BONGKREK ACID AND TOXOFLAVIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC method for the determination of bongkrek acid and toxoflavin extracted from laboratory made tempe bongkrek is described. The method appeared to be reliable and simple; samples containing 0·5–80 μg/ml of bongkrek acid or 1–300 μg/ml of toxoflavin could be analysed.

INTRODUCTION

Bongkrek acid and toxoflavin are toxins which became well-known in connection with cases of food poisoning by the consumption of tempe bongkrek, one of the many fermented foods in Indonesia. It is made by fermentation of partially defatted coconut with *Rhizopus oligosporus*. Such products are wholesome and are regularly made by traditional manufacturers. Due to inadequate hygienic conditions during storage of the raw material and during processing into tempe bongkrek, contamination with *Pseudomonas cocovenenans* may take place. Under conditions of the manufacturing process this bacterium could produce bongkrek acid and toxoflavin in the coconut substrate. These two toxins make the food poisonous and consumption of such food can be fatal (van Veen, 1966).

Bongkrek acid is a colourless toxin of which the chemical structure is shown in Fig. 1 (de Bruyn *et al.*, 1973). It was suggested that bongkrek acid inactivates the translocation of adenine nucleotides into mitochondria (Henderson & Lardy, 1970).

Toxoflavin is a yellow coloured toxin with the chemical structure shown in Fig. 2 (van Damme *et al.*, 1960; Hellendoorn *et al.*, 1961). It was suggested that toxoflavin may act as an electron carrier which enables a bypassing of the cytochrome system. Under aerobic conditions, the electron transfer results in the production of H_2O_2.

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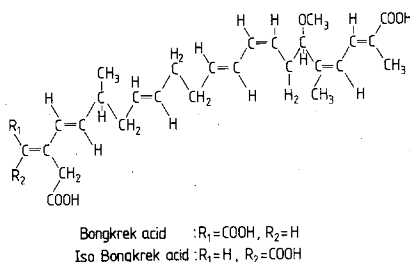

Fig. 1. Structures of bongkrek acid and *iso*-bongkrek acid (de Bruyn *et al.*, 1973; Lauquin *et al.*, 1976).

This might be the cause of the poisonous effect of toxoflavin (Latuasan & Berends, 1961). Bongkrek acid is more dangerous than toxoflavin (van Veen, 1966).

The determination of bongkrek acid and toxoflavin has been carried out by various methods, e.g. bio-assays (toxicity to animals, Mertens & van Veen, 1933; antibiotic properties, Nugteren, 1956), spectrophotometry, paper chromatography (Nugteren, 1956) and thin layer chromatography (Lijmbach, 1969). In the spectrophotometric method the amount of bongkrek acid or toxoflavin is calculated from the absorptions at 267 nm and 258 nm, respectively, using their molar absorptivities at these wavelengths (36,700 and 19,305, respectively; (Lijmbach *et al.*, 1970; van Damme *et al.*, 1960). It is, however, not possible to determine bongkrek acid in the presence of toxoflavin, or vice versa, because of the interference of their absorption spectra. The spectrophotometric method may also be affected by the presence of impurities which absorb at λ_{max} of bongkrek acid or toxoflavin. Laborious extraction procedures are required to obtain separate extracts. This paper describes some possibilities for the determination of these toxins by high pressure liquid chromatography in a simple and less elaborate way.

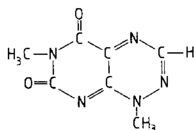

Fig. 2. Structure of toxoflavin (van Damme *et al.*, 1960; Hellendoorn *et al.*, 1961).

MATERIALS AND METHODS

Bongkrek acid extracts were isolated as described by Ko *et al.* (1979) from contaminated tempe bongkrek made in the laboratory from coconuts. The ether extracts were dried in a stream of N_2 and redissolved in methanol. A bongkrek acid standard was prepared from a pool of various extracts. A reference sample of bongkrek acid in ammonia was kindly supplied by Dr Berends, Laboratory of

Biochemistry and Biophysics, Technical University, Delft, The Netherlands. The purity of the laboratory preparation, as well as the reference sample, was determined by thin layer chromatography on Merck Silica 60F254 plates, art. No. 5715 (Lijmbach, 1969). The density at 270 nm of the various spots was measured with a Shimadzu Dual wavelength TLC scanner CS-910, temporarily made available by Pleuger, Inc., The Netherlands. Toxoflavin extracts were isolated as described by van Damme *et al.* (1960) from contaminated tempe bongkrek made in the laboratory from coconuts. The water extracts were dried and redissolved in methanol. A toxoflavin standard was prepared from a pool of various extracts. For HPLC analysis a Spectra Physics Liquid Chromatograph 3500 B with a Schoeffel 770 variable wavelength detector was used. The 250 × 4·6 mm inside diameter columns were made in our laboratory by upward slurry packing. For the determination of bongkrek acid, as well as toxoflavin, a LiChrosorb 10RP18 (Merck) column, in combination with a guard column (100 × 2·1 mm inside diameter), filled with CoPell ODS (37–44 μ, Chrompack Nederland BV) was used. Bongkrek acid was eluted with a methanol–water–acetic acid mixture (80:19:1) using a flow rate of 2 ml/min.

The compound was detected by its ultraviolet absorbance at 267 nm. Toxoflavin was determined in a separate run with a methanol–water–acetic acid mixture (8:91:1) as the eluent using a flow rate of 1·6 ml/min; it was detected by its ultraviolet absorbance at 258 nm. Toxoflavin can also be determined on a Partisil 5 (6 μ, Chrompack Nederland BV) column (100 × 4·6 mm inside diameter) with chloroform–*n*-hexane–methanol (73:25:2) as the eluent and a flow rate of 1·4 ml. Quantification of the chromatograms was achieved by determining the peak areas (peak width at one-half height times height).

RESULTS

The purities of both the laboratory stock of bongkrek acid and the reference sample were verified by TLC. Detection under short ultraviolet light showed a main spot of bongkrek acid with $R_f = 0{\cdot}26$ and some minor spots of impurities from which *iso*-bongkrek acid ($R_f = 0{\cdot}12$) is most evident. Spraying with bromocresolgreen (Lijmbach, 1969) did not reveal additional spots. The densitograms of the TLC plates are shown in Fig. 3; from these curves it can be calculated that the laboratory stock contained 85 % bongkrek acid and the reference sample, 95 %; this last percentage is in accordance with the specifications of Dr Berends.

A typical HPLC chromatogram for a bongkrek acid extract is shown in Fig. 4; next to the bongkrek acid a small peak for another compound was detected. This compound had the same retention time as *iso*-bongkrek acid (see Fig. 1), a product obtained from bongkrek acid by alkaline conversion according to the procedure of Lauquin *et al.* (1976). The presence of *iso*-bongkrek acid was also confirmed by

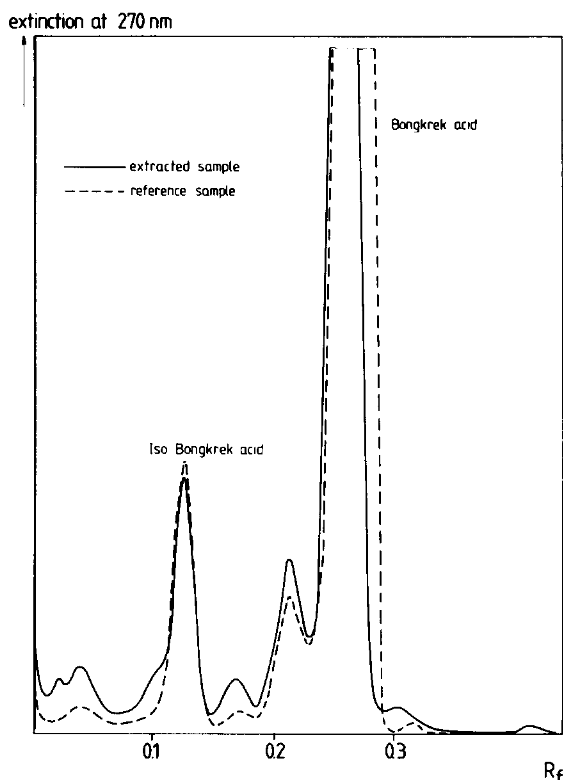

Fig. 3. Densitograms of an extracted sample and a reference sample of bongkrek acid. TLC on Silica 60F254 with chloroform–methanol–acetic acid (94:5:1) as the solvent.

TLC. For the reference sample a similar HPLC chromatogram was found including a small peak for *iso*-bongkrek acid.

The linearity of the HPLC analysis was tested by injecting 10 and 50 μl of standard solutions of bongkrek acid in methanol. The concentration of bongkrek acid in the standard solutions was calculated from their ultraviolet absorbance at 267 nm. With 10 μl injections a linear range was found for 2–160 μg/ml and with 50 μl injections the linear range was between 0·5 and 80 μg/ml. For 50 μl injections of samples containing 41 μg bongkrek acid per millilitre a standard deviation of 2·2 % was calculated.

The effect of increased temperature, sunlight and oxidation by air on the stability of bongkrek acid was tested with this HPLC method. Bongkrek acid solutions in methanol were aerated for 4 h at ambient temperatures (approximately 20 °C), stored at 50 °C for 4 h or placed in sunlight behind a glass window for several days. It was found that bongkrek acid was affected strongly by sunlight but not by aeration or storage at 50 °C. Figure 5 shows the changes in bongkrek and *iso*-bongkrek acid

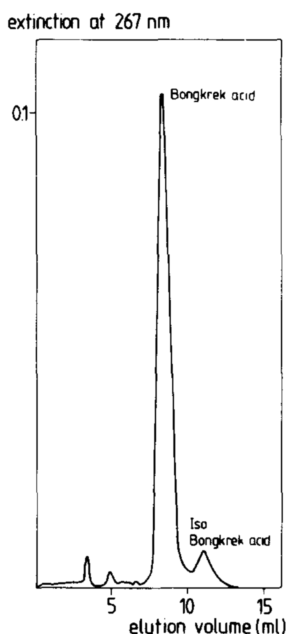

Fig. 4. HPLC chromatogram of a bongkrek acid extract on a LiChrosorb 10RP18 column. The extract was prepared from contaminated tempe bongkrek as described by Ko *et al.* (1979). Mobile phase, methanol–water–acetic acid = 80:19:1; flow, 2 ml/min; injection, 10 μl.

concentrations under the effect of light. The initial concentrations of both compounds were considered as 100 %. This sensitivity of bongkrek acid to light was also reported by Lijmbach (1969).

Our toxoflavin preparation was found to be pure on analysis by HPLC and by thin layer chromatography on Silica 60F254 plates with benzene–acetone–methanol (10:10:1) as the solvent. Figure 6 shows a typical HPLC chromatogram

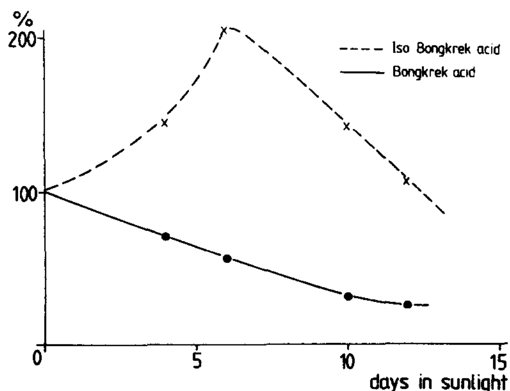

Fig. 5. The effect of sunlight on the composition of a methanolic solution of a bongkrek acid sample.

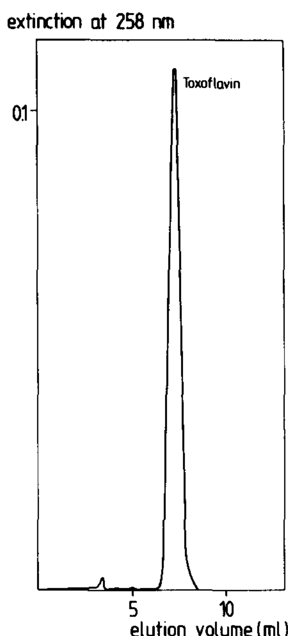

Fig. 6. HPLC chromatogram of a toxoflavin extract on a LiChrosorb 10RP18 column. The extract was prepared from contaminated tempe bongkrek as described by van Damme *et al.* (1960). Mobile phase, methanol–water–acetic acid = 8:91:1; flow, 1·6 ml/min; injection, 10 μl.

for a toxoflavin extract. With 10 μl injections of standard solutions in water a linear range was found between 1 and 300 μg/ml; with 50 μl injections the linear range was between 1 and 60 μg/ml. The standard deviation for 50 μl injections of a sample containing 3·1 μg/ml was 4·4 %. For the Partisil 5 column a linear range was found between 0·5 and 60 μg/ml for 10 μl injections of standard solutions in chloroform.

DISCUSSION

The described HPLC method for the analysis of bongkrek acid and toxoflavin appeared to be simple and reliable. Samples containing 2 to 160 μg bongkrek acid per millilitre can be determined with 10 μl injections, samples containing 0·5 to 80 μg/ml bongkrek acid with 50 μl injections. For toxoflavin these figures are 1–300 μg/ml.

The method can be used to determine bongkrek acid, as well as toxoflavin, in a mixture of the two compounds without separating and purifying each. This is an advantage over the commonly used spectrophotometric method by which bongkrek acid can only be determined in toxoflavin-free extracts, and vice versa, and where

one assumes that the extracts are free of impurities which might absorb at λ_{max} of bongkrek acid or toxoflavin. Figures 7(a) and (b) show the HPLC chromatograms of a chloroform/petroleum ether extract of a coconut medium inoculated with *Pseudomonas cocovenenans* containing both compounds. Ten-microlitre samples in methanol were injected. This procedure for the simultaneous extraction of bongkrek acid and toxoflavin from fermented products is currently under investigation.

From the chromatograms it can be seen that some ultraviolet absorbing impurities in the extract elute from the column; they are, however, well separated from bongkrek acid and toxoflavin. Other ultraviolet absorbing impurities were found to accumulate on the guard column; they can be washed out with methanol. Under the HPLC conditions for the determination of bongkrek acid, toxoflavin passes through the column unretained and under the HPLC conditions for the determination of toxoflavin, bongkrek acid remains on the guard column.

It was found that our laboratory stock preparation, the reference sample and also the chloroform/petroleum ether extract contained small amounts of presumably *iso*-bongkrek acid; this compound was also found to accumulate in the initial phase of the exposure of bongkrek acid to sunlight (Fig. 5).

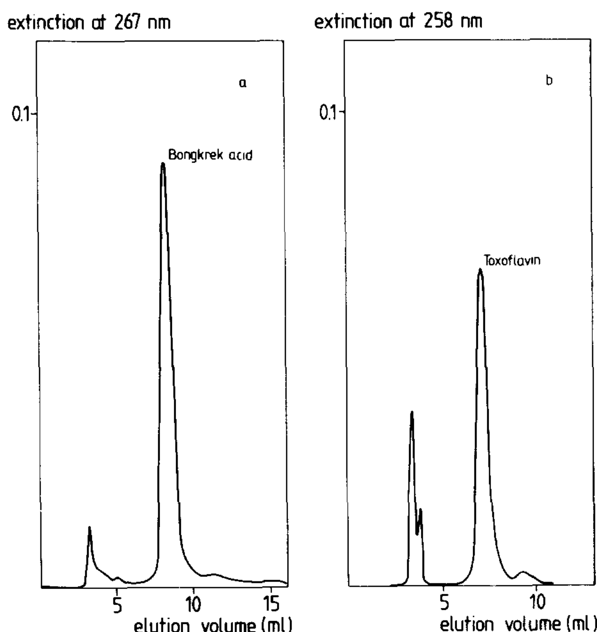

Fig. 7. HPLC chromatograms of bongkrek acid (a) and toxoflavin (b) of a chloroform/petroleum ether extract from a culture of *Pseudomonas cocovenenans* on a coconut medium. (a) HPLC conditions as in Fig. 4. (b) HPLC conditions as in Fig. 6.

An attempt was made to analyse for bongkrek acid and toxoflavin in one run by applying a wide range gradient, i.e. from 10% methanol in water to 80% methanol in water on a SP8000 chromatograph. This system had, however, two serious drawbacks; first, the gradient caused a drift of the baseline at higher methanol concentration and, secondly, the gradient run and the re-equilibration of the system is time consuming. For these reasons we prefer separate analyses for bongkrek acid and toxoflavin.

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AMINO ACID PROFILE OF THE SEED AND OTHER PARTS OF THE WINGED BEAN

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(Received: 11 May, 1982)

ABSTRACT

The chemical composition of the seeds and other parts of the winged bean shows some variation in their nutrient content. The protein content in the seeds is similar to that in soybean while the roots have a protein level higher than that of any known tropical root crop. The amino acid composition of the different parts of the winged bean indicates high amounts of lysine and threonine with total essential amino acids similar—or even superior—to that of soybean seeds. All the parts studied except the roots, the unripe pods and the entire plant contain less total S-amino acids than soybean seeds. In terms of their percentage of FAO values adjusted for digestibility, all the different parts show good quality protein which can be utilised in supplementing cereal-based diets.

INTRODUCTION

In some parts of the developing world, animal protein is scarce while plant proteins are found in sufficient quantities. Animal proteins are an expensive source of dietary protein. Legumes form one of the main sources of protein, mainly because of their high protein content. Unless the seeds are processed (home cooking or roasting), they are generally poor sources of dietary protein.

The winged bean is a tropical legume whose seeds are high in protein and oil, with 71 % of the fatty acids being unsaturated (Pospisil *et al.*, 1971; Claydon, 1975; Ekpenyong & Borchers, in press). The plant has exceptional nutritional value and is different from most legumes in that every part of it is consumed and some parts have medicinal properties (Cerny *et al.*, 1971; Cerny & Addy, 1973). In terms of human

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nutrition, the young pods are used in vegetable salad; the old pods are steam-cooked in drums and eaten; the seeds, with their sweet, nutty taste, are eaten in many countries; leaves, flowers and roots are also used as food (Cerny & Addy, 1973; Claydon, 1975).

The amino acid composition of the winged bean seed has been reported to compare favourably with that of the soybean which is, in turn, generally regarded as the legume with the best quality protein (Pospisil *et al.*, 1971; Cerny *et al.*,1971). However, there is no indication yet in the literature as to the amino acid composition of the other parts of the winged bean plant and these other parts are commonly consumed by man. If the winged bean were to be taken as a substitute for soybean, information would then be necessary not only about the amino acid composition of the seeds, but also about other consumable parts of the crop.

MATERIALS AND METHODS

Materials

Seeds of variety TPt-2 of *Psophocarpus tetragonolobus* were obtained from the International Institute of Tropical Agriculture in Nigeria. These seeds were planted in triplicate in clay pots in the University of Nebraska greenhouse. Ten plants were pooled from which was obtained a representative sample for further study.

From each representative pool, samples of immature and mature pods, young and mature leaves, shoots, dry seeds and roots were taken and stored in the cold room (5 °C) to await analysis.

At harvest the entire plant was separated from the roots. The roots were washed, dried and stored in the cold room.

Methods

The samples were milled after drying to a fine powder. Proximate analysis was carried out in duplicate on each milled sample using the AOAC procedure (1970). The amino acid content of the different duplicate samples was determined by the method of Spackman *et al.* (1958). An alkaline hydrolysis using $Ba(OH)_2$ was used to determine tryptophan (Pataki, 1968).

Methionine and cystine

These sulphur amino acids were determined as cystine and methionine sulphone using a performic acid oxidation procedure followed by an acid hydrolysis (Moore, 1963).

All the acidic, neutral and basic amino acids except tryptophan, methionine and cystine, were released from the protein by hydrolysis under vacuum in 6N HCl at 110 °C for 24 h. The hydrolysates so obtained were injected into a Beckman-120C amino acid analyser and the data obtained were computed automatically (Cavins & Friedman, 1968).

RESULTS AND DISCUSSION

Considerable variation exists among the different parts in terms of moisture, crude protein, fat, fibre and other chemical nutrients (Table 1). The moisture content of the vegetative parts was almost similar. The seeds had a high protein content, as did the roots—higher than that of any known edible root crop. The total carbohydrate of the dry seeds was the highest, followed by that of the roots. The same pattern is shown in the fibre content. This high fibre content of the seeds and roots may influence the availability of the nutrients in animal feeds and human nutrition. The samples were, in most cases, rich in calcium and potassium while the dry seeds alone were rich in all the elements determined. Whilst the other parts might be low in iron content, the level of iron in the dry seeds just about meets the recommended daily allowance for iron.

The results of the amino acid composition of the winged bean and soybean samples have been reported previously (Table 2). The superiority of winged bean over soybean and casein in terms of its lysine content is evident from the results previously obtained. Casein in Table 2 was used as the baseline for amino acids. The lysine content in soybean seems lower than that found in the literature. No explanation could be found for this except that such lower results could be due to the protein being precipitated by acid. Methionine seems to be the most limiting amino acid in winged bean samples with tryptophan the next.

The various parts of the winged bean are utilised in animal and human nutrition. The amino acid profiles of these different parts have not been investigated. Table 3 presents the amino acid composition of the seeds, immature pods, mature pods, young and mature leaves, shoots and roots. The results show that tryptophan is the first limiting amino acid in the pods, the entire plant and the roots; cystine is

TABLE 1
CHEMICAL ANALYSIS OF THE SEEDS AND OTHER PARTS OF THE WINGED BEAN (g/100 g EDIBLE PORTION)

% Composition	*Dry seeds*	*Immature pods*	*Mature pods*	*Shoots*	*Leaves*	*Roots*
Moisture	10·4	80·6	88·2	77·9	75·8	57·4
Fat	15·8	2·8	0·3	1·1	1·1	0·9
Crude protein	35·9	2·9	3·0	5·9	5·8	11·6
Carbohydrates	23·9	9·2	6·5	8·2	—	20·7
Fibre	9·2	2·4	2·2	5·0	—	7·4
Ash	4·9	2·1	0·8	1·9	2·3	2·0
Calories	381·4	73·6	40·7	66·3	33·1	137·3
Minerals (mg/100 g)						
Calcium	275·0	240·2	66·0	263·0	184·0	30·0
Phosphorus	315·0	52·7	29·0	51·8	73·0	45·0
Potassium	117·0	208·0	195·0	176·0	—	—
Iron	10·2	7·3	0·1	2·3	5·8	2·1
Sodium	35·0	2·7	3·0	—	—	—
Magnesium	178·0	—	—	—	8·2	—

TABLE 2
AMINO ACID COMPOSITION OF WINGED BEAN, SOYBEAN AND CASEIN (g/100 g PROTEIN)

Amino acid	*Winged bean*[a]	*Winged bean*[b]	*Soybean*[a]	*Soybean*[b]	*Casein*[a]
Aspartic acid	6·93	11·5–12·5	11·0	8·3	6·1
Threonine	3·91	4·3–4·5	3·9	3·8	3·9
Serine	4·56	4·9–5·2	4·5	5·6	3·2
Glutamic acid	14·99	15·3–15·8	17·2	18·5	13·5
Proline	5·97	6·9–7·6	5·1	5·4	5·6
Glycine	4·07	4·3	5·0	3·8	2·0
Alanine	4·11	4·3	3·9	4·5	3·2
Cystine	1·41	1·6–2·6	2·3	1·2	0·5
Valine	4·91	4·9–5·7	4·4	5·2	5·6
Methionine	0·86	1·2	1·9	1·1	3·5
Isoleucine	4·60	4·9–5·1	4·4	5·8	6·6
Leucine	8·32	8·6–9·2	7·0	7·6	10·1
Tyrosine	4·74	3·2	3·7	3·2	7·7
Phenylalanine	4·64	4·8–5·8	5·1	3·8	6·9
Lysine	7·91	7·4–8·0	4·1	6·6	5·7
Histidine	2·99	2·7	2·2	2·5	4·1
Arginine	7·54	6·5–6·6	6·3	7·0	4·3
Tryptophan	0·94	1·0	1·0	1·2	1·4

[a] Source: Ekpenyong & Borchers, in press.
[b] Source: Cerny *et al.* (1971) and Kapsiotis (1968).

limiting in the pods and mature leaves while histidine is limiting in young leaves. The total essential amino acids of all the different parts of the winged bean compare favourably with that of soybean while some are better than soybean. Except for the immature pods, whole plant and roots, the total sulphur amino acids in the other parts of the winged bean are low. This gives an indication that the S-amino acids are more readily available in some parts than in others.

Except for the young leaves, the lysine content in all the parts analysed is similar to, or higher than, that reported for whole egg and soybean (FAO, 1970).

All parts of the plant contain sufficient levels of threonine which, along with lysine, participates in protein synthesis. Arginine, of which the plant has a high content, although not essential for adults, is essential for children and young people. Our results further show that, not only are the essential amino acids present in sufficient amounts, but that the non-essential amino acids are also adequate. This is important because protein synthesis is limited not only by the availability of essential amino acids but also by the speed and efficiency with which the non-essential amino acids are supplied. Again, since the different parts of the winged bean are rich in lysine and threonine, the winged bean can be used effectively in supplementing cereal diets which serve as the principal sources of proteins and energy but are deficient in these two amino acids.

One striking result is the amino acid profile of the roots (Table 3). The roots contain high levels of lysine and tryptophan (5·62 g/100 g and 4·31 g/100 g, respectively) and much higher levels than are found in soybean seeds (4·09 g/100 g

TABLE 3
AMINO ACID COMPOSITION OF DIFFERENT PARTS OF THE WINGED BEAN PLANT (g/100 g PROTEIN)

Amino acid	*Different botanical parts*								
	WB[a]	*SB*[b]	*Immature pods*	*Mature pods*	*Young leaves*	*Mature leaves*	*Shoot no pods*	*Whole plant*	*Roots*
Aspartic acid	6·93	11·0	12·2	9·13	10·4	8·59	10·7	12·1	11·3
Threonine	3·91	3·79	3·70	2·80	4·80	3·96	4·38	3·90	4·31
Serine	4·36	4·47	4·20	3·05	4·22	5·97	4·41	4·57	6·35
Glutamic acid	15·0	17·2	11·3	21·7	11·5	14·8	10·6	10·1	9·86
Proline	5·97	5·09	5·26	5·96	5·30	7·12	5·15	5·33	6·14
Glycine	4·07	5·03	4·01	6·74	5·93	3·49	5·21	4·72	4·72
Alanine	4·11	3·92	4·43	3·71	6·45	4·16	5·72	5·37	5·12
Cystine	1·41	2·27	1·93	2·06	1·35	0·67	1·02	2·67	2·62
Valine	4·91	4·38	5·11	4·93	6·44	6·10	6·30	5·86	6·74
Methionine	0·86	1·93	3·43	1·16	1·63	1·90	1·10	2·34	1·96
Isoleucine	4·60	4·38	4·25	4·04	5·51	5·33	4·81	4·42	4·32
Leucine	8·32	7·00	6·88	7·05	9·52	9·48	8·47	7·71	7·37
Tyrosine	4·74	3·66	3·89	4·77	4·74	4·66	3·32	3·36	4·27
Phenylalanine	4·64	5·12	4·54	4·61	6·62	4·70	4·25	3·90	3·45
Lysine	7·91	4·09	6·66	4·05	2·59	4·67	4·23	4·71	5·62
Histidine	2·99	2·19	3·17	2·10	1·26	2·27	2·10	2·49	2·53
Arginine	7·54	6·27	6·21	4·75	3·28	4·11	5·12	5·47	4·29
Tryptophan	0·94	0·97	0·95	0·66	2·09	0·93	4·94	2·26	1·13
Total essential	42·2	37·5	40·3	35·9	43·4	42·4	42·8	41·1	35·1
Total sulphur	2·27	4·20	3·36	3·22	3·98	2·57	2·12	5·01	4·58
Total aromatic	9·38	8·78	8·43	9·08	11·36	9·36	7·57	7·25	7·72

[a] WB = Raw Winged Bean Seeds.
[b] SB = Raw Soybean Seeds.

TABLE 4

THE ESSENTIAL AMINO ACID CONTENTS OF WINGED BEAN, RAW SOYBEAN AND CASEIN AS PERCENTAGE OF FAO[a]

Protein source	Essential amino acid (g/100 g of protein)							
	Lysine	*Methionine and cystine*	*Threonine*	*Isoleucine*	*Leucine*	*Valine*	*Tyrosine and phenylalanine*	*Tryptophan*
Raw soybean	4·09 (53)	4·20 (84)	3·79 (67)	4·38 (77)	7·00 (70)	4·38 (62)	8·78 (102)	0·98 (71)
Casein	5·72 (95)	4·01 (102)	3·84 (86)	6·59 (148)	10·1 (127)	5·62 (102)	14·6 (216)	1·43 (134)
Winged bean[b]	7·91 (102)	2·27 (45)	3·91 (68)	4·60 (80)	8·32 (83)	4·91 (69)	9·38 (108)	0·94 (69)
Winged bean[c]	5·24 (76)	3·19 (72)	4·15 (82)	4·57 (90)	7·93 (89)	4·95 (79)	10·3 (134)	1·13 (93)
Immature pods	6·66 (88)	4·35 (89)	3·70 (66)	4·25 (76)	6·88 (70)	5·11 (74)	8·43 (100)	0·95 (71)
Mature pods	2·59 (34)	2·99 (60)	4·80 (85)	5·51 (97)	9·52 (95)	6·44 (92)	11·4 (132)	2·09 (154)
Young leaves	2·59 (34)	2·99 (60)	4·80 (85)	5·51 (97)	9·52 (95)	6·44 (92)	11·4 (143)	2·09 (154)
Mature leaves	4·67 (54)	2·57 (46)	3·96 (63)	5·33 (85)	9·48 (85)	6·10 (78)	9·36 (98)	0·93 (61)
Shoot (no pods)	4·23 (53)	2·13 (42)	4·38 (75)	4·81 (83)	8·47 (83)	6·30 (87)	7·57 (86)	2·26 (169)
Entire plant	4·71 (59)	5·01 (102)	3·90 (70)	4·42 (79)	7·71 (89)	5·86 (85)	7·25 (86)	2·26 (169)
Roots	5·62 (71)	4·58 (89)	4·31 (74)	4·32 (74)	7·37 (72)	6·74 (93)	7·72 (87)	1·13 (80)

[a] Values in parentheses represent percentage of FAO adjusted for digestibility.
[b] Winged bean seeds subjected to 24 h hydrolysis.
[c] Winged bean autoclaved at 120°C for 30 min at 15 lb pressure before the 24 h hydrolysis.
Source: Ekpenyong & Borchers, in press.

and 3·79 g/100 g). Even the total S-amino acids are higher in the roots (4·56 g/100 g protein) than soybean or any other part of the winged bean except the whole plant. Tyrosine is present in sufficient quantity. Tyrosine is non-essential since it can be formed from phenylalanine and both are found in sufficient amounts in all parts of the winged bean studied.

Table 4 shows the essential amino acid composition of the different parts of the winged bean, soybean and casein as percentages of FAO values adjusted for digestibility. The values in parentheses represent the percentages of FAO values and these values were computed from a predetermined model (Kendrick *et al.*, 1976). These computed data show that the sulphur amino acids are the first limiting amino acids in the winged bean. The values in parentheses indicate that, although there is a high lysine content in the seeds, young pods and roots of the winged bean compared with casein, autoclaving tends to reduce the lysine content of heated seeds. On the other hand, when compared as percentages of FAO figures adjusted for digestibility, heat treatment improves the content in S-amino acids. Threonine is improved with heating in the seeds and so also are the other essential amino acids when these values are computed as percentages of FAO figures, adjusted for digestibility. The different essential amino acids in the winged bean are present at different levels in the different parts of the plant.

Generally, the computed values for lysine and threonine, when adjusted for their digestibilities, compare favourably with—and in some cases are superior to—the values for soybean.

It appears from the results that not only the seeds, but also the other edible portions of the winged bean, are high in nutrients, especially proteins. The amino acid composition indicates that, while lysine and threonine appear in adequate amounts in all the different parts of the plant, the sulphur amino acids, methionine and cystine, are deficient. However, such deficiency can be overcome when this legume is used as a supplement in cereal diets which contain sufficient amounts of methionine and cystine. Since there is no available information in the literature on the amino acid composition of these parts, comparison with other research results is impossible and this therefore raises the need for feeding experiments to be able to confirm the results of chemical analysis. It is also clear from the FAO values adjusted for digestibility that the different parts of the winged bean are of good nutritive value since they are sources of good quality protein. A mixture of some or all of these parts with cereal-based diets would yield a mutual protein supplementation effect in terms of their essential amino acids, especially lysine and threonine on one hand with methionine and cystine on the other.

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OXIDATION OF METHIONINE. EFFECTS OF HYDROGEN PEROXIDE ALONE AND IN COMBINATION WITH IODIDE AND SELENITE

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ABSTRACT

The oxidation of methionine by hydrogen peroxide, and the influence of iodide, pH, amino acids and selenite were studied with free methionine and with casein and fish fillet protein. The concentration levels tested ranged from 0·05 mM to 3·0 mM. Hydrogen peroxide oxidation was not influenced by pH in the range 5·0 to 8·0; at pH 8·5 the rate of oxidation was increased. When iodide was added in amounts equivalent to or less than the amounts of H_2O_2, the reaction was accelerated with free but not protein-bound methionine. At higher levels iodide inhibited the oxidation. An amino acid mixture and proteins inhibited the effect of iodide; this effect seemed to be due to tryptophan. Selenite also accelerated the effect of H_2O_2, both with free and with protein-bound methionine. Cu^{++} catalysed the oxidation by H_2O_2 at low reactant concentration but not at the higher levels. The reaction between methionine and H_2O_2 seemed to be of first order with respect to both reactants.

INTRODUCTION

Methionine, in free form and in proteins, is oxidised to methionine sulphoxide by hydrogen peroxide and by other chemical oxidants (Shechter *et al.*, 1975). Foods and feeds occasionally contain appreciable amounts of methionine sulphoxide (Njaa, 1980) which may be due to naturally occurring oxidants like H_2O_2, superoxide anion and singlet oxygen (Aksnes & Njaa, 1981). There are few systematic studies of the formation of methionine sulphoxide from free or protein-bound methionine by these oxidants. In the present paper the effects of H_2O_2 on free and protein-bound methionine are studied in some detail. As there are conflicting results on the effect of iodide in combination with oxidants like H_2O_2

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and chloramine-T on methionine oxidation (Rosenberg & Murray, 1979; Heward *et al.*, 1979) the effects of H_2O_2 in combination with iodide were also studied. A few observations on the effect of selenite on the oxidation with H_2O_2 were also included.

MATERIALS AND METHODS

The chemicals used were of analytical grade, the two protein sources were casein (Hammarsten, Merck, Darmstadt, West Germany) and fish fillet from Saithe bought at the local fish market. The reactions were carried out in phosphate buffers (0·05 mM) at pH 5·5–6·0, except when pH effects were studied. The components were mixed and diluted to near the final volume (50 ml) before the addition of H_2O_2 or other oxidants. The samples were kept at 20° for the times indicated in the Tables and Figures, and portions (2–6 ml) were taken for analysis at intervals or at one specified time. Methionine concentrations (unoxidised or total) were determined by the method described by Njaa (1980). Decrease in the concentration of unoxidised methionine was taken to indicate oxidation. As a measure for the mean oxidation rate the numerical value of the linear regression coefficient relating concentration of unoxidised methionine ([Met]) to the logarithm of the reaction time in hours ($\log t$) was arbitrarily used. Concentrations obtained before 2 h tended to be erratic and were therefore not used in the calculations. The regression equations calculated are given in the legends to the Figures.

A freshly made solution of 0·2 M hydrogen peroxide was prepared from Perhydrol (Merck, Darmstadt, West Germany) and appropriate amounts of this were taken for the oxidation experiments. Similarly, a freshly made 80 mM stock solution of iodine in ethanol was used when iodine was used as an oxidant. When iodine was dissolved in potassium iodide equimolar amounts were used (80 mM I_2 in 80 mM KI).

EXPERIMENTAL METHODS AND RESULTS

Effects of varying either methionine or H_2O_2 concentration

Methionine or H_2O_2 (2·0 mM) was reacted with varying concentrations of the other component (0·6, 1·2, 1·8, 2·4 and 3·0 mM). The mean oxidation rates were very similar for the same product [Met][H_2O_2] (Fig. 1). In both cases they increased approximately linearly (Fig. 2) and the regression coefficients were practically equal. Thus, the reaction between methionine and H_2O_2 seems to be of first order with respect to both reactants.

Effect of Cu^{++}

Four experiments were run to test whether Cu^{++} catalysed the oxidation of methionine with H_2O_2. For levels of methionine between 1·0 and 2·0 mM and of

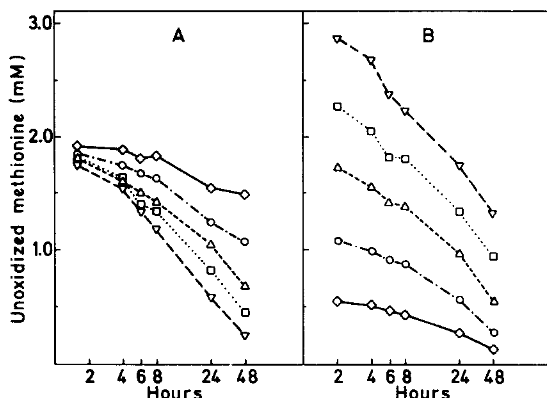

Fig. 1. Effect of varying concentrations of methionine or H_2O_2 on the oxidation rate of methionine. Initial concentrations: A. [Met] 2·0 mM, [H_2O_2] 0·6 (◇), 1·2 (○), 1·8 (△), 2·4 (□) and 3·0 (▽) mM. B. [Met] 0·6 (◇), 1·2 (○), 1·8 (△), 2·4 (□) and 3·0 (▽) mM, [H_2O_2] 2·0 mM. Linear regression equations:

A.	B.
[Met] = 2·1 − 0·35 log t	[Met] = 0·68 − 0·35 log t
[Met] = 2·1 − 0·60 log t	[Met] = 1·3 − 0·59 log t
[Met] = 2·1 − 0·80 log t	[Met] = 2·0 − 0·84 log t
[Met] = 2·2 − 1·01 log t	[Met] = 2·6 − 0·95 log t
[Met] = 2·2 − 1·14 log t	[Met] = 3·3 − 1·14 log t

H_2O_2 between 0·6 and 1·2 mM no effects were observed with Cu^{++} concentrations ranging from 1·5 to 500 μM. At a lower level of methionine (0·1 mM) with low H_2O_2 levels (0·05–0·2 mM) effects of Cu^{++} were seen. Figure 3 gives the results obtained in two 72-h experiments. They show that the mean oxidation rate for 0·1 mM methionine increased with increased H_2O_2 concentrations (0·05, 0·1 and 0·2 mM), that addition of 10 μM Cu^{++} to 0·10 mM H_2O_2 had an equal, or greater, effect on the mean oxidation rate than doubling the H_2O_2 concentration and that increasing the

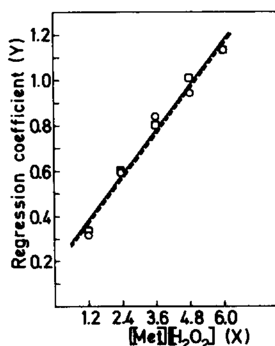

Fig. 2. Relationship between the product [Met] [H_2O_2] (X) and the regression coefficients (Y) given in the legend to Fig.1. (□) Initial methionine concentration, 2·0 mM: $Y = 0{\cdot}167X + 0{\cdot}183$ ($r = +0{\cdot}995$). (○) Initial H_2O_2 concentration, 2·0 mM: $Y = 0{\cdot}167X + 0{\cdot}168$ ($r = +0{\cdot}989$).

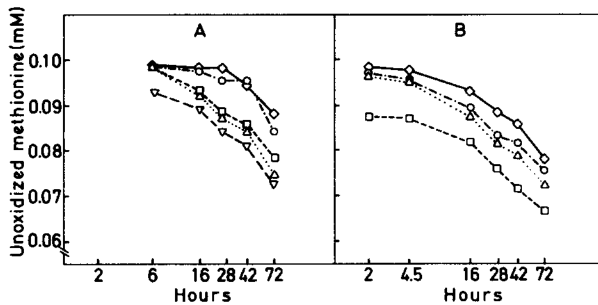

Fig. 3. Effect of Cu^{++} on the oxidation rate of 0·10 mM methionine. The concentrations of H_2O_2 (mM) and added Cu^{++} (μM), respectively, are given within parentheses, together with the symbols used.

A. (0·05, 0, ◇) [Met] = 0·11 − 0·009 log t
(0·10, 0, ○) [Met] = 0·11 − 0·012 log t
(0·10, 10, △) [Met] = 0·11 − 0·020 log t
(0·20, 0, □) [Met] = 0·11 − 0·017 log t
(0·20, 10, ▽) [Met] = 0·11 − 0·020 log t
B. (0·20, 0, ◇) [Met] = 0·10 − 0·012 log t
(0·20, 5, ○) [Met] = 0·10 − 0·014 log t
(0·20, 10, △) [Met] = 0·10 − 0·016 log t
(0·20, 50, □) [Met] = 0·09 − 0·013 log t

Cu^{++} concentration from zero over 5 μM to 10 μM increased the mean oxidation rate linearly. However, at 50 μM Cu^{++} the mean oxidation rate after 2 h was low. In this case the initial oxidation during the first 2 h was obviously greater than for the other Cu^{++} concentrations. The effects of adding Fe^{++}, Fe^{+++} or EDTA, all at the 10 μM level, were also studied but they had no effect on the oxidation.

Effect of iodide in combination with H_2O_2

Samples with the methionine and H_2O_2 concentrations of 2·0 mM and 1·2 mM, respectively, were tested with graded concentrations of added iodide (0·6, 1·2, 2·4, 3·6 and 4·8 mM). The reaction was followed for 48 h, but as there was a tendency for it to reach completion at 24 h at the higher iodide concentrations, mean oxidation rates were calculated between 2 and 24 h. The results are given in Fig. 4. Each increment in the iodide concentration increased the initial oxidation during the first 2 h. Up to 1·2 mM iodide the mean oxidation rate after 2 h was also increased, but above this level it tended to decrease again. This may be significant as iodide seems to inhibit the oxidation due to iodine (Table 1).

Effect of pH on oxidation with H_2O_2 alone and in combination with iodide

The previous experiments were run at pH 5·5–6·0. The effect of pH was studied in the pH range from 5·0 to 8·5, with 0·5 intervals. The oxidation was followed for 48 h, both with and without iodide added to H_2O_2. The methionine and H_2O_2 concentrations were 2·0 mM and 1·2 mM, respectively; the iodide concentration was

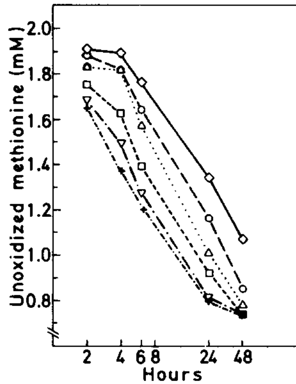

Fig. 4. Effect of varying iodide concentration on the oxidation rate of 2·0 mM methionine with 1·2 mM H_2O_2.

(◇) negative control: [Met] = 2·2 − 0·64 log t
(○) 0·6 mM I^-: [Met] = 2·2 − 0·78 log t
(△) 1·2 mM I^-: [Met] = 2·2 − 0·83 log t
(□) 2·4 mM I^-: [Met] = 2·0 − 0·77 log t
(▽) 3·6 mM I^-: [Met] = 1·9 − 0·72 log t
(+) 4·8 mM I^-: [Met] = 1·9 − 0·66 log t

2·4 mM. The mean oxidation rate between 2 and 24 h was practically unaffected by pH up to pH 8·0 when iodide was not present, but was appreciably greater at pH 8·5. When iodide was present pH did not seem to affect the mean reaction rate, but it was generally higher without iodide added. The rates with iodide were of about the same mangnitude as the rate calculated for pH 8·5 when iodide was not present. Because the differences were slight, only the results obtained at pH 5·0 and 8·5 are given in Fig. 5.

TABLE 1

OXIDATION OF METHIONINE WITH H_2O_2 AND I_2, ALONE, COMBINED AND IN COMBINATION WITH IODIDE, WITH AND WITHOUT ADDED AMINO ACID MIXTURE

Meth (*m*M)	I_2 (*m*M)	*KI* (*m*M)	H_2O_2 (*m*M)	*Percent unoxidised methionine* −*aa*	+*aa*
1	0·6	—	—	35 (40)*	78 (40)
1	—	1·2	0·6	51 (40)	82 (40)
1	0·6	1·2	—	64 (40)	82 (40)
1	—	—	0·6	72 (40)	78 (40)
1	0·6	—	0·6	0 (0)	58 (0)
1·34	0·32			75 (76)	
1·34	0·64			52 (52)	
1·34	0·32 in KI			90 (76)	
1·34	0·64 in KI			87 (52)	
1·34	0·96 in KI			95 (28)	

* Theoretical values in parentheses.

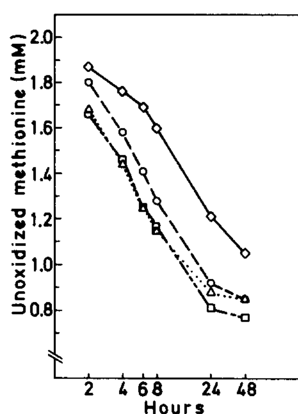

Fig. 5. Effect of pH on the oxidation rate of 2·0 mM methionine with 1·2 mM H_2O_2 with and without 2·4 mM iodide.

(◇) neg. control, pH 5·0 [Met] = 2·14 − 0·62 log *t*
(○) neg. control, pH 8·5 [Met] = 2·05 − 0·83 log *t*
(□) with iodide, pH 5·0 [Met] = 1·90 − 0·80 log *t*
(△) with iodide, pH 8·5 [Met] = 1·87 − 0·75 log *t*

Effects of amino acids on oxidation with H_2O_2 in combination with iodide

Solutions of an amino acid mixture (Gjøen & Njaa, 1977) or some of the constituent amino acids in that mixture were added to samples containing methionine (1·0 mM) and H_2O_2 (0·6 mM) both with and without added iodide (1·2 mM). The amounts used are indicated in Table 2. Relative to methionine the amount of amino acid mixture was about as in fish meal and the amounts of the amino acids were the same as in the mixture. Table 2 gives the concentrations of unoxidised methionine found after 24 hours' reaction time. The amino acid mixture

TABLE 2
EFFECTS OF AMINO ACIDS ON THE OXIDATION OF 1·0 mM METHIONINE WITH 0·6 mM H_2O_2 WITH AND WITHOUT 1·2 mM IODIDE

Addition	*Percent unoxidised methionine*	
	$-I^-$	$+I^-$
None	71	47
Amino acid mixture*	75	80
Tyr, Phe, His, Trp**	70	72
Tyr (0·69 mmol)	66	44
Phe (1·83 mmol)	69	49
His (0·68 mmol)	64	45
Trp (0·30 mmol)	66	78
Ser (1·69 mmol)	69	46
Thr (1·77 mmol)	61	49

* 4·8 g/mM methionine.
** The amounts used were the sum of the single ingredient given below.

inhibited the oxidation due to iodide addition and the experiments with the amino acids show that tryptophan was responsible for this.

Effects of H_2O_2 and I_2, alone, combined and in combination with iodide

The effects of iodine were studied in two experiments. In one, 1 mM methionine was oxidised with 0·6 mM I_2 or with 0·6 mM H_2O_2. Further, the combined effects of I_2 and H_2O_2 and the effects of I_2 with 1·2 mM iodide or H_2O_2 with 1·2 mM iodide were studied. All these treatments were tested both with and without added amino acid solution as in Table 2. Table 1 records the percentages of unoxidised methionine analysed after 24 h. In the other experiment the effects of I_2 dissolved in ethanol and I_2 dissolved in equimolar potassium iodide were compared. In this case the initial methionine concentration was 1·34 mM and iodine 0·64 mM (dissolved in ethanol) and 0·32, 0·64 and 0·96 (dissolved in potassium iodide).

Amino acids inhibited oxidation in all cases when iodine or iodide plus H_2O_2 were involved but not oxidation due to H_2O_2 alone. Without amino acids iodine was a more effective oxidant than H_2O_2, but it was inhibited by iodide. Also, without amino acids, iodine in ethanol was a more potent oxidant than iodine in iodide. Tests not detailed here showed that the oxidation of methionine with iodine was as effective at pH 6·0 as at pH 8·5. In fact, the oxidations with iodine seemed to be so fast that they had usually gone to completion before 1 hour's reaction.

Effect of selenite in combination with H_2O_2

It seems reasonable to explain the accelerating effect of iodide on the H_2O_2 oxidation of methionine by assuming that H_2O_2 oxidises iodide to iodine and that iodine reacts rapidly with methionine. Similarly, it is possible that other substances may be oxidised by H_2O_2 and the oxidised product might, in turn, oxidise methionine. We have tested some substances for this effect but so far we have only found it in the case of selenite. The results of one such experiment are shown in Fig. 6. Here methionine and H_2O_2 (2·0 mM and 1·2 mM, respectively) were tested with graded concentrations of selenite (0·6, 1·2 and 1·8 mM). The mean oxidation rate increased with the selenite concentration up to 1·2 mM (equivalent to the H_2O_2 concentration). The rates were comparable with those found for iodide (Fig. 5). Selenite alone had no effect. It may be premature to conclude that the effect of selenite proceeds as indicated above, but it indicates that some naturally occurring substances may accelerate the effect of naturally produced H_2O_2. In Fig. 6 only results obtained without selenite and with 1·2 mM selenite are given.

Experiments with casein

Casein solutions (50 mg/ml) in phosphate buffer, pH 6, were used in tests with H_2O_2, H_2O_2 plus KI, H_2O_2 plus Na_2SeO_3, I_2 and $I_2 + KI$ as oxidants.

Ten-millilitre portions of the casein solution (about 500 mg casein) containing about 80 μmol of unoxidised methionine were treated in polypropylene weighing

TABLE 3

EFFECTS OF I_2 ALONE AND OF H_2O_2 ALONE AND IN COMBINATION WITH IODIDE AND SELENITE ON THE OXIDATION OF METHIONINE IN CASEIN

H_2O_2 (*μmol*)	*KI* (*μmol*)	SeO_3^{--} (*μmol*)	I_2 (*μmol*)	*Unoxidised methionine* (*μmol*)
—	—	—	—	83
60	—	—	—	52
60	30	—	—	55
60	60	—	—	58
60	120	—	—	60
60	180	—	—	61
60	240	—	—	64
60	—	30	—	37
60	—	60	—	34
—	—	—	30	80
—	60	—	30	81
—	—	—	60	72
—	120	—	60	72

bottles with the amounts of oxidants indicated in Table 3. The mixtures were kept at 20° for 24 h and analysed for unoxidised and total methionine after hydrolysis with $Ba(OH)_2$. Results for unoxidised methionine are given in Table 3; the values for total methionine were practically the same (mean value 96 μmol) in all cases. In contrast to the results obtained with free methionine, iodine did not oxidise the protein-bound methionine to any great extent. Also in contrast to the results with free methionine, addition of iodide to H_2O_2 inhibited the oxidation. On the other hand, addition of selenite to H_2O_2 increased the oxidation appreciably (70% unoxidised with 60 μmol H_2O_2, 40% unoxidised with 60 μmol H_2O_2 plus 60 μmol Na_2SeO_3).

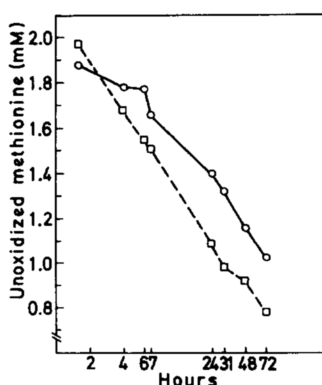

Fig. 6. Effect of selenite on the oxidation rate of 2·0 mM methionine with 1·2 mM H_2O_2. (○) neg. control: [Met] = 2·12 − 0·56 log *t*; (□) 1·2 mM Na_2SeO_3: [Met] = 2·15 − 0·75 log *t*.

Experiments with fish fillet protein

Fish fillets were minced and 2 g portions were weighed into polypropylene weighing bottles. Ten millilitres of boiling water were added and the samples were kept in a hot water bath for 30 min. After cooling, the appropriate amounts of oxidants were added as indicated in Table 4. The mixtures were kept at 20° for 24 h, after which they were hydrolysed with $Ba(OH)_2$ and analysed for unoxidised and total methionine. Results for unoxidised methionine are given in Table 4; the amount of total methionine showed little variation (mean value 82 μmol per 2 grammes of fish). The results obtained with iodine and iodide together with H_2O_2 were similar to those obtained with casein; that is, practically no oxidation with iodine and inhibition of H_2O_2 oxidation by iodide. Also, for selenite the results were similar to those obtained with casein (66 % unoxidised with 60 μmol H_2O_2, 52 % with 80 μmol H_2O_2 and 45 % with 80 μmol H_2O_2 plus 80 μmol selenite).

TABLE 4

EFFECTS OF IODINE ALONE AND OF H_2O_2 ALONE AND IN COMBINATION WITH IODIDE AND SELENITE ON THE OXIDATION OF METHIONINE IN FISH FILLET PROTEIN. EFFECT OF AZIDE IN RAW FISH

H_2O_2 (μmol)	KI (μmol)	SeO_3^{--} (μmol)	I_2 (μmol)	NaN_3 (μmol)	Unoxidised methionine (μmol)
—	—	—	—	—	78
40	—	—	—	—	62
60	—	—	—	—	52
80	—	—	—	—	44
100	—	—	—	—	41
40	80	—	—	—	74
60	120	—	—	—	74
80	160	—	—	—	72
100	200	—	—	—	71
80	—	10	—	—	41
80	—	20	—	—	39
80	—	40	—	—	39
80	—	80	—	—	35
—	—	—	60	—	76
—	—	—	120	—	70
40	—	—	—	+	66* (66)
80	—	—	—	+	55* (52)
—	—	—	—	+	79*
80	—	80	—	+	42*

* Raw fish, cooked fish in parentheses.

H_2O_2 did not oxidise methionine in raw fish. When azide was added to inhibit catalase, oxidation took place to about the same extent as in cooked fish (Table 4).

DISCUSSION

Compared with experiments reported in the literature the oxidation of methionine by H_2O_2 was slow. This was probably due to the low concentrations and to the

rather low temperature (20°) used. The levels of H_2O_2 used in most experiments were 60% of the methionine levels.

Experiments reported in the literature were run at higher concentrations (Toennies & Callan, 1939) or with excess H_2O_2 over methionine (Yang, 1970; Rosenberg & Murray, 1979). In experiments with proteins elevated temperatures were often used (Rasekh *et al.*, 1972; Cuq *et al.*, 1973; Slump & Schreuder, 1973).

The present results showed no effect of pH on the mean oxidation rate between pH 5·0 and 8·0, but an increased rate at pH 8·5. Toennies & Callan (1939) found no effect on the oxidation rate between pH 1 and pH 5. As the results obtained at pH 5·5–6·0 indicated that the reaction was of first order with respect to both reactants, activation of H_2O_2 does not seem to be involved in acid solutions and up to pH 8·0. If this were the case one would have expected zero order reaction with respect to methionine provided that H_2O_2 activation was rate limiting. Activation of H_2O_2 at alkaline pH (formation of hydroxyl radical, superoxide anion and singlet oxygen) was reported by Agnemo & Gellerstedt (1979). This may explain the increased oxidation observed at pH 8·5. Toennies & Callan (1939) found increased rate of oxidation in 1M–4M perchloric acid solutions and stated that the product obtained was methionine sulphoxide only. Njaa (1962) found that methionine applied to filter paper in hydrochloric acid solution and treated with H_2O_2 oxidised during drying to a mixture of sulphoxide and sulphone.

In agreement with Toennies & Callan (1939) we found no catalytic effect of cupric ions when the concentrations of methionine and H_2O_2 were 1·0–2·0 mM and 0·6–1·2 mM, respectively. However, at lower concentrations (0·1 mM methionine, 0·05–0·2 mM H_2O_2) cupric ions catalysed the reaction (Fig. 3). The reason for this difference is not clear.

When iodide was present together with H_2O_2 the initial oxidation during the first 2 h was increased (Fig. 4). Also, the rate of oxidation increased from zero iodide concentration through 0·6 mM to 1·2 mM potassium iodide. Above this (2·4, 3·6 and 4·8 mM) the oxidation rate seemed to decrease. Presumably this activation of H_2O_2 is due to H_2O_2 oxidising iodide to iodine. This, in turn, could react fast with methionine to give dehydromethionine followed by hydrolysis to methionine sulphoxide (Gensch & Higuchi, 1967). During these reactions iodide is reformed and might act catalytically as long as there is H_2O_2 present. However, side reactions consuming iodide and iodine may occur (Young & Hsieh, 1978). The activating effect of iodide was completely inhibited with sodium sulphite, presumably by reacting with the iodine formed. The reaction between iodine and methionine was very fast. This reaction—and the reaction due to H_2O_2 combined with iodide—were inhibited by a solution of an amino acid mixture having an amino acid composition similar to fish protein. We found that this effect was due to the tryptophan in the mixture, tyrosine, phenylalanine and histidine having no effect (Tables 1 and 2). Potassium iodide inhibited oxidation of methionine by iodine (Table 1). Toennies & Callan (1939) found oxidation of methionine by aqueous iodine to be negligibly

slow. Presumably they dissolved iodine in iodide and thus experienced the iodide inhibition on iodine oxidation of methionine.

Rosenberg & Murray (1979) also reported that iodine in iodide solution did not cause significant methionine oxidation, but that iodine *in statu nascendi* was a potent oxidant. Gensch & Higuchi (1967) oxidised methionine with iodine in iodide solution and concluded that the rate of reaction was inversely proportional to the iodide concentration. From this they inferred that free iodine is involved in the reaction. This seems to be in accordance with the present results which were obtained with iodine in ethanol solution. The inhibition by tyrosine and iodide of methionine oxidation with H_2O_2 reported by Rosenberg & Murray (1979) could not be reproduced.

The principle that H_2O_2 may oxidise methionine by first oxidating another compound which then acts as the final oxidant may also apply to the results obtained with selenite. In this case one could visualise selenite–selenate as part of a catalytic system. However, increased oxidation was only observed with amounts of selenite which were nearly equivalent to the hydrogen peroxide used.

In the experiments with casein and fish fillets the amounts of unoxidised methionine which theoretically could be oxidised are given by the amounts of H_2O_2. Thus, in the experiments with casein, H_2O_2 alone oxidised about 50% of the theoretical amount. The effectiveness of oxidation decreased with increasing levels of iodide to about 30% at the highest level. Selenite, on the other hand, increased the effectiveness of oxidation to about 80% at the two levels tested. Iodine alone oxidised only small amounts of methionine and the effects of iodide could not be observed (Table 3).

With fish protein, H_2O_2 alone was about equally effective at the four levels tested, oxidising about 40% of the theoretical amounts. When equivalent amounts of iodide were added the effectiveness of oxidation was reduced to 7–10%. Selenite increased effectiveness (46–54%), but not so much as for casein. Again, iodine alone gave little oxidation. With raw fish there was no oxidation of methionine by H_2O_2, but when azide was added to inhibit catalase, H_2O_2 was about equally effective as with cooked fish (Table 4).

There seem to be no reports in the literature with which the results obtained with casein and fish protein may be compared. Cuq *et al.* (1973, 1978), working with casein, used great excesses of H_2O_2 and elevated temperature and obtained between 20% and 100% oxidation of methionine in 30 min. Rasekh *et al.* (1972) working with FPC (fish protein concentrate) also used elevated temperature and short time, but also had low H_2O_2 levels included. They did not, however, determine the degree of methionine oxidation. They had a slight decrease in total methionine at the highest H_2O_2 levels. As methionine sulphoxide reverts to methionine during acid hydrolysis (Njaa, 1962) the rather constant methionine content at the lower H_2O_2 levels gives no indication about the oxidation.

Sjøberg & Bostrøm (1977) found that fish meal treated with H_2O_2 at pH 8·5

contained increasing amounts of methionine sulphone as the excess of H_2O_2 over methionine was increased. Methionine sulphone has not been found in the proteins oxidised under the conditions used in the present experiments.

Iodine did not oxidise methionine under the conditions used in the present experiments. Presumably this is due to iodine reacting preferably with other amino acids in the proteins.

The present experiments were performed to gather information on factors which might influence oxidation of methionine to methionine sulphoxide in foods and feeds. Results obtained in our Institute with fish flours, fish meals and some other protein sources show that this oxidation, if it occurs at all, is a very slow process. Presumably, the oxidant responsible is formed slowly so that methionine is in excess over the oxidant. Such conditions are not realised in the present experiments. They show, however, that oxidation with H_2O_2 can take place at low concentrations and that this oxidant may be active over a long period of time.

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CYANIDE, PROTEIN AND IODINE INTERACTION IN THE PHYSIOLOGY AND METABOLISM OF RATS

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ABSTRACT

Performance and metabolic traits were measured in sixty-four growing rats fed on varying levels of dietary cyanide, protein and iodine.

The presence of cyanide in the diets caused a non-significant reduction in both feed consumption and growth rate. Moreover, on protein deficient diets, the lowest body weight gain was obtained in the animals with 750 ppm cyanide. Iodine deficiency did not have any marked influence on performance.

Dietary cyanide significantly increased serum and urinary thiocyanate concentration while iodine deficiency caused significant reductions in serum Protein Bound Iodine (PBI).

Interactions of protein deficiency and dietary cyanide also significantly reduced serum thiocyanate concentrations, while interactions of the three dietary variables significantly reduced kidney protein content.

INTRODUCTION

Chronic cyanide intoxication in the cassava-consuming population is complicated by gross nutrient imbalance in patients and accompanying physiological deficiency states usually associated with the observed toxicity symptoms. This can be inferred from the work of Ekpechi *et al.* (1966), Osuntokun (1968), Maner & Gomez (1973) and Delange (1974).

The bulk of metabolic evidence points to three principal nutritional factors implicated in the human illnesses or reduced animal performance that are observed

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with cassava consumption; these are (1) cyanide, (2) iodine deficiency and (3) protein deficiency.

This experiment, which was carried out with Sprague–Dawley rats, had the following objectives:

(1) To investigate the effects of cyanide, iodine and protein as they individually and collectively influence the performance of rats.
(2) To evaluate the influence of these dietary factors on thiocyanate, protein, protein bound iodine and rhodanese activity of various tissues.

EXPERIMENTAL PROCEDURE

Sixty-four weanling albino rats of the Sprague–Dawley strain were allotted factorially on the basis of sex and body weight to eight treatments groups.

The gross and proximate compositions of the diets are given in Tables 1 and 2. Six replicates of each treatment were individually maintained in metabolic growth cages where they had access to feed and demineralised water *ad libitum*. The remaining two replicates, one of each sex, were housed in stainless steel metabolic cages where a urine sample was collected daily for the first month of the study period. Body weights and feed intake were recorded twice weekly. Weekly urine samples of rats in the metabolic cages were pooled by treatment and analysed for thiocyanate content by the method of Bowler (1944) and for iodine by the Hycel (1967) cuvette method for iodine determinations.

TABLE 1
GROSS COMPOSITION OF DIETS FED TO RATS ON CYANIDE, PROTEIN AND IODINE DEFICIENCY TRIALS

Ingredients	*Treatments*							
	1 %	*2* %	*3* %	*4* %	*5* %	*6* %	*7* %	*8* %
Maize	71·24	71·24	71·24	71·24	89·00	89·00	89·00	89·00
Soybean	17·76	17·76	17·76	17·76	—	—	—	—
Corn oil	5·00	5·00	5·00	5·00	5·00	5·00	5·00	5·00
Mineral mix (+1)[a]	4·00	—	4·00	—	4·00	—	4·00	—
Mineral mix (−1)[b]	—	4·00	—	4·00	—	4·00	—	4·00
Vitamin mix[c]	2·00	2·00	2·00	2·00	2·00	2·00	2·00	2·00
Total	100·00	100·00	100·00	100·00	100·00	100·00	100·00	100·00
Cyanide (ppm)	—	—	750	750	—	—	750	750
KCN (mg/100 g)	—	—	187·5	187·5	—	—	187·5	187·5

[a] The mineral mix, commonly known as the John Foster salt mixture, contained per kilogramme diet 100 g: NaCl, 14·04 g; KH_2PO_4, 39·20 g; $MgSO_4$, 5·17 g; $CaCO_3$, 38·24 g; $FeSO_4 . 7H_2O$, 2·72 g; KI, 0·08 g; $MnSO_4 . 2H_2O$, 0·45 g; $ZnCl_2$, 0·03 g; $CuSO_4 . 5H_2O$, 0·05 g; $CoCl_2 . 6H_2O$, 0·02 g.
[b] In the iodine deficient diets, KI was eliminated from the mineral mix.
[c] Vitamin premix was obtained from the Nutritional Biochemical Corporation, Ohio, USA.
Cyanide was added to the rations in the form of KCN. Quantities added are shown in Table 1.

TABLE 2

PROXIMATE COMPOSITION OF DIETS FED TO RATS ON VARYING LEVELS OF CYANIDE, PROTEIN AND IODINE

Composition (%)		*Adequate protein*				*Protein deficient*			
	Added cyanide (ppm)	0	0	750	750	0	0	750	750
	Potassium iodide (ppm)	(32)	(—)	(32)	(—)	(12)	(—)	(12)	(—)
Dry matter		86·73	87·03	87·17	86·86	86·85	87·05	87·13	86·99
Crude protein		14·63	14·44	14·88	15·31	8·00	8·06	8·13	8·06
Ether extract		5·78	6·57	6·15	6·79	3·53	2·53	2·10	1·75
Crude fibre		2·35	2·39	2·35	2·21	1·80	1·66	1·80	1·63
Nitrogen-free extract		57·91	56·40	57·79	56·90	68·95	70·23	69·79	70·89
Ash		6·06	7·23	6·00	5·65	4·57	4·57	5·31	4·66

Values shown are the means of all determinations carried out on different batches of feed samples offered to the rats throughout the experiment period.

After 56 days on the trials, the rats were anaesthetised with chloroform and blood obtained by heart puncture for determination of serum thiocyanate, proteins using a concentrimeter (product of the American Optical Company instrument Division Buffalo, New York, USA) and protein bound iodine (PBI) by the method of Barker *et al.* (1951). The livers and kidneys were removed, weighed and kept frozen for rhodanese activity determinations by Sorbo's (1951) method as modified by Tewe (1975).

Factorial analysis was carried out on the measured parameters by the method described by Steel & Torrie (1960).

RESULTS

The physiological parameters in rats on the various treatments are presented in Table 3. Metabolic changes are presented in Table 4. A summary of the factorial analysis is shown in Table 5.

Feed consumption, body weight gain and protein efficiency ratio were significantly lowered by protein deficiency. Feed/gain ratio was also significantly increased by protein. Dietary cyanide caused non-significant reductions in feed intake and growth rate while iodine alone had no marked effect on the performance of rats. No effect of the interactions of the various dietary factors was manifested in the performance of the rats.

Statistical analysis on the fresh weight of livers obtained from the rats showed that a deficiency of protein caused a significant ($P < 0{\cdot}05$) reduction in the weight of this organ when expressed as a percentage of total body weight. Weights of kidneys were also significantly ($P < 0{\cdot}01$) reduced by protein deficiency. An interaction of iodine and protein deficiency also caused a significant ($P < 0{\cdot}05$) reduction in the fresh weight of kidneys. Dietary cyanide did not affect the weight of these organs.

Feeding of a protein deficient diet caused a significant reduction in serum protein values, but no effect was observed on serum PBI. PBI values were, however, significantly reduced by iodine deficiency. Dietary cyanide had no significant effect on either of these parameters.

Dietary cyanide significantly increased serum thiocyanate concentration. Protein deficiency significantly reduced this metabolite. Significant reduction in serum thiocyanate was also caused by the interaction of dietary cyanide and iodine deficiency. The interaction of cyanide and protein also has the same effect.

Urinary thiocyanate excretion during the first week of the trial was remarkably reduced by protein deficiency while dietary cyanide had the opposite effect. Only the urine samples of rats on adequate protein diets were analysed in the fourth week of the trial due to the fact that urine excretion was considerably reduced in dietary protein-deficient rats as from the second week of the trial. Chemical analysis of samples collected from rats with adequate protein showed a remarkable increase in

TABLE 3

PHYSIOLOGICAL PARAMETERS IN RATS ON VARYING DIETARY CYANIDE, PROTEIN AND IODINE

Parameters		*Dietary variables*							
	Cyanide (ppm)	0	0	750	750	0	0	750	750
	Potassium iodide (ppm)	(32)	(—)	(32)	(—)	(32)	(—)	(32)	(—)
	Crude protein	15%	15%	15%	15%	8%	8%	8%	8%
Daily feed intake (g)		15·6 ± 1·2	16·0 ± 1·0	15·3 ± 1·0	15·0 ± 0·8	8·3 ± 0·5	7·9 ± 0·4	6·2 ± 0·5	6·2 ± 0·5
Daily weight gain (g)		4·3 ± 0·5	4·5 ± 0·6	4·1 ± 0·4	4·1 ± 0·4	0·63 ± 0·05	0·59 ± 0·06	0·41 ± 0·06	0·50 ± 0·05
Feed efficiency		3·6 ± 0·19	3·6 ± 0·23	3·7 ± 0·17	3·7 ± 0·22	13·2 ± 0·68	13·4 ± 1·49	15·1 ± 1·90	12·4 ± 0·91
Protein efficiency ratio		1·9 ± 0·09	2·0 ± 0·11	1·8 ± 0·08	1·8 ± 0·10	0·93 ± 0·01	0·92 ± 0·10	0·82 ± 0·19	1·00 ± 0·08
Fresh weight of organs (as percent of body weight)									
Liver		4·0 ± 0·1	3·9 ± 0·1	3·8 ± 0·08	3·9 ± 0·09	4·2 ± 0·2	4·0 ± 0·2	4·1 ± 0·1	4·1 ± 0·07
Kidney		0·60 ± 0·02	0·59 ± 0·01	0·59 ± 0·01	0·62 ± 0·03	0·87 ± 0·03	0·82 ± 0·08	0·93 ± 0·05	0·85 ± 0·02

56-day growth study.
Each value represents mean of eight rats per treatment ±SEM.

TABLE 4

METABOLIC CHANGES IN RATS FED VARYING LEVELS OF DIETARY CYANIDE, PROTEIN AND IODINE

Parameters	Dietary variables								
	Cyanide (ppm)	0	0	750	750	0	0	750	750
	Potassium iodide (ppm)	(32)	(—)	(32)	(—)	(32)	(—)	(32)	(—)
	Crude protein	15%	15%	15%	15%	8%	8%	8%	8%
Serum protein (g/100 ml)		6·5 ± 0·07	6·8 ± 0·08	6·4 ± 0·18	6·5 ± 0·18	4·4 ± 0·09	4·4 ± 0·04	4·5 ± 0·04	4·3 ± 0·20
Serum protein bound iodine (PBI) (μg/100 ml)		5·7 ± 0·28	3·7 ± 0·15	5·8 ± 0·20	3·7 ± 0·11	6·1 ± 0·29	3·9 ± 0·13	6·2 ± 0·25	3·9 ± 0·23
Serum thiocyanate (mg/100 ml)		1·70 ± 0·05	1·69 ± 0·08	2·36 ± 0·10	2·73 ± 0·14	2·35 ± 0·04	2·17 ± 0·05	2·50 ± 0·19	2·59 ± 0·17
Urinary thiocyanate* (milligrammes excreted/100 g feed intake)		2·13 ± 0·21	1·96 ± 0·05	5·66 ± 0·96	5·34 ± 0·53	0·75 ± 0·11	0·75 ± 0·34	2·86 ± 0·86	2·07 ± 0·79
Rhodanese activity of tissues (mg thioc./min/g protein)									
Liver		31·4 ± 2·5	31·1 ± 1·9	31·4 ± 1·5	30·2 ± 1·7	32·2 ± 1·8	31·1 ± 1·8	30·5 ± 1·5	31·8 ± 1·5
Kidney		26·2 ± 1·0	24·7 ± 0·9	24·3 ± 1·2	25·0 ± 0·8	23·0 ± 1·0	23·0 ± 1·0	24·4 ± 1·4	23·2 ± 1·0
Protein content of tissues (mg/100 g fresh tissue weight)									
Liver		25·8 ± 0·9	24·0 ± 1·1	23·6 ± 0·6	26·1 ± 1·1	23·6 ± 1·2	24·1 ± 1·2	23·0 ± 0·9	22·3 ± 0·8
Kidney		23·0 ± 1·2	21·9 ± 1·2	21·7 ± 1·0	21·7 ± 0·6	17·7 ± 0·9	19·2 ± 1·4	19·9 ± 1·2	19·0 ± 1·2

* Urinary thiocyanate values are those recorded during the first week of the study period.
± = SEM.

urinary thiocyanate caused by dietary cyanide. The weekly thiocyanate excretions of rats on adequate protein diets are shown in Fig. 1.

Rhodanese activity in the liver was not inhibited by any of the dietary variables or their interactions. In the kidney, the activity of this enzyme was significantly reduced by a deficiency of protein in the diets. Protein concentration of the liver was also significantly reduced by dietary protein deficiency. In the kidney, protein concentration was not only significantly reduced by a deficiency of protein or iodine or by the presence of cyanide in the diets, but also by interactions of iodine and protein deficiencies and interaction of the three dietary variables. No significant differences between sexes was observed in any of the performance and metabolic parameters evaluated in this study.

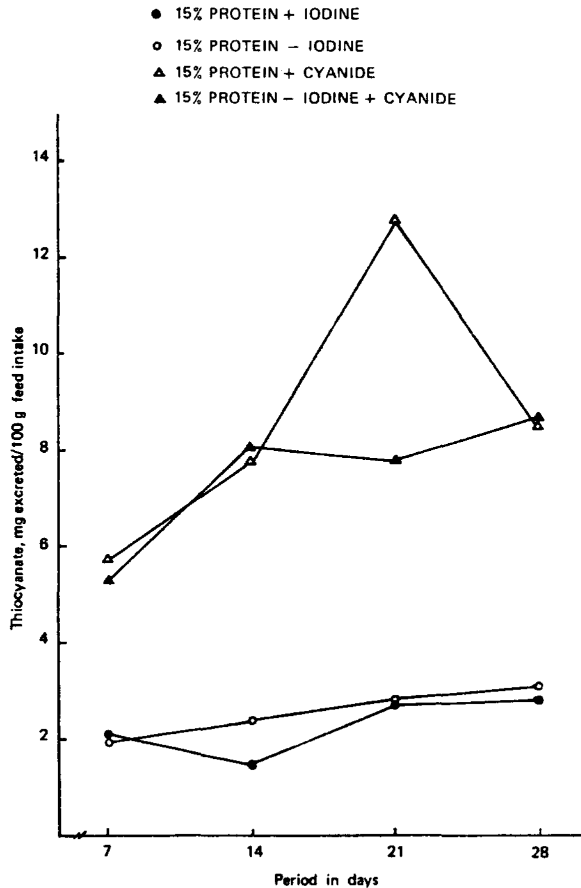

Fig. 1. Thiocyanate excretion of rats on varying dietary cyanide and iodine levels.

TABLE 5
SUMMARY OF FACTORIAL ANALYSIS OF PHYSIOLOGICAL AND METABOLIC TRAITS OF GROWING RATS FED VARYING LEVELS OF CYANIDE, PROTEIN AND IODINE

	Physiological changes			*Metabolic changes*							
Sources of Variation	*Feed intake*	*Body weight gain*	*Feed efficiency*	*Protein efficiency ratio*	*Serum proteins*	*Serum PBI*	*Serum thiocyanate*	*Rhodanese activity*		*Tissue protein*	
								Liver	*Kidney*	*Liver*	*Kidney*
Iodine (A)						**					*
Protein (B)		**	**	**	**		**		*		
Cyanide (C)							**				*
AB							*				
AC							*				
BC							**				
ABC											

56-day growth study.
Blank spaces indicate that means are not significant at the 5 % level.
* Means significant at the 5 % level.
** Means significant at the 1 % level.

DISCUSSION

The results indicate that a sublethal dose of cyanide at the 750 ppm level causes slight depressions in feed consumption and growth rate of rats. Work carried out in the CIAT laboratory by Maner & Gomez (1973) also shows that slight depressions in performance were observed at a level of 480 ppm cyanide in diets for rats. Higher levels (960 ppm and above), however, caused significant changes in feed consumptions and growth rate. Deaths were observed on levels of cyanide above 1480 ppm. The deficiency of iodine in the diets also gave no indication of reduced performance in the rats. Indeed, daily weight gain was slightly higher on the iodine deficient ration compared with the control (15 % protein + iodine). These results are confirmed by that of Hill *et al.* (1974). These workers demonstrated that dietary cyanide up to a level of 1500 ppm KCN (600 ppm cyanide ion) caused growth depressions. These were, however, not significant. They also found that the response of rats to potassium iodide was erratic and seemingly without relation to KCN ingestion.

Protein deficiency appears to be the principal nutritional factor which affects the performance of the rats. It is also remarkable that when protein is adequate in the diets, the lowest weight gain was observed when low iodine level was interacted with cyanide in the feed. Also, on the protein-deficient diet, the lowest body weight gain was in the 750 ppm cyanide + low KI treatment group. The interaction of dietary cyanide, protein and iodine deficiency therefore constitutes a more potent factor in influencing the performance of rats than any of these factors considered singly.

The metabolic studies revealed many interesting relationships. Table 4 shows the serum and urinary thiocyanate concentration in the rats. There was a rise in urinary thiocyanate excretion when the serum thiocyanate concentration was higher. This shows that ingestion of cyanide not only causes an increased excretion of

thiocyanate, but also a corresponding increase in the serum thiocyanate level. Maner & Gomez (1973) observed a rise in urinary thiocyanate of rats on dietary cyanide while Osuntokun (1970) observed a rise in serum thiocyanate in rats on cassava based diets. The observation that protein deficiency significantly lowered urinary thiocyanate excretion and serum thiocyanate concentration confirms the necessity for proteins in the detoxification of cyanide in rats.

Figure 1 shows that the thiocyanate excretion of rats on 15 % protein with dietary cyanide in the diet consistently exceeded the values in corresponding diets with no added cyanide. This suggests that, over this 28-day urine collection period, the processes of cyanide metabolism and thiocyanate excretion were in no way inhibited when 750 ppm cyanide diets were fed. As shown in the same Figure, the excretion of thiocyanate in the group receiving protein + cyanide was much higher than in that which additionally suffered iodine deficiency. The significance of this is, however, not known.

Serum protein was significantly lower on the protein deficient diets. Corresponding protein bound iodine (PBI) values, as shown in Table 4 and statistical analysis, however, indicate that, when dietary iodine is adequate, a reduced serum protein level does not influence the serum PBI. It is known that thyroxine released from the thyroid gland in man and animals is attached to some thyroxine-binding serum protein (Bustad & Fuller, 1970). The normal level of serum PBI observed in dietary protein-deficient rats therefore suggests that the rat can undergo some physiological adaptation which ensures that the thyroid hormone is not excessively lost from the circulatory system even when serum proteins are reduced. The reduction in urine excretion by these groups of rats may aid this retention of PBI in the serum. A low dietary level of iodine, however, caused a significant reduction of the serum PBI, while dietary cyanide had no effect on this parameter.

Rhodanese activity values show that, while the liver was not affected, the rhodanese activity of the kidney was significantly reduced by dietary protein deficiency. This shows variations in the susceptibility of different organs to the influence of dietary factors. Reduction in protein content was found in the liver and kidney. This was caused by protein deficiency in the former while the three dietary variables produced the effect in the latter. While the metabolism of KCN may not give the same quantitative effect as intact cyanogenic glucosides and their hydrolytic products (HCN) from cassava, it should be noted that the metabolism of HCN is closely related to that of inorganic cyanide in mammalian physiological systems (Ermans *et al.*, 1972, 1973).

The result of this experiment therefore suggests the probable importance of the interaction of dietary variables in a number of illnesses observed in areas where cyanogenic plants are widely consumed. It also suggests that, if animals are maintained on adequate protein and iodine in their diets, symptoms of chronic cyanide toxicity may be significantly alleviated should the diets contain ingredients prepared from cyanogenic plants.

ACKNOWLEDGEMENTS

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OPTIMUM CONDITIONS FOR DETERMINING DEPOLYMERISATION OF PECTIC SUBSTANCES WITH THE SUMNER REAGENT*

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ABSTRACT

Using Sumner reagents for determining reducing groups following polygalacturonase activity, high degradation of the polyuranoid polymers was observed. The time, the NaOH concentration and the polyuranoid materials influenced the rate and intensity of colour development. Low esterified and high esterified pectin did not show any difference in colour intensity during Sumner reaction. As the Sumner reaction did not include buffer capacity, the reaction mixture for polygalacturonase activity—which included pectic and organic acids—influenced the colour intensity. $CaCl_2$ *at low concentrations (0·5–2·5 mM) accelerated the degradation of pectin and higher concentrations (2·5–10 mM) inhibited the reaction.*

INTRODUCTION

Polygalacturonase is a well-known and important enzyme in vegetable and fruit processing (Rombouts & Pilnik, 1978). The dinitrosalicylic acid in the Sumner reagent (Sumner & Sisler, 1944) is a useful compound in alkaline media for reducing sugar determination and for following the activity of carbohydrate-hydrolysing enzymes (Riov, 1974; Kanner *et al.*, 1978). A comparison of several methods for measuring end groups of pectin degradation, including the dinitrosalicylic acid/phenol reagent, was carried out by Voragen *et al.* (1971). The polyuranoid polymers, unlike other carbohydrates, are very susceptible to β elimination degradation at alkaline pH levels (Vollmert, 1950; Neukom & Deuel, 1958). Unsaturated compounds, formed by the *trans* elimination reaction, result from the

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removal of the hydrogen atom at C-5 and the glycosidic residue at C-4 (Albersheim *et al.*, 1960).

Studying the activity of polygalacturonase on polygalacturonic acid (PGA) with dinitrosalicylic acid reagent, non-enzymatic degradation of the substrate was observed during the colour reaction. The aim of the present work was to determine the conditions and factors suitable for following polygalacturonase activity with the Sumner reagent.

MATERIALS AND METHODS

Chemicals

Glucose, galacturonic acid, $CaCl_2$, phenol, Rochelle salt, bisulphite and NaOH were obtained from the Sigma Chemical Co. Polygalacturonic acid was obtained from Sigma and ICN and citrus pectin 64 % esterified were obtained from Yakhin Ltd., Israel.

Colour reaction

The colour reaction was developed according to the modified method of Sumner (Miller, 1959). The stock solution included 1 % dinitrosalicylic acid, 0·2 % phenol and concentrations of NaOH between 1 % and 2·5 % (as noted). Bisulphite 0·05 % was added just prior to the addition of the reagent. The colour tests were done with 2 ml of stock solution reagents, 0·1 ml of sodium bisulphite and 2 ml of the carbohydrate solution. The mixtures were heated at 100 °C for 3–15 min, and then 2 ml of 18 % Rochelle salt was added prior to cooling. The colour intensity was measured in a Varian model 635 spectrophotometer at 575 nm. Degradation of pectin and PGA by Sumner reagent as a function of time, NaOH, calcium ions and purity of the material (details given below) were followed at 575 nm and expressed as A (absorbance) 575 nm.

RESULTS AND DISCUSSION

The development of the colour with glucose in the Sumner reaction as a function of time and NaOH concentration is shown in Fig. 1. The intensity of the colour was dependent on the concentration of the NaOH. At all concentrations of NaOH, the colour reaction reached a maximum after 3 min and remained constant for 15 min. Development of the colour with galacturonic acid under the same conditions showed (Fig. 2) that in the Sumner reagent, which contains 1 % NaOH, the maximum intensity of the colour occurred only after 9 min instead of 3 min, as 1·5 % and 2 % NaOH were used. Checking the pH of the reaction mixture in the presence of galacturonic acid showed that the pH dropped at 1 % NaOH but changed slightly at 1·5 % and 2 % NaOH. As the dinitrosalicylic acid reagent developed by Sumner

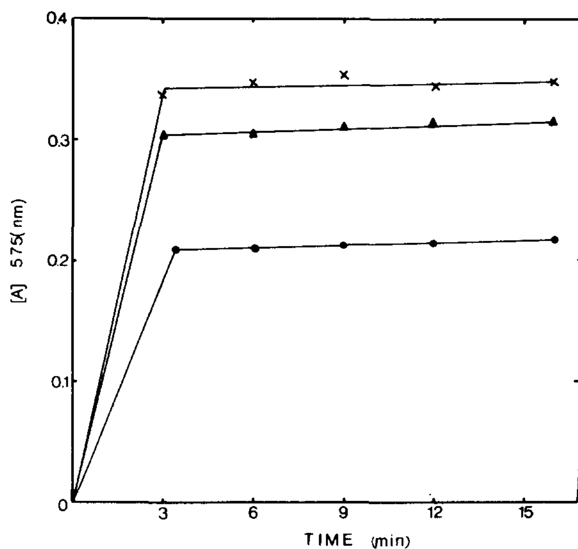

Fig. 1. Effect of time and concentration of NaOH on the development of colour with glucose in the Sumner reaction (see 'Materials and Methods' section). ●, 1 % NaOH; △, 1·5 % NaOH; ×, 2 % NaOH.

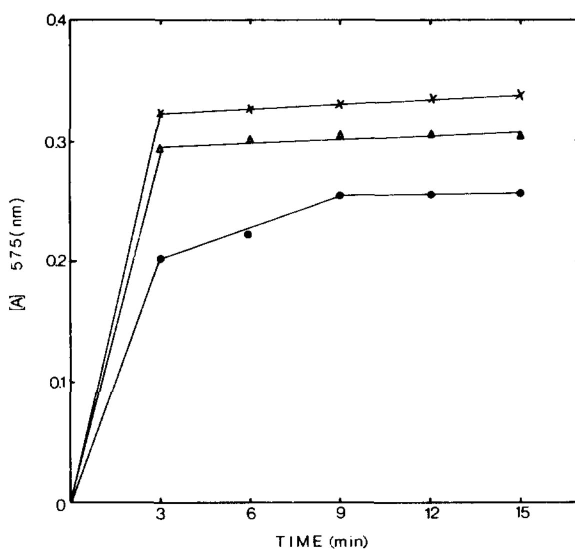

Fig. 2. Effect of time and NaOH concentration on the development of colour with galacturonic acid in the Sumner reaction. ●, 1 % NaOH; △, 1·5 % NaOH; ×, 2 % NaOH.

& Sisler (1944) did not include any buffer capacity, the final pH in the medium was determined by the balance between the concentration of the acidic group in the medium (galacturonic acid) and the basic groups (NaOH).

Miller (1959) found that, when using a neutral sugar, like glucose, 1 % NaOH was the most suitable for colour development. From our results (Figs 1 and 2) it seems that, when working with galacturonic acid instead of glucose, as little as 1·5 % NaOH is sufficient to obtain a colour reaction in a short period.

As plant polygalacturonase activity is determined mainly at mild acidic pH with polyuranoid substrate and a suitable buffer capacity, it seems to be important to check the effect of NaOH on the degradation of pectin and polygalacturonic acid in this medium. Pectin degradation in the presence of 0·1 M sodium acetate at various times and NaOH concentrations is shown in Fig. 3. The rate of pectin degradation increased significantly as a function of time and NaOH concentration. After 3 min the absorbances were 0·34 and 0·53 in 1·5 % and 2·5 % NaOH, respectively, and changed to 0·87 and 1·3 after 12 min. In 1·25 % NaOH, and especially in 1 % NaOH, the colour did not increase at the same rate as at the other concentrations. Checking the final pH of the reaction mixture showed that, in the presence of 1·25 % NaOH, the pH dropped from 12·3 to 10·0 and from 12·0 to 9·0 with 1 % NaOH, but did not drop lower than 12·0 at 1·5 % or higher NaOH concentrations.

Degradation of pectin resulting from a base-catalysed β-elimination reaction is a well known mechanism (Miller, 1959; Rombouts & Pilnik, 1978). Although splitting of pectin chains can occur at neutral pH by the β-elimination reaction (Albersheim *et al.*, 1960), the intensity of colour in the Sumner reaction is strongly dependent on the final pH of the reaction mixture (Miller, 1959). From these results it seems that 1·5 % NaOH is the most suitable concentration to promote the Sumner

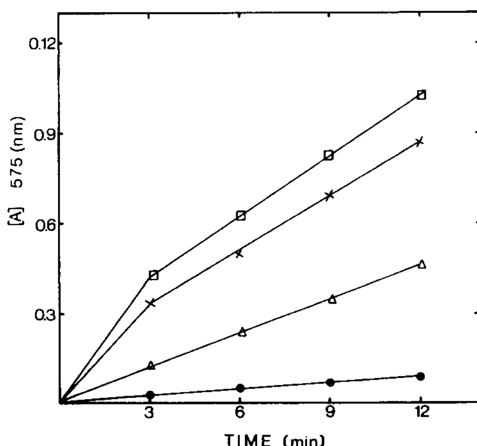

Fig. 3. Effect of time and NaOH concentration on the degradation of pectin in the presence of the Sumner reagent. ●, 1 % NaOH; △, 1·25 % NaOH; ×, 1·5 % NaOH; □, 2 % NaOH.

reaction in the presence of pectin and acetate buffer. Studying the effect of time and of pectin concentration on the intensity of colour in the presence of 1·5% NaOH showed (Fig. 4) that colour increased linearly in relation to the concentration of pectin in the medium and to the time for which the pectin was exposed to NaOH. The absorbance after 15 min rose to 0·17 with 0·1% pectin and to 1·0 with 0·5% pectin.

From these results it seems important to reduce the reaction time for colour development to 3 min and the concentration of pectin to a minimum in order to determine breakdown of the substrate by the *trans* elimination reaction.

Various esterified pectic substances at a concentration of 0·2% were prepared with pectin esterase as described in the 'Materials and Methods' section. The samples, dialysed against buffer citrate-phosphate, pH 6·1, were heated for 30 min at 100 °C and then the absorption was measured at 235 nm using Sumner reagent with NaOH 1·5% for 3 min at 100 °C, and colour intensity was measured at 575 nm.

Exposure of pectic substances with various degrees of esterification to heat treatment at 100 °C for 30 min showed (Table 1) that the intensity of β-elimination increased in relation to the methyl ester content. A significant difference in the absorbance at 235 nm was observed between the non-heated and heated samples. The Sumner reaction, which was carried out with the same pectic samples, did not show any differences in colour intensity.

It has been demonstrated (Albersheim *et al.*, 1960) that esterification is a prerequisite for the rapid degradation of polyuronic acids in neutral solution. These authors also showed that, by heating pectin at neutral pH, de-esterification is also taking place. It seems that, in the medium with the Sumner reaction at high

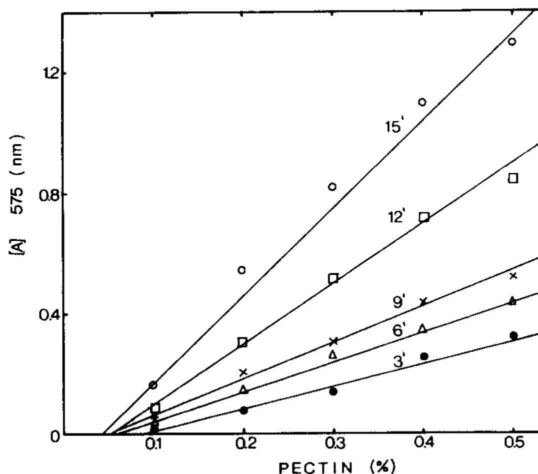

Fig. 4. Effect of pectin percentage and of time on the rate of colour development in the presence of 1·5% NaOH and the Sumner reagent (see 'Materials and Methods' section).

TABLE 1
β-ELIMINATION OF ESTERIFIED CITRUS PECTIC SUBSTANCES

Degree of esterification (%)	*Δ235 nm*[a]	*Absorbance at 575 nm*
21	1·126	0·120
42	1·402	0·115
61	1·491	0·117
73	1·584	0·122

[a] Δ235 nm = Difference in absorbances at 235 nm between heated and unheated samples.

temperature and basic pH, the rate of de-esterification is very high and it is very difficult to show the difference in the overall colour reaction among the samples with various ester contents.

Studying the effect of NaOH on the splitting of the polygalacturonic chain in relation to the source of the material showed (Fig. 5) a great difference in the rate of colour formation between the two samples (white powder with DE 24·4% and yellow powder with DE 16·2%). After 3 min in the presence of 1·5% NaOH and Sumner reagents, the white powder reached an absorbance of 0·02 and the yellow powder, 0·25. Between 3 and 15 min, the slope of the white PGA absorbance curve

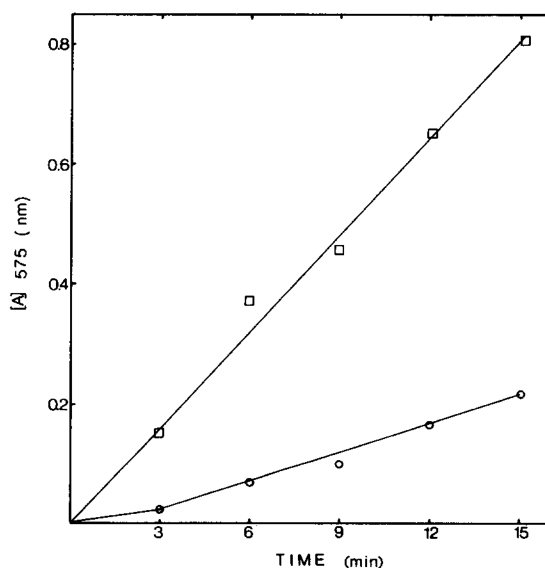

Fig. 5. Effect of two different batches of polygalacturonic acid (○, white powder from ICN with DE 25·4%; □, yellow powder from Sigma with DE 16·2%) on the rate of colour development in the Sumner reaction.

against time (0·015 units/min) was about three times less steep than that of the yellow PGA. As all the initial reducing groups of the powders were reacting during the first 3 min (as shown in Fig. 2), we cannot explain the difference between the two slopes after that period.

The calcium ion is used as a co-factor for the activity of the enzyme polygalacturonase (Pressy & Avants, 1976). It was important to study its rôle during the Sumner reaction. From Fig. 6 it can be seen that a concentration lower than 0·5 mM $CaCl_2$ did not influence the reaction. 2·5 mM accelerates the degradation of

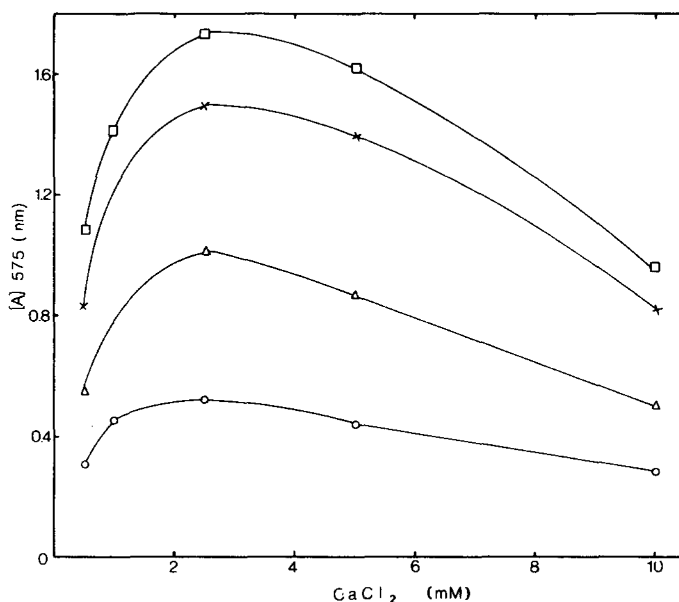

Fig. 6. Effect of calcium ions on the degradation of different concentrations of polygalacturonic acid in the presence of 1·5% NaOH. ○, 0·2% PGA; △, 0·3% PGA; ×, 0·4% PGA; □, 0·5% PGA.

pectin, but a concentration higher than 2·5 mM (as high as 10 mM) inhibited it. This phenomenon was reproducible with a pectin concentration between 0·2% and 0·5% (Fig. 6). Keijbets & Pilnik (1974) showed that the *trans* elimination reaction of pectin at pH 6·1 was accelerated in the presence of calcium ions and that the reaction was more intense as the ratio between the calcium and the pectin increased.

CONCLUSIONS

In order to follow the degradation products of polygalacturonase with the Sumner reagent, it is important to work in a medium which contains not less than 1·5% NaOH and to develop the colour for no longer than 3 min. It does not make any

difference whether low esterified or high esterified pectin is used in terms of colour intensity in the Sumner reaction. As the impurity of the substrate and calcium ions were found to affect the degradation of the polyuranoid polymers, it is important to use the right blanks and to choose the correct substrates.

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TRIMETHYLAMINE *N*-OXIDE DEMETHYLASE: ITS OCCURRENCE, PROPERTIES, AND RÔLE IN TECHNOLOGICAL CHANGES IN FROZEN FISH

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ABSTRACT

Trimethylamine N*-oxide (TMAO) is a natural, characteristic component of muscle tissues and visceral organs of sea fish and invertebrates. In about thirty species of fish and eight species of invertebrates TMAO is broken down post mortem to dimethylamine (DMA) and formaldehyde (FA). The enzyme catalysing this reaction is present in the muscles, skin and visceral organs. The available published information indicates that the enzymes differ in molecular weight and requirements of cofactors, depending upon the source of isolation. However, glutathione, ferrous chloride, ascorbic acid, methylene blue and flavonucleotide activate the demethylase from various sources. The optimum pH for TMAO demethylation is from 5·0 to 7·5.*

Freezing and frozen storage do not destroy the activity of TMAO demethylase. The rate of enzymatic cleavage of TMAO to FA and DMA depends on the substrate and FA.

In numerous observations it was found that during frozen storage of fish belonging to the Gadoid family a progressive accumulation of DMA and FA takes place and is accompanied by a decrease in protein extractability and deterioration of the textural properties of the flesh.

INTRODUCTION

Trimethylamine-*N*-oxide (TMAO) is a characteristic component of muscle tissues and visceral organs of sea fish and invertebrates. In fish flesh its concentration depends on the fish species and size, as well as on the season and region of catch (Dyer, 1952). The highest content of TMAO was found in tissues of elasmobranch

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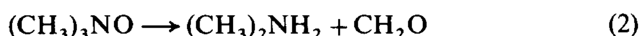
fishes—from 500 to 1500 mg/100 g; large amounts were reported also in the muscles of squid and Gadidae (Ikeda, 1980). TMAO is synthesised in the fish bodies but can possibly be also absorbed via marine animals from the lower end of the food chain (Bickel, 1969; Ikeda, 1980); there is published evidence of high concentrations of both TMAO and trimethylamine (TMA) in some species of seaweed. In fish tissues TMAO plays the rôle of an osmotic pressure regulator (Bentley, 1971; Love, 1970) and in the dark lateral muscles of some pelagic fish it takes part in oxido-reduction processes (Kawabata, 1959; Tokunaga, 1970*b*).

The post-mortem cleavage of TMAO proceeds in two directions: reduction (eqn. (1)) and demethylation (eqn. (2)):

$$EH_2 + (CH_3)_3NO \longrightarrow (CH_3)_3N + H_2O + E \quad (1)$$



$$(CH_3)_3NO \longrightarrow (CH_3)_2NH_2 + CH_2O \quad (2)$$

TMAO reductase is present in the dark lateral muscles of several fish species (Kawabata, 1959; Tokunaga, 1970*b*) and in the cells of some species of bacteria within the family Enterobacteriaceae and other Gram-negative facultatively anaerobic rods. The rate of TMA formation is a function of the number of bacteria and is reflected in the change of sensory quality of ice-stored fish (Farber, 1965). The products of TMAO demethylation, i.e. dimethylamine (DMA) and formaldehyde (FA), occur in several species of marine organisms. The presence of TMAO-demethylase in marine fish and invertebrates has aroused the attention of food technologists and chemists. One of the products of the enzyme catalysed reaction, DMA, may be a useful criterion for following deterioration in a few species of frozen-stored fish. On the other hand, FA can be a factor accelerating 'freezing denaturation' and drastic decrease in the technological value of frozen fish meat (Gill *et al.*, 1979; Sikorski *et al.*, 1976).

OCCURRENCE AND ACTIVITY OF TMAO-DEMETHYLASE IN FISH AND SHELLFISH

Occurrence in different marine organisms

TMAO demethylation products were found in different fish and invertebrates belonging to many species (Amano & Yamada, 1964, 1965; Bremner, 1977; Castell *et al.*, 1970; Dingle & Hines, 1975; Flores & Crawford, 1973; Harada, 1975; Sikorski *et al.*, 1975; Tokunaga, 1964; Tokunaga, 1970*a*; Yamada, 1968; Yamada & Amano, 1965*a*). However, so far no systematic research has been carried out aimed at disclosing the reasons for the presence of TMAO-demethylase in various marine organisms. Investigators were mainly interested in commercially important species of fish and invertebrates, abundant in the Atlantic and Pacific, off the coast of Japan, Canada and the USA. Thus, the TMAO-demethylase was found in

representatives of thirty species of fish belonging to ten families and eight species of invertebrates.

The most numerous groups of fish, in which the products of TMAO demethylation accumulate post mortem, belong to the species Gadidae, Myctophidae, Thunnidae and Scombridae (thirteen, four, three and three representatives, respectively).

The activity of the TMAO-demethylase depends on the fish species and the season of the year. Castell *et al.* (1970) demonstrated that the quantities of DMA produced in fish belonging to the Gadidae species were small in cod and haddock, and increasingly larger in pollock, cusk and hake. The rate of DMA formation was about twice as high in mince prepared from large, spawning, summer-caught silver hake than in comparable material from smaller, winter-caught fish (Lall *et al.*, 1975). However, the age, sex and body length of the fish had no influence on DMA accumulation during frozen storage of Alaska pollock (Tokunaga, 1964).

The published information regarding the occurrence of DMA and FA in marine invertebrates is incomplete and inconsistent. Castell *et al.* (1970) found that DMA was not produced in frozen lobster, shrimp and scallop muscles stored at −5 °C for 50 days. However, other authors indicated that at 0 °C DMA accumulated in the muscle of Pacific shrimp (Flores & Crawford, 1973), in the visceral homogenates of two species of bivalvian molluscs (Harada, 1975) and in the hepatopancreas of five species of shellfish, including abalone, oysters and blue crab (Yamada & Amano, 1965*a*).

Anatomical parts

The products of TMAO demethylation have been found in the viscera, in the muscles and in the skin of sea fish and invertebrates. The activity of the TMAO-demethylase is different in particular organs or tissues. Harada (1975) indicated that in the case of some species of fish the content of DMA and FA was 40–100 times higher in the viscera than in the muscles. Amano & Yamada (1965) and Yamada & Amano (1965*a*) reported that in several species of the Gadoid family the difference in the content of demethylation products between the viscera and the muscles was 2–20 fold. Similarly, in five species of shellfish the demethylation products were detected in the hepatopancreas only but not in the muscles (Yamada & Amano, 1965*a*). The activity of the TMAO-demethylase is significantly greater in the dark lateral muscles than in the ordinary muscles in different species of fish. During storage at −6 °C and 0 °C the content of DMA increased only in dark muscles of some species of Thunnidae and Scombridae, being constant at a very low level in the white muscles (Tokunaga, 1970*b*). In Gadidae there appeared to be a correlation between the concentration of DMA formed in the frozen stored fillets and the amount of dark lateral muscles. When the latter were removed from the fillets before freezing the formation of DMA during frozen storage was either inhibited or greatly reduced (Castell *et al.*, 1971).

Protein fraction

The TMAO-demethylase from the muscle and intestines of fish and shellfish occurs in the water-soluble protein fraction. DMA and FA accumulate very rapidly in the presence of TMAO in the supernatant obtained by centrifuging homogenates of cod pyloric caeca with water, of the liver of *Lotella phycis* (Yamada & Amano, 1965*b*,*c*) or of the viscera of the bivalvian mollusc *Barbatia virescens* (Harada & Yamada, 1971). On the other hand, in cod mince washed three times with water before freezing and containing added TMAO, no FA and DMA were observed during frozen storage at $-18\,°C$ (Sikorski *et al.*, 1975). Similarly, the DMA level after 17 days at $-5\,°C$ was six times lower in washed than in unwashed minces from cod frames (Dingle & Hines, 1975).

Enzymes isolated from various sources can be detected in different fractions of water-soluble proteins, as, depending upon the kind of tissue and organism, different amounts of $(NH_4)_2SO_4$ are required to precipitate them. The enzyme isolated from the pyloric caeca of Alaska pollock (Yamada *et al.*, 1969) or the muscle tissue of cod (Kostuch & Sikorski, 1977) precipitated when the degree of saturation with $(NH_4)_2SO_4$ was 33 % and 66 %, respectively.

STRUCTURE AND PROPERTIES OF THE ENZYME

Cofactor

Investigations of the structure of the TMAO-demethylase are fragmentary and refer only to some of the known sources of the enzyme. They deal mainly with the presence and characteristics of a non-protein cofactor. Only Harada (1975) isolated and partially purified the enzyme from the liver of lizardfish. As the result of separation on Sephadex G-50, two active protein fractions and a non-protein cofactor were obtained.

The presence of a cofactor was found in various tissues of different marine animals: in the pyloric caeca of Alaska pollock (Yamada *et al.*, 1969), in the liver of some teleosteans, and in the mid-gut gland of some species of molluscs belonging to Bivalvia or Gastropoda (Harada, 1975) and in the liver of shark which has no TMAO-demethylase (Yamada *et al.*, 1969). However, the participation of a cofactor was not recognised in the biological formation of DMA and FA from TMAO by the crude enzyme isolated from *Barbatia virescens* (Harada & Yamada, 1971). The cofactor was weakly bound to the enzyme isolated from the pyloric caeca or the muscle tissue of Alaska pollock as it was possible to separate it from the protein by dialysis (Tokunaga, 1964; Yamada & Amano, 1965*c*). The cofactor isolated from the pyloric caeca of Alaska pollock is of a heat tolerant nature, as heating at 100 °C for 30 min destroyed none of its activity (Yamada *et al.*, 1969). The investigations by Harada (1975) suggested that the cofactor isolated from the liver of lizardfish might be a flavonucleotide. The activity of TMAO-demethylase was found to increase in the presence of $FMNH_2$ and $FADH_2$.

Effect of activators and inhibitors

The demethylation of TMAO to DMA and FA does not depend on the presence of oxygen (Babbitt *et al.*, 1972; Harada & Yamada, 1971). Different compounds are activators and inhibitors of enzymes isolated from various sources. In fish and shellfish tissues methylene blue enhances the enzyme activity (Harada, 1975; Kostuch & Sikorski, 1977; Yamada & Amano, 1965*c*). In 0·001 M concentration in homogenates of pyloric caeca, of muscle tissue of Alaska pollock and of the liver of *Lotella phycis* it caused a considerable increase in enzyme activity, the effect depending on the kind of tissue (Yamada & Amano, 1965*c*).

Sarcoplasmic proteins isolated from cod muscle decomposed TMAO appreciably faster in the presence of ferrous chloride, ascorbic acid and methylene blue at concentrations from 0·5 to 5 mmoles/100 mg protein than in control samples. The effect of these activators increased very significantly with their concentration (Kostuch & Sikorski, 1977). The addition of cod blood to minced cod fillet caused a small but consistent increase in the rate of DMA production (Dingle & Hines, 1975). The fact that compounds changing the redox potential accelerate the biological decomposition of TMAO and that the cofactor is probably a flavonucleotide suggests that the reaction takes place with the participation of oxidoreductases.

Cyanide in 1 μM concentration and 0·1 M riboflavin, sodium azide and oxaloacetate completely inhibit the production of FA and DMA from TMAO by a crude enzyme preparation from the viscera of *Barbatia virescens* (Harada & Yamada, 1971). In smoked cod the rate of FA production during frozen storage was found to decrease with the concentration of phenols in the tissues and was, in the surface layers of the fish, very significantly lower than in the deeper parts and in unsmoked samples (Moini & Storey, 1980). There is an apparent strong relationship between the reciprocal of the rate of accumulation of FA and the concentration of phenol in smoked frozen cod, although non-phenolic smoke components, not determined in the experiments described, may also have contributed to the results.

The variety of compounds changing the reaction course suggests that demethylation of TMAO proceeds in several stages.

Effect of pH and temperature

The optimum pH for demethylation of TMAO is between 5·0 and 7·5 and depends on the kind of organism and tissue (Fig. 1). The influence of pH is also very distinct in frozen media.

The TMAO-demethylase is resistant to freezing and frozen storage but is inactivated by heating. In the viscera of Alaska pollock and cod stored for a year at −80 °C no significant loss in the enzyme activity was found after thawing (Yamada & Amano, 1965*b*); however, the demethylation did not occur below −30 °C (Harada, 1975; Tokunaga, 1974).

Optimum temperature for enzymatic production of DMA and FA depends on

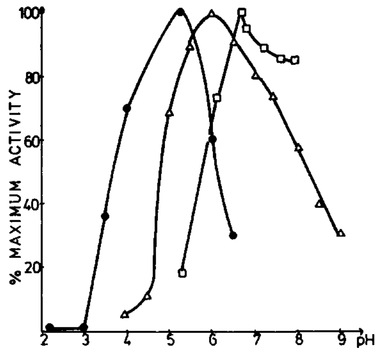


Fig. 1. Effect of pH on the activity of trimethylamine *N*-oxide demethylase. ● *Barbatia virescens* visceral homogenate, 45 °C, 120 min (Harada & Yamada, 1971). △ *Pseudomonas aminovorans*, 0 °C, 30 min (Large, 1971). □ Cod muscle albumins, 20 °C, 24 h (Kostuch & Sikorski, 1977).

the origin of the enzyme (Fig. 2). Heating the muscle tissue of Alaska pollock for 30 min at 40 °C before freezing caused a 20 % decrease in FA production after 4 weeks at −17 °C to −19 °C and diminished almost totally when preheating was carried out at 50 °C (Tokunaga, 1964). Preheating of fillets and minced flesh of silver hake up to 60 °C had little or no effect on the rate of DMA formation during subsequent storage for 1 month at −10 °C while heating to 80 °C was found to arrest DMA formation totally (Lall *et al.*, 1975).

Effect of concentration of the substrate and the reaction products on activity

The enzymatic cleavage of TMAO depends on the concentration of the substrate and one of the products—FA.

In crude enzyme extract isolated from the liver of lizardfish it was found that there

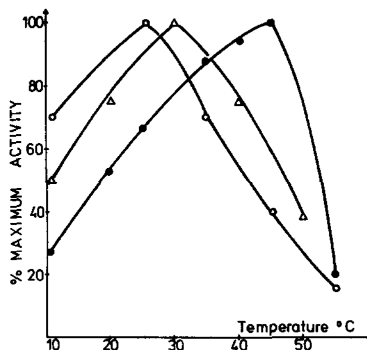


Fig. 2. Effect of temperature on the trimethylamine *N*-oxide demethylase. ○ Alaska pollock pyloric caeca (Yamada & Amano, 1965*b*). ● *Barbatia virescens* visceral homogenate (Harada & Yamada, 1971). △ Lizard fish, liver (Harada, 1975).

is a maximum on the curve reflecting the influence of TMAO concentration on the accumulation of DMA and FA (Harada, 1975). No similar type of dependence was found in the muscle tissue of many other species of fish. The addition of TMAO in amounts of 500 and 1000 mg/100 g to minced cod flesh did not influence the rate of DMA and FA accumulation during frozen storage (Kostuch & Sikorski, 1977). Tokunaga (1970*b*) found no correlation between the natural TMAO concentration and the accumulation of DMA of different species of fish. On the other hand, in our experiments a depletion of TMAO before freezing to about 30 mg/100 g by prolonged ice storage caused a significant decrease of DMA accumulation compared with results obtained in control samples (Kostuch & Sikorski, 1977).

The addition of DMA to minced cod flesh or to crude enzyme isolated from the liver of lizardfish in concentrations from 50 to 150 mg/100 g and from 7 to 28 μg/100 g, respectively, did not influence the rate of TMAO cleavage (Harada, 1975; Kostuch & Sikorski, 1977). However, similar amounts of FA considerably decreased the TMAO-demethylase activity. The addition of FA to cod flesh (from 10 to 100 mg/100 g) and to the crude enzyme preparation from lizardfish (from 4·5 to 18 μg/100 g) decreased the enzyme activity by 25 % to 95 % and 30 % to 50 %, respectively (Harada, 1975; Kostuch & Sikorski, 1977). These results suggest that increasing the concentration of products does not influence the equilibrium while FA changes the enzyme properties, probably by denaturation.

THE RÔLE OF TMAO-DEMETHYLASE IN CHANGES OF THE TECHNOLOGICAL PROPERTIES OF FROZEN FISH

Numerous observations revealed that during frozen storage of Gadidae the progressive accumulation of DMA and FA is accompanied by a decrease in protein extractability (PE). In the fillets of Alaska pollock stored for 60 days at temperatures ranging from −5 °C to −40 °C the correlation coefficients between FA concentration and PE are from −0·73 to −0·81. At DMA concentrations from 6·4 to 8·0 mg/100 g or FA content from 4·0 to 5·0 mg/100 g the extractability of myosin decreased by about 50 % (Tokunaga, 1974). There was a strong correlation between the level of FA and DMA and the textural changes in minced blocks of hake flesh after 6 months at −26 °C (Crawford *et al.*, 1979).

It has been demonstrated in model investigations that DMA does not influence the properties of proteins whilst, in the presence of FA, aggregation of fish muscle proteins and a drastic change in their technological value take place. After 24 hours' storage of cod flesh with 5 to 20 mg of FA per 100 g the myofibrillar PE decreased by 5 % and 40 %, respectively (Castell *et al.*, 1973). When the concentration of added FA in the flesh was 100 mg/100 g the emulsifying capacity dropped by 30 % (Childs, 1974) and the solubility in SDS by 55 % (Connell, 1975). Electrophoretic investigations demonstrated that tropomyosin and heavy meromyosin totally lost

their mobility at an FA concentration of 40 mg/100 g (Childs, 1973), while electron microscopy studies showed changes in the shape of muscle proteins from filamentous at FA concentrations between 0 and 0·2 g/100 g to granular at FA 0·4 g/100 g (Ohnishi & Rodger, 1980). In addition, FA produced in red hake induced covalent cross-linking of tropomyosin and myosin light chains and formation of higher molecular weight aggregates (Gill *et al.*, 1979). In numerous experiments a strong correlation between the rate of FA accumulation in fish flesh and the freezing changes in proteins has been demonstrated. In minced cod flesh containing added kidney homogenate DMA accumulated after 6 days at −5 °C in six times higher concentration and PE was four times lower than in the control samples (Dingle & Hines, 1975). Similar results were obtained when cod kidney homogenate was added to the flesh of flounder and stored at −5 °C. There was a strong production of DMA and a sharp drop in PE in this mixture within 10 days whilst, in the samples without cod kidney, no changes were noticed (Dingle & Hines, 1975). In cod flesh containing very little TMAO (about 30 mg/100 g) the accumulation of DMA was very low and PE did not change considerably (Fig. 3). The production of DMA in such flesh was 6 mg/100 g and PE was reduced by 20 %, while in samples containing 230 mg TMAO/100 g after 40 days at −18 °C the concentration of DMA was 17 mg/100 g and PE was lowered by 80 % (Kostuch & Sikorski, 1977).

The results of biochemical and technological investigations indicate that TMAO and its enzymatic cleavage before and after freezing have a very significant influence on the technological value and texture of the flesh of several commercially

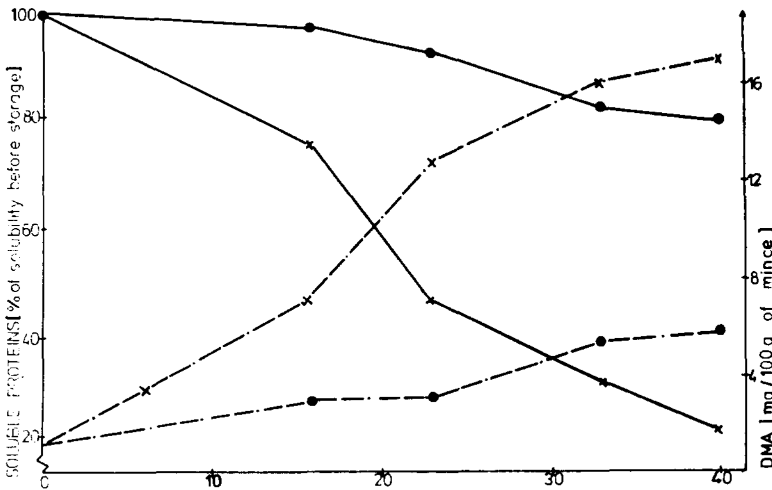


Fig. 3. The influence of the initial concentration of TMAO on the accumulation of DMA (– – · – – ·) and protein solubility (——) in frozen stored cod mince. ●—30, ×—230 mg TMAO/100 g of mince (Kostuch & Sikorski, 1977).

important species of marine fish. By applying proper handling procedures it is possible to minimise the adverse effects exhibited by the TMAO demethylation product.

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INTENSITY/TIME STUDIES OF SWEETNESS: PSYCHOPHYSICAL EVIDENCE FOR LOCALISED CONCENTRATION OF STIMULUS

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ABSTRACT

Salivary glucose concentrations, following tasting of a 10 ml glucose solution, declined to sub-threshold levels sooner than the elicited persistence of sweetness response. Thus persistence cannot be explained by residual stimulus in the oral fluid. Panellists experience 'saturation of sweetness intensity' at lower concentrations of sugar than those which cause 'saturation of persistence', which suggests that separate mechanisms are responsible for the intensity and persistence effects. Increases of volume from 10^{-2} ml to 1 ml of tasted solution, as well as increases of concentration, cause elevation of both intensity and persistence of sweetness although volume change does not affect intensity of response at low concentrations. These observations can be explained by assuming that the persistence of sweetness response is caused by localised concentrations of stimulus at or near the receptor while intensity is governed by the accessibility of receptors to stimuli at these sites.

INTRODUCTION

The problem of persistence of sweet response is of practical and theoretical interest (Birch, 1979; Birch *et al.*, 1980; Hough *et al.*, 1978; Dubois *et al.*, 1977; Larson Powers & Pangborn, 1978; Lawless & Skinner, 1979; Swartz, 1980). Many new intense sweeteners exhibit long persistence of response which may preclude their use in foods without modification. On the other hand, this temporal characteristic may constitute an important clue as to the mechanism of taste chemoreception (Birch, 1980; Birch *et al.*, 1980). One explanation of persistence of taste might simply be the

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physical retention of stimulus in oral fluid whilst another might be the binding of the stimulus, with greater or less affinity, at the receptor. The former should be directly examinable by correlation of the kinetics of taste persistence with the rate of disappearance of stimulus from saliva whilst the latter demands some form of stimulus/receptor interaction which should result in discrete types of response. In either case, careful study of the kinetics of sweet taste response should allow tentative deductions about the physical process of sweet taste chemoreception in terms of the concentration and volume of stimulus and the intensity and time of the taste experience.

It is known that 'saturation of sweetness intensity' (i.e. point of maximum intensity of sweetness discernible by panellists) occurs in sucrose solutions of about 35–40 % w/v concentration (Birch *et al.*, 1978; Schutz & Pilgrim, 1957) but it is not known whether a similar effect might be derived by increasing volume of tastant solution at a fixed concentration. Such a result might allow deduction of receptor availability in terms of the diffusion characteristics of stimuli and have importance for the determination of taste threshold. With this in mind we have measured the intensity and persistence of sucrose sweetness over a range of concentrations and volumes. Separately we have compared the persistence of sweetness of glucose solutions with the rate of disappearance of glucose from saliva.

MATERIALS AND METHODS

Glucose used in this study was A. R. Grade (Fisons, Loughborough, Great Britain). Sucrose was AR grade (May and Baker, Dagenham, Great Britain). Glucose determinations in saliva were carried out by the Boehringer GOD Perid method after deproteinising with 0·33M perchloric acid (Birch & Ray, 1979). Saliva was collected from subjects by asking them to spit into a small empty beaker once every 15 s for 5 min, then once every 30 s for a further 5 min, then once every minute for a further 10 min and finally once every 5 min for a further 10 min. Saliva (0·1 ml) was then added to perchloric acid (1·0 ml, 0·33M) for deproteinisation, then assay. Tap water was used for all ingested solutions. All experiments involved solutions at room temperature (17–22 °C).

Panellists for the glucose threshold studies, persistence studies and the salivary glucose concentration studies consisted of the same four subjects (three males and one female). All four were selected for their familiarity with sweet taste experiments. For threshold studies each panellist (previously fasted for 3 h) was presented with 3·0 ml of glucose solution at five different concentrations (0·25, 0·5, 1·0, 2·0 and 4·0 % w/v) at one session. Panellists tasted each sample in turn by the sip and swallow method, tap water being used for all tasted solutions; panellists tasted samples (clean white plastic cups) in order of concentration, weakest first, pausing

one minute with a tap-water rinse between samples. Each panellist returned for a total of four sessions in this experiment, making sixteen judgements of sweetness, each sample being categorised on the scale (1–7) where 1 = same as water and 7 = clear–sweet. Threshold was then calculated by the method of Gregson (1962) using cumulative proportions.

Salivary glucose concentration studies involved the same four panellists as in the threshold studies. Panellists tasted and swallowed 10 ml of glucose solution at concentrations of 10, 20, 40 and 70 % w/v concentration. Each concentration required one complete session for each panellist and panellists returned at weekly intervals for testing each concentration. Samples were presented in clean white plastic cups identified by a three-digit random code number.

An initial saliva sample was always taken before ingestion of the glucose solution, then at timed intervals afterwards as described above. Results were calculated as mean concentrations above the mean initial level.

For the persistence time of glucose sweetness the same four panellists were used as for the threshold and salivary glucose concentration studies. Panellists tasted and swallowed 10 ml of glucose solution at concentrations of 10, 20, 40 and 50 % w/v and recorded the end of sweetness sensation with the aid of a stop-clock. Samples were again presented in clean white plastic cups identified by a three-digit random code number and the order of presentation was randomised using a 4 × 4 Latin square design. All four concentrations were tested by a panellist at one session with a 5 min statutory pause with a tap-water rinse between samples.

For the concentration/volume studies using sucrose, ten panellists were selected from a pool of fifteen on a random basis. All panellists were screened only for their ability to rank 10, 20 and 30 % w/v sucrose solutions in the correct order of sweetness. The ranges of concentrations investigated were from 15–55 % w/v sucrose (tap water) for intensity studies and 1–70 % w/v sucrose (seven steps of 10 %) (tap water) for persistence studies. Volumes of solution tested ranged from 1×10^{-2} ml to 10 ml (seven steps). Order of presentation of samples for a given set of tests was randomised. For the reaction time (beginning of sweetness) and persistence time (total duration of sweetness) panellists signified the two parameters (measured from administration of stimulus) with the aid of a stop-clock. All solutions were pipetted directly onto the anterior third of the extended tongue as quickly as possible. Panellists were asked to taste and swallow as normal. Each panellist tested all forty-nine solutions at one session pausing 1 min with a tap-water rinse between samples. For the intensity determinations a form of category scaling was employed. Panellists were presented with 10 % w/v and 20 % w/v sucrose solutions at the same volume as the test samples under investigation. They were told to categorise the intensities of these solutions at 10 and 20, respectively. They then were asked to categorise two unknown solutions on the scale 0 = zero sweetness up to 100 = intensely sweet in relation to the two previous sucrose solutions. Only two test solutions were determined in this way at each session by a panellist.

RESULTS AND DISCUSSION

Persistence of sweetness and residual glucose in saliva

Table 1 lists the individual results of four panellists for determination of the threshold of D-glucose solution. Plotting of total scores for the category scale (1–7) at each of the tested concentrations on arithmetic probability paper by the method of cumulative proportions (Gregson, 1962) gave a threshold for the fiftieth percentile of 0·85 % w/v of D-glucose. The concentration of the glucose remaining in the saliva of each of the four panellists was then determined as a function of time, following tasting and swallowing 10 ml of D-glucose solution at concentrations of

TABLE 1

THRESHOLD DETERMINATION OF D-GLUCOSE SWEETNESS. RATINGS ARE ON SCALE 1 = SAME AS WATER TO 7 = CLEAR–SWEET TASTE

Subjects	*Concentration of glucose solution (% w/v)* 0·25	0·5	1·0	2·0	4·0
	Ratings given on four occasions				
A	2, 1, 2, 3	1, 6, 3, 3	4, 2, 4, 3	5, 3, 6, 5	7, 7, 7, 7
B	2, 1, 2, 1	3, 2, 5, 2	5, 6, 3, 3	6, 5, 7, 5	7, 6, 7, 7
C	1, 2, 1, 1	2, 1, 1, 2	1, 3, 4, 3	4, 4, 6, 3	7, 4, 8, 7
D	1, 1, 2, 1	1, 1, 3, 2	3, 4, 2, 4	4, 6, 4, 6	6, 7, 7, 7
Number of panellist judgements with a rating ≥4	0	2	7	14	16
Cumulative proportion (%)	0	12·5	43·75	87·5	100

10 %, 20 %, 40 % and 70 % w/v. Means of the four panellists are illustrated in Fig. 1 which show that sub-threshold concentrations of glucose occur in saliva almost immediately following the drinking of 10 % or 20 % glucose solutions whereas concentrations in saliva following 40 % and 70 % glucose solutions take less than 1 min to reach sub-threshold levels. The persistence time increases with concentration (Fig. 2) from 20 s (at 10 % w/v of glucose in the test solution) up to 106 s (at 50 %). More concentrated solutions (e.g. 70 %) were not tested due to the nauseous reaction which they caused in panellists. Comparison of Figs 1 and 2 shows that persistences of sweetness of 10, 20 or 40 % w/v of glucose continued after the salivary glucose concentration had declined to sub-threshold levels. For example, after the 20 % w/v glucose solution both the 15 s (first) and 30 s (second) saliva samples were below 0·35 % w/v glucose (*cf.* threshold concentration = 0·85 % w/v glucose) although the persistence continued for 33 s (Table 2) after tasting and drinking this sample.

These results show that, if stimulus/receptor interaction is responsible for persistence, a localised suprathreshold concentration of glucose must still exist in the microenvironment of the receptor during the period of persistence of sweetness. This conclusion supports the idea of an ordered localisation of stimuli near the

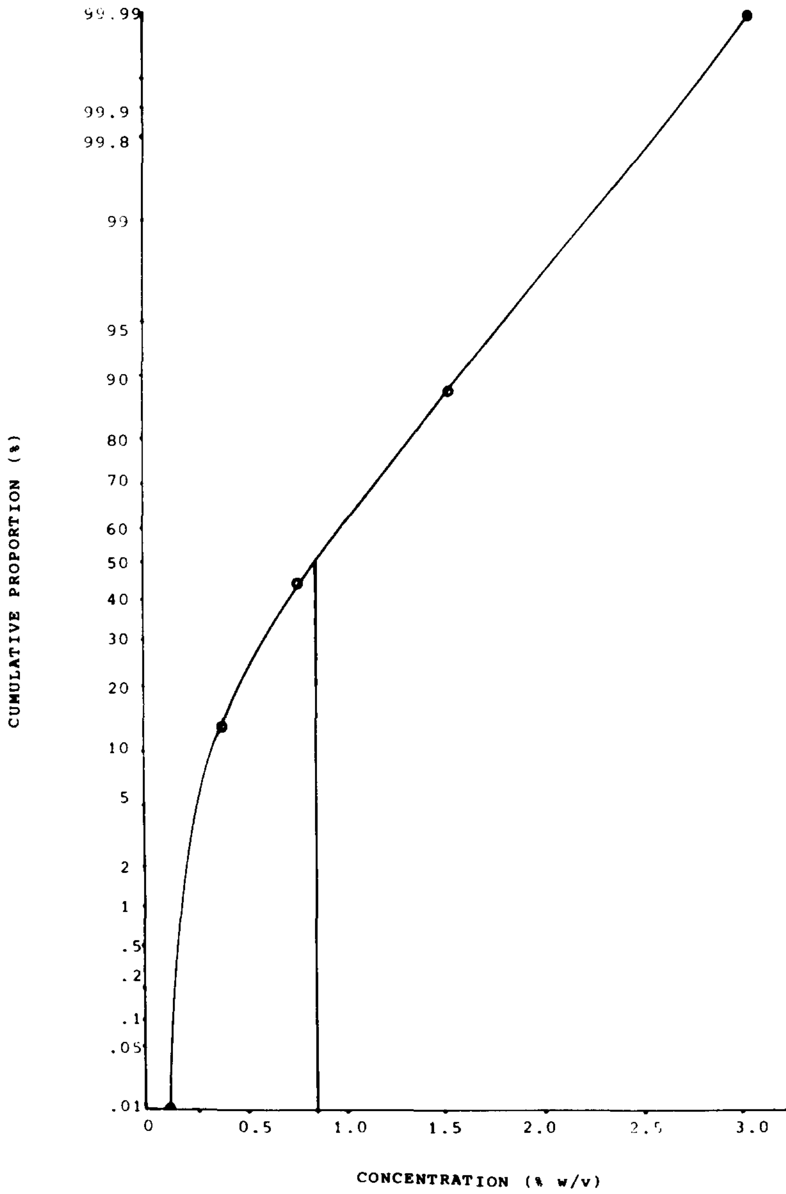
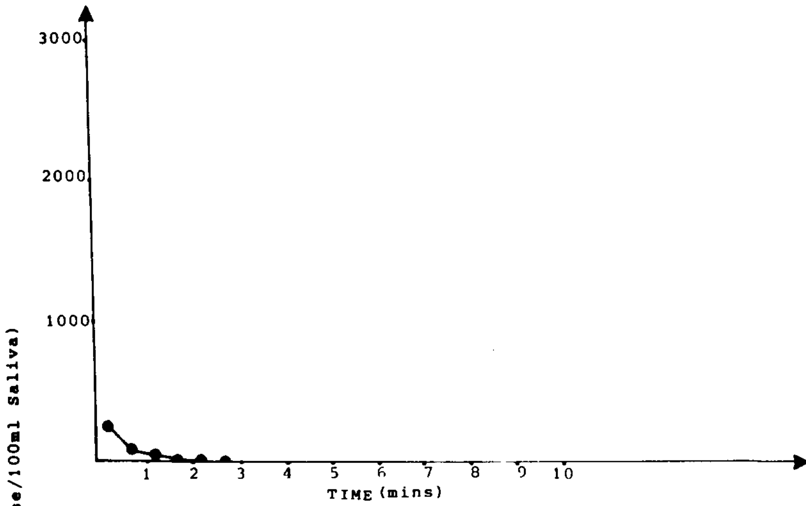


Fig. 1. Determination of glucose threshold by cumulative proportions (Gregson, 1962) (four panellists).



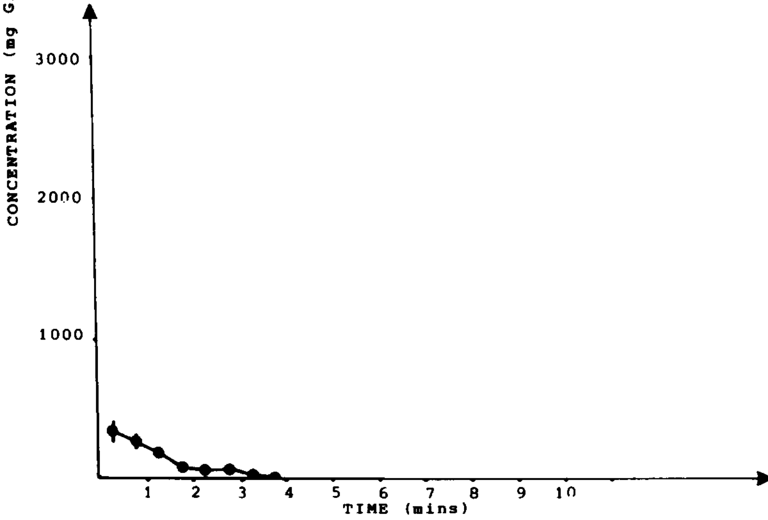
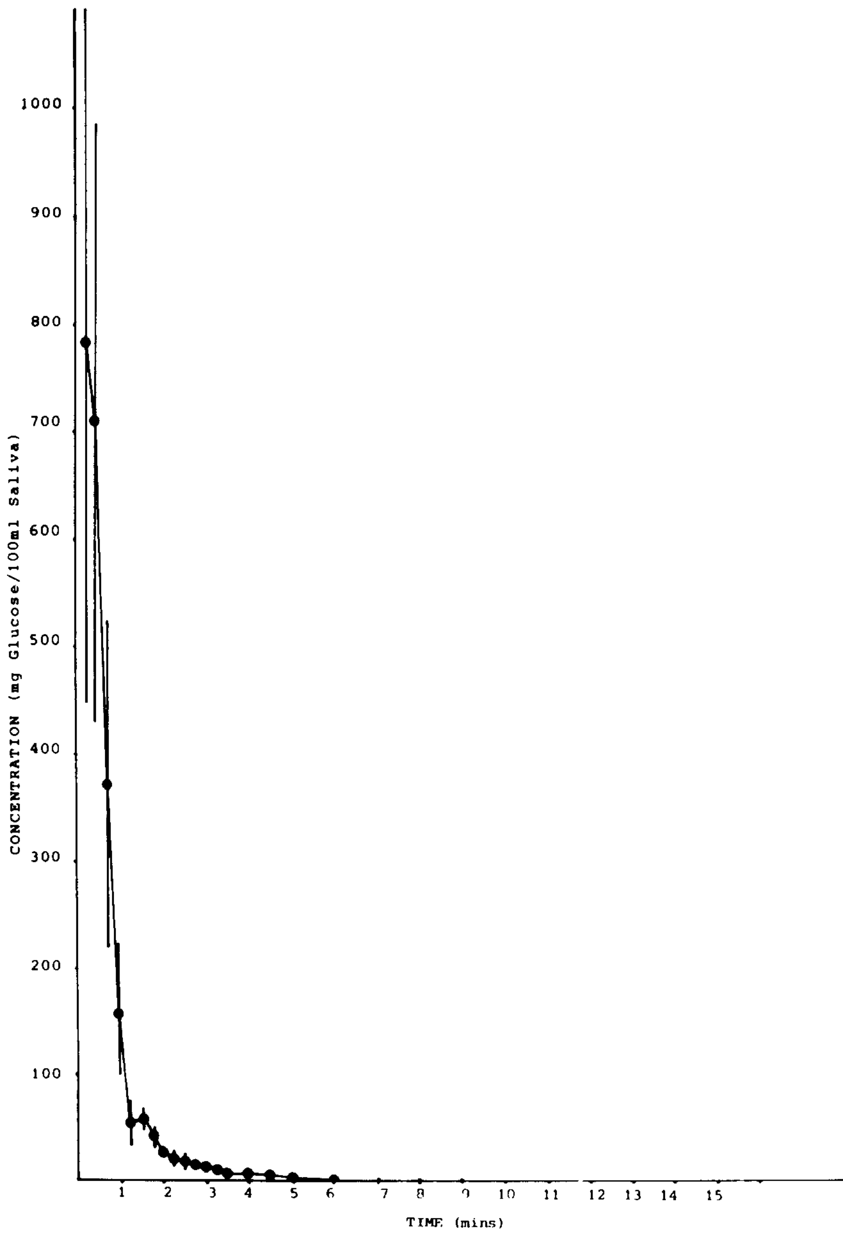
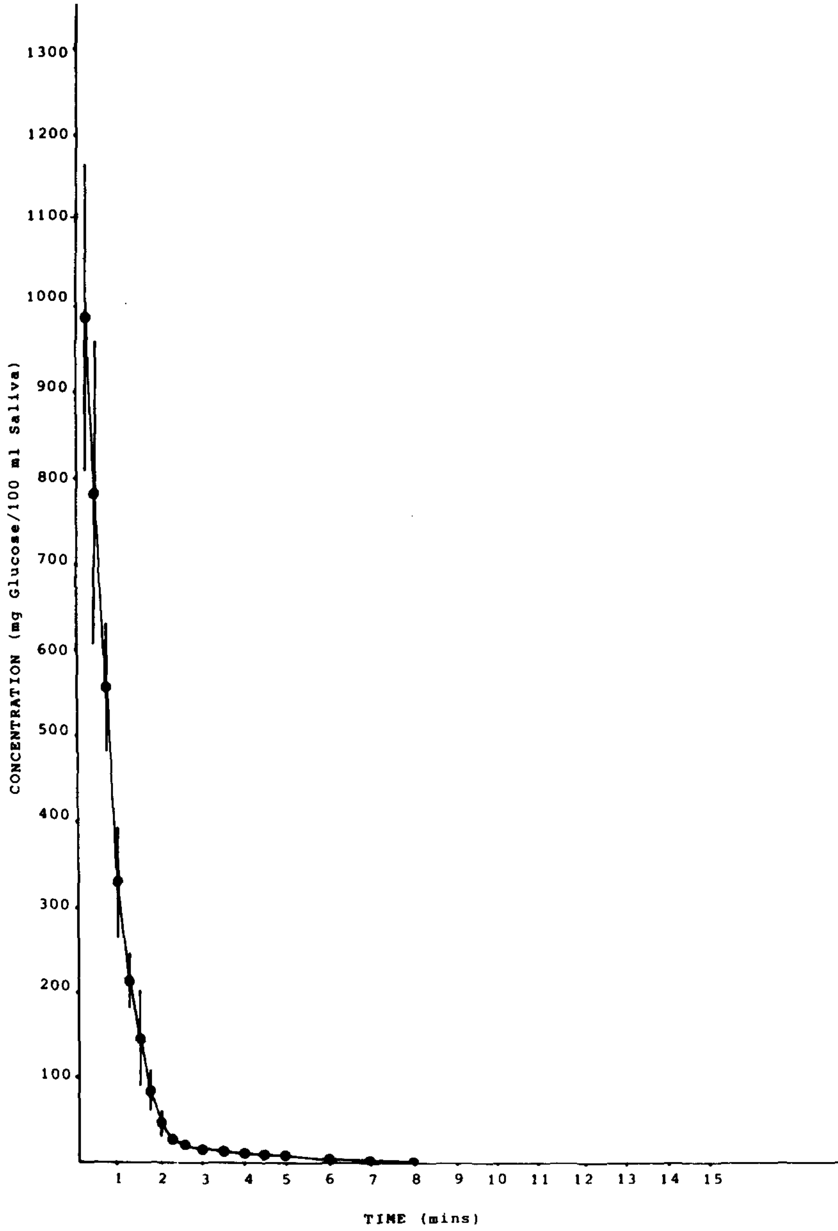


Fig. 2. Concentration of glucose in saliva against time after tasting and swallowing samples (four panellists). ((a), (b), (c) and (d) are 10, 20, 40, 70 % w/v concentrations, respectively.)



C

Fig. 2.—*contd.*



d

Fig. 2.—*contd.*

TABLE 2

PERSISTENCE OF GLUCOSE SWEETNESS (s) AFTER TASTING AND DRINKING 10 ml SOLUTIONS OF GLUCOSE AT 10, 20, 40 AND 50 % w/v

Conc. of glucose solution	*A*	*B*	*Subject C*	*D*	*Overall mean*
10%	20	18	22	20	
	22	16	26	24	
	20	18	26	22	
	8	14	22	23	
Mean	17·5	16·5	24	22	20
20%	25	32	30	28	
	35	30	32	48	
	40	31	30	36	
	35	30	34	29	
Mean	33·3	30·75	31·5	35·25	32·7
40%	60	39	120	140	
	45	38	92	94	
	65	35	98	120	
	50	33	120	85	
Mean	55	36·25	107	109	76·8
50%	70	48	220	160	
	60	48	140	100	
	75	49	100	200	
	60	47	130	180	
Mean	66·25	48	147·5	160	105·4

receptor advanced earlier from this laboratory (Birch *et al.*, 1980). If such localised concentration sites do exist a question of sample volume emerges because insufficiency of sample volume is likely to restrict the number of accessible localised concentration sites. If larger volumes (>10 ml) were tasted different results might have been obtained but in support of the present observations a previous experiment (Birch & Ray, 1979) has shown that even massive volumes (50 ml) of ingested 20 % glucose solutions exhibit exponential falls in salivary glucose levels.

Effects of sample volume and concentration on intensity and persistence of sucrose sweetness

Table 3 lists the reaction times of sweetness response as the means of ten panellists over a range of sucrose concentrations and volumes and, whilst increase in volume from 10^{-2} ml to 10 ml caused a clear drop in reaction time over all concentrations, no such trend was apparent with increase in concentration. Presumably this reflects the greater opportunity of sweetness recognition when the sample volume is large enough to affect much of the oral cavity. Reaction time seems to approach a limiting value at volumes over 10 ml which may represent a property of the taste receptor system. A limiting value of reaction time has been reported also from neurophysiological studies of rat chorda tympani (Marowitz & Halpern, 1977).

TABLE 3
REACTION TIMES OF SUCROSE SOLUTIONS AFTER TASTING DIFFERENT CONCENTRATIONS AND VOLUMES OF SAMPLE

Sucrose sample	*Volume (ml)*						
Concentration % (*w/v*)	1×10^{-2}	5×10^{-2}	1×10^{-1}	5×10^{-1}	1	5	10
1	2·1	1·6	1·3	1·0	1·2	1·1	1·1
10	2·0	1·6	1·9	0·9	1·0	1·3	1·0
20	2·7	2·0	1·9	1·2	0·8	0·6	0·8
30	2·5	1·8	1·5	1·3	1·1	1·3	1·2
50	2·2	1·4	1·4	1·5	0·9	0·7	1·3
60	2·8	2·1	2·0	1·0	0·9	1·1	0·7
70	2·6	1·6	1·6	1·2	0·9	0·9	0·6

Figure 3 illustrates how persistence time varies with volume of sample over a range of concentrations and shows that 'saturation' of persistence (i.e. maximum persistence effect attainable) requires at least 10 ml of sample at 70%w/v concentration. Although our results indicate that 'saturation' was not achieved, the proximity of the three upper curves means we were close to it. Higher concentrations

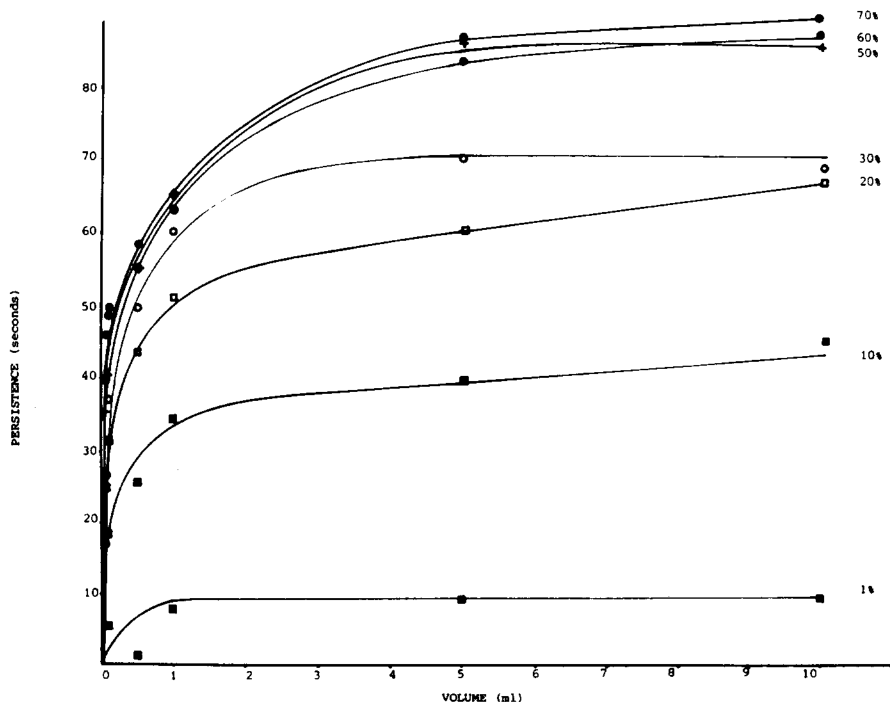


Fig. 3. Persistence of sucrose sweetness (s) against volume of sample tasted (ml) at different concentrations (ten panellists).

and volumes were not tested because of the nauseous effect which they cause in panellists. Figure 4 shows the effect of concentration on persistence time over a range of volumes and indicates the same 'saturation' region as before. It is thus clear that both concentration and volume of sample will affect the persistence of sweetness response. Figure 5 shows how intensity of sweetness varies with concentration over a range of volumes. A family of curves again results at higher concentrations but their coincidence at lower concentrations means that in this region volume does not affect intensity. This seems to suggest that the outermost receptors are easily accessible at a low concentration and a low volume of sample and accords with a previous report of constant numbers of sweetness receptors available near threshold (Birch & Munton, 1981). Comparison of Fig. 5 with Fig. 4 shows that 'intensity saturation' seems to occur at lower concentration of sample than 'persistence saturation'. This

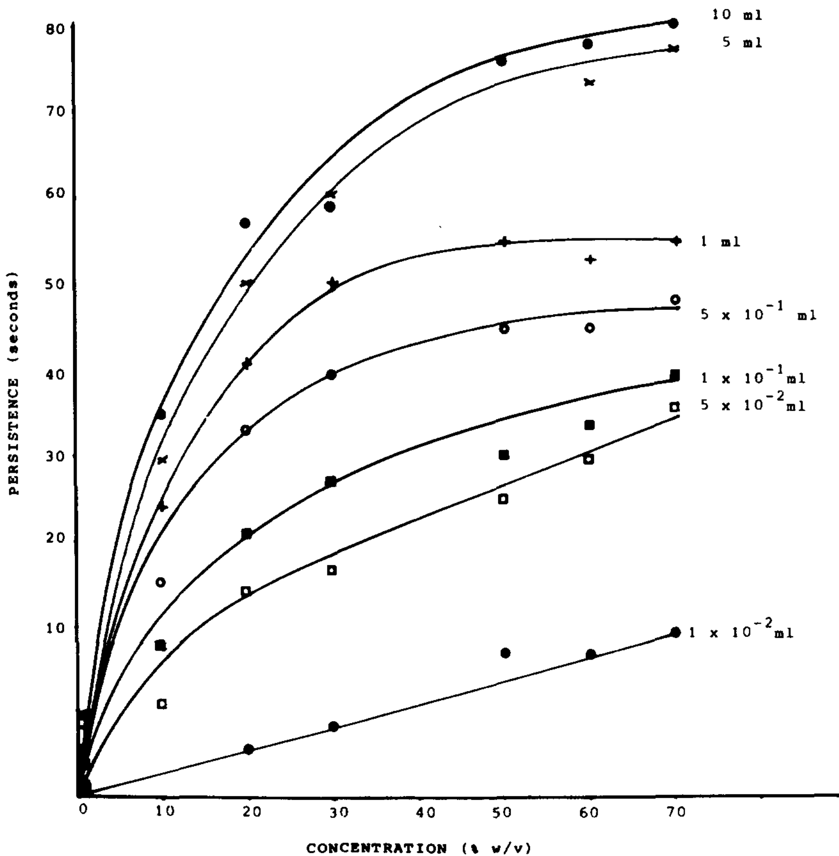


Fig. 4. Persistence of sucrose sweetness (s) against concentration of sample tasted (% w/v) at different volumes (ten panellists).

leads to the conclusion that separate molecular chemoreception mechanisms are responsible for these two facets of the taste response. Related evidence for separate stages of chemoreception has been recently reported from neurophysiological studies (Marowitz & Halpern, 1977; Senf *et al.*, 1980) and accords with the idea of an initial localised concentration of stimulus at or near the receptor, followed by an ionophore trigger process. Persistence of response is then explained by the continuation of the localised store of stimuli whereas intensity is determined largely by numbers of operating ionophore trigger mechanisms (Birch *et al.*, 1980). Figure 6 expresses the data of Fig. 5 in an alternative form. A noticeable feature of Fig. 3 is the similar initial slope of all the curves depicted. On the basis of the localised concentration hypothesis, this means that the initial localised concentration site occupation increases with increasing volume but is independent of concentration. This does not conflict with the conclusion from Fig. 5 that the number of occupied sites depends on concentration of stimulus. No such conclusion can be drawn from Fig. 4 which indicates that, for a given increase in concentration, the site occupancy is highly dependent on volume.

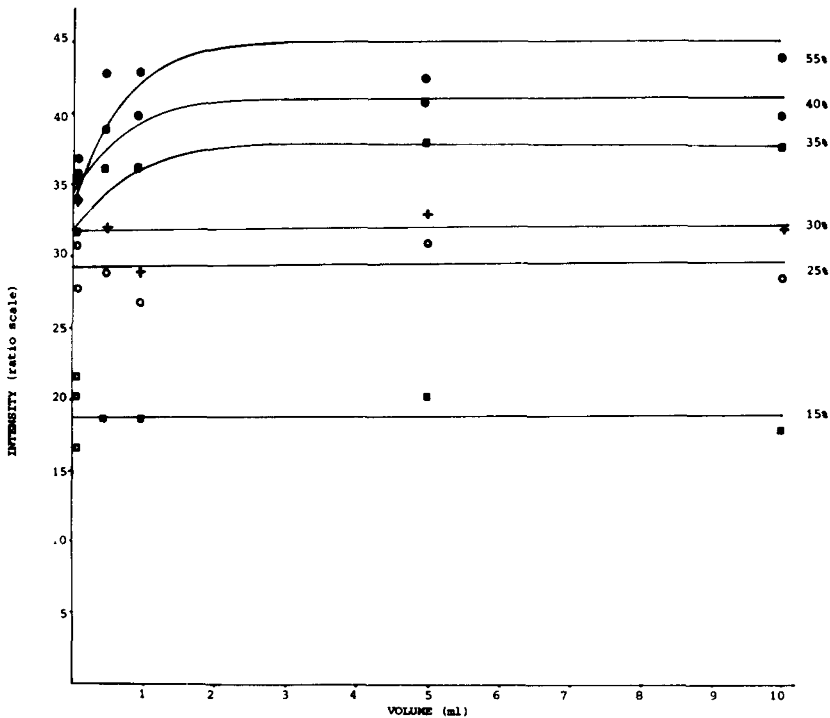


Fig. 5. Intensity of sucrose sweetness (arbitrary ratio scale) against concentration of sample tasted (% w/v) at different volumes (ten panellists).

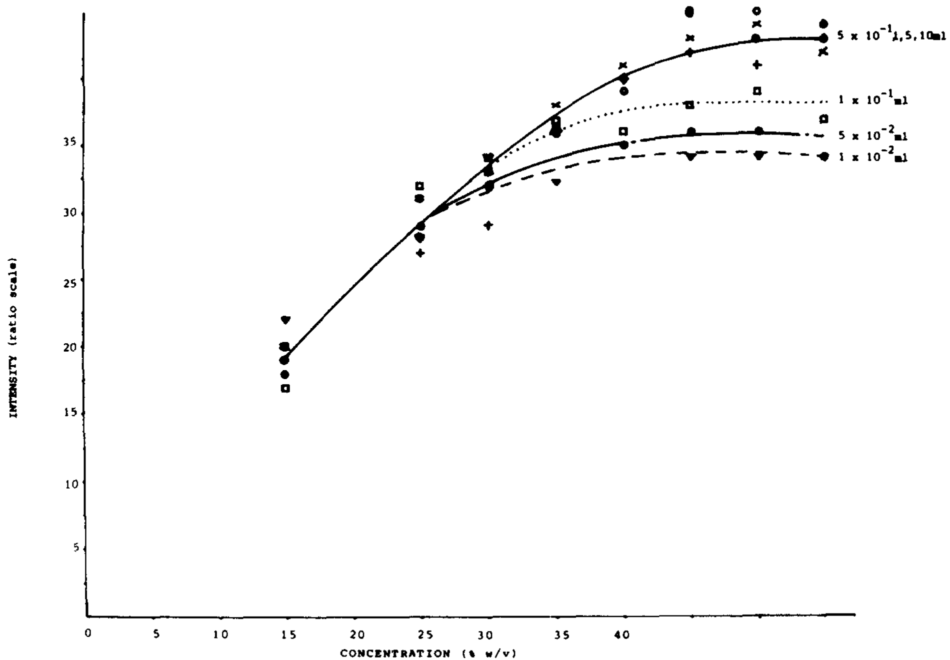


Fig. 6. Intensity of sucrose sweetness (arbitrary ratio scale) against volume of sample tasted (ml) at different concentrations (ten panellists).

The curves in Fig. 3 show that, for any particular concentration, there is a limiting volume above which no increase in persistence is possible. Presumably this means that volumes in excess of this limited amount will be 'wasted' and swallowed by the panellists. Volumes of administered solution *below* the limiting value may be assumed to be affecting receptor sites by displacing oral fluid in the vicinity of these sites and thus Fig. 3 gives an understanding of the accessibility of receptors to increasing concentrations and volumes of stimulus solution. It seems likely that increasing concentrations of stimulus allow the recruitment of deeper layers of receptors but only if the stimulus volume is sufficient to displace the oral fluid surrounding the sites.

Inspection of Figs 3, 4 and 5 leads us to conclude that the influence of volume on persistence of sweetness is not of the same order as concentration and this is illustrated by the log/linear plot of concentration$\sqrt{\text{volume}}$ against persistence (Fig. 7) which gives a straight line.

In summary, our results demonstrate that both concentration and volume of stimulus each contribute to the intensity and persistence of sweet response. The response data seem best explained by a two-stage mechanism of taste chemoreception, the first involving a localised concentration of stimulus at or near the

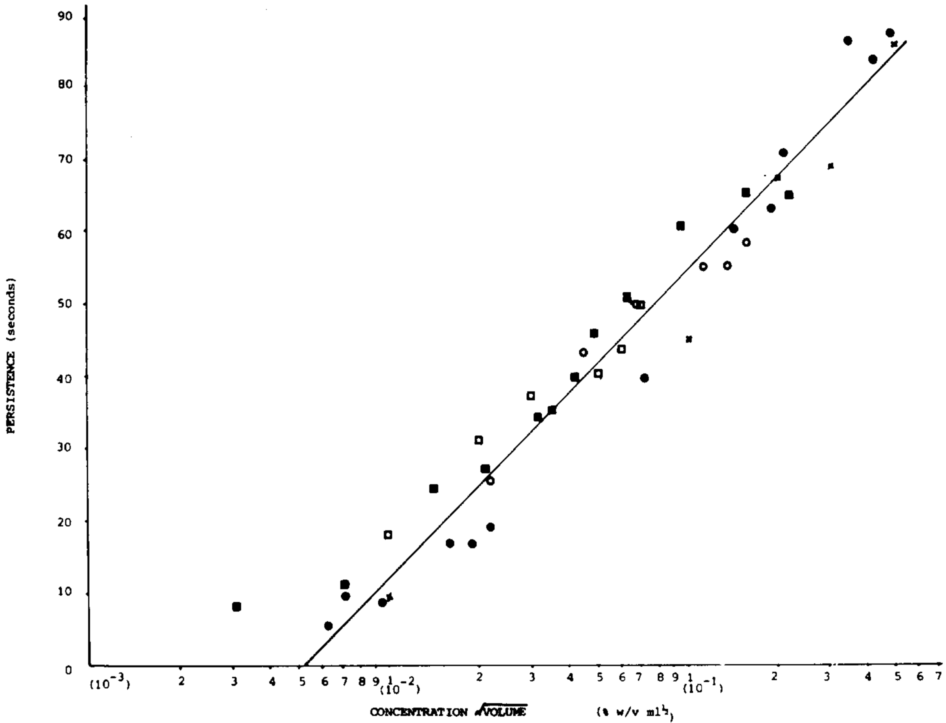


Fig. 7. Persistence of sucrose sweetness (s) against concentration$\sqrt{\text{volume}}$ (%w/v ml$^{1/2}$) (ten panellists).

receptor and the second an ionophore trigger mechanism whereby the taste neuron becomes activated. The localised concentration step itself is best envisaged in two ways—occupancy and capacity. Occupancy alone would constitute a primary determinant of taste intensity whereas capacity would determine the maximum possible contribution to persistence. On this basis 'intensity saturation' is governed by the maximum possible number of sites occupied while 'persistence saturation' is governed by the maximum possible filling (i.e. capacity) of these sites.

An earlier paper (Meiselman & Bose, 1977) on taste has noted that intensity is dependent on flow rate of stimulus solution passed across the tongue and this may be regarded as an analogy of our current experiment. By elevating flow rate of sample Meiselman & Bose (1977) were, in fact, elevating volume of tastant and their experimental results concur with our own, i.e. weaker solutions may be made to taste sweeter than stronger solutions if volume is increased (flow rate) (Fig. 5). However, we disagree with the conclusion of Meiselman & Bose (1977) that flow rate mimics concentration and instead offer the hypothesis of localised concentration of stimulus outlined in this paper.

ACKNOWLEDGEMENTS

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Food Chemistry 9 (1982) 239–242

BOOK REVIEWS

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The book is profusely illustrated, but the quality of photograph reproduction is poor. Evidence of editorial pressure is hard to find, and far too much of the text is characterised by diffuseness, repetitiveness and diversions into peripheral matters. It is not a scholarly work, the style being that of the trade press. Only some chapters have bibliographies and the references included tend to be from the 'fifties and 'sixties. There are many errors, particularly in the underlying science. Students and the inexperienced need to be warned.

Even so, there is a great deal of useful information in the book, and all professionally concerned with aspects of coffee technology will need to possess a copy.

HARRY E. NURSTEN

Fundamentals of Food Chemistry. By W. Heimann. Ellis Horwood. Avi. Chichester and Westport, Connecticut. 1980. 344 pp. Price: £22·50 (Hardback) £7·50 (Cloth covers).

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This is a very readable book. The chapters are short and well written and the material they contain is often challenging. In a real sense the book deals with the continually developing inter-relationship between the food and catering industries, the future demands of the educated food consumer and the need for an efficient as well as personally acceptable food system.

A useful acquisition for those teaching in the area of food and catering technology and food production and development but at £44·00 likely to spend most of its time on a library shelf often referred to but seldom bought.

J. THOMSON

Selective Toxicity (The Physico-chemical Basis of Therapy). By Adrian Albert. (Sixth edn) Chapman & Hall, London. ix + 662 pp. 1979. Price: £15·00.

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Food chemists will be able to recognise much of importance amongst this impressive array of headings and the book presents a complete and up-to-date review of theories relating to drug action, enzymes and receptors. Although the title of the book appears more attractive to the drug therapist than the food chemist, the contents are, in fact, indispensable to research workers concerned with the metabolic fate of food components. Even more valuable are the sections on occupation theory, rate theory, etc., of receptors which put modern concepts of these entities entirely into perspective and can thus add much to the working knowledge of taste chemists. Other sections of particular interest to food scientists are those on stereochemistry and surface chemistry.

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G. G. BIRCH

Food Chemistry **9** (1982) 243–252

DETERMINATION OF ACRYLONITRILE MONOMER IN FOOD PACKAGING MATERIALS AND IN FOODS

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ABSTRACT

An automated headspace gas chromatographic method is described using nitrogen specific detection for the quantification of acrylonitrile monomer (AN) in acrylonitrile–butadiene–styrene (ABS) food packaging tubs and in the contained foods. For a limited number of retail foods, levels of AN in ABS tubs (ranging from 2 to 10 mg/kg) and their contents (soft margarine, butter and shortening) are reported (ranging from 0·01 to 0·05 mg/kg), the results being confirmed by mass spectrometry (low and medium resolution selected ion monitoring). The latter technique was also used to quantify the levels of AN in the coatings of retail packaging films on a range of substrates, which were found to be between $<0{\cdot}001$ *and* $0{\cdot}02\,mg/m^2$.

INTRODUCTION

The detection of vinyl chloride in foodstuffs packaged in poly (vinyl chloride) (PVC) (Schaffner & Lombardo, 1975), coupled with concern over its toxicity, has led to a number of surveys of vinyl chloride in PVC (Eckert, 1975; Breder *et al.*, 1975; Ministry of Agriculture, Fisheries and Food, 1978) and in foods (Ehtesham-Ud Din *et al.*, 1977; Fuchs *et al.*, 1975; Williams, 1976; Page & O'Grady, 1977) and the subsequent imposition of legislative controls within the EEC for residual levels of vinyl chloride (Anon, 1980). Interest has extended to other monomers with reports in the literature of the levels of vinylidene chloride (VDC) in packaging materials, simulants and foods (Gilbert *et al.*, 1980; Salvatore *et al.*, 1977; Hollifield & McNeal, 1978) and of residual styrene levels in polystyrene (Watanabe *et al.*, 1977) and in certain wines (Piccinini & Bonciani, 1979) and foods (Withey & Collins, 1978; Gawell *et al.*, 1979) which have been in contact with styrene-containing polymers. Attention has also been directed towards acrylonitrile (AN) with the

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levels of monomer being reported in polymers of acrylonitrile–butadiene–styrene (ABS) (Di Pasquale *et al.*, 1978) and styrene–acrylonitrile (SAN) (Chopra *et al.*, 1978) used for food packaging, and in food simulants (Brown *et al.*, 1978; Di Pasquale *et al.*, 1978) and certain beverages (Gawell, 1979). However, to date there have been no reports of residual AN in films where AN is present in the coating, nor has there been any published overall survey of AN levels in retail packaging and foods within the United Kingdom where food packaging practices may differ significantly from those elsewhere.

The food contact uses of AN within the UK are diverse and range from packaging materials to kitchenware and household appliances. For ABS, which offers the advantage of resistance to environmental stress cracking, the major packaging use is in the fabrication of tubs for soft margarine and 80% of the packed soft margarine in the UK is sold in ABS tubs. Other minor uses are for frozen desserts, prepared salads, shortening and for some dairy products, including concentrated butter. Polymers containing high levels of AN suitable for carbonated beverage bottles have not so far been used in the UK. However, as a co-monomer with VDC, AN is used extensively in the preparation of co-polymer coatings for regenerated cellulose and plastics films, for a variety of applications which include packaging for biscuits, snack foods, confectionery and cooked meats.

For the analysis of AN in polymers direct injection of solutions of polymer into a gas chromatograph (GC) has been successfully employed (Brown *et al.*, 1978; Chopra *et al.*, 1978). However, headspace GC does offer significant advantages in speed of analysis and sensitivity and has been employed very successfully in this laboratory for the past three years and elsewhere (Gawell, 1979; Di Pasquale *et al.*, 1978) for routine AN analysis. A headspace method, once developed, has the additional attraction that it can also be successfully applied with little modification to the analysis of AN in foods. In this case the advantages over alternative techniques, for example, the direct injection of food simulants (Brown *et al.*, 1978) or azeotropic distillation, (McNeal *et al.*, 1979), become even more significant than for the plastics. There are further practical advantages if the headspace analysis can be automated (Kolb, 1976; Kolb *et al.*, 1979); not least the increased precision obtained and the convenience of unattended operation.

For AN measurements in ABS tubs and in foods by headspace GC, the alkali flame ionisation detector (AFID) (nitrogen/phosphorus mode) offers increased sensitivity compared with an FID, together with additional selectivity. However, in contrast, for the analysis of coated films we have used a 'hot-jar' technique as opposed to dissolution (heating the film in a sealed container and manually sampling the headspace gas) with single ion monitoring as the detection method with its increased sensitivity over the AFID, a requirement for the lower levels of AN present in films.

Similarly, for the foods, quantitative confirmation was obtained by single ion monitoring at both low and medium resolution, the good agreement with nitrogen

detector quantification showing the latter method to be free of any AN interferences. As an illustration of these techniques a limited number of results are reported for levels of AN in films, in ABS tubs and the contained foods (soft margarine, concentrated butter and shortening) purchased from retail outlets; the full survey results for the soft margarines over the past 4 years are published elsewhere (Ministry of Agriculture, Fisheries and Food, 1982).

MATERIALS AND METHODS

Materials

AN was purchased from Koch-Light Laboratories (Colnbrook, Great Britain) and propionitrile and 1,2-dichlorobenzene from BDH Chemicals Ltd (Poole, Great Britain). Soft margarine samples and food packagings (films and tubs) representative of the wide variety of different brands were obtained from normal retail outlets whilst the concentrated butter and shortening were samples provided by the manufacturers (Co-operative Wholesale Society Ltd, Manchester, Great Britain) and had been subjected to prolonged refrigerated storage.

Determination of AN in ABS tubs

(*a*) *Sample preparation:* Immediately after purchase samples were stored overnight at −15 °C to enable easy removal of the contents from the tub which was then wiped carefully to remove grease. The tub was cut into small pieces, samples (*ca.* 0·5 g) were accurately weighed into glass vials (24 ml capacity) and 5 ml of propionitrile (internal standard) in 1,2-dichlorobenzene (1·0 μg/ml) was added. After sealing with a butyl rubber septum and an aluminium ring, the sample was allowed to stand overnight to effect dissolution prior to water bath equilibration at 70 °C for 2 h.

As ABS containing zero residual AN was not available for the preparation of standards, polystyrene was employed as the polymer. The procedure was identical to the above except that microlitre amounts of a standard solution of AN in dichlorobenzene were added by syringe to the vials containing the dissolved polymer, to give the required range of levels of AN (0–12 mg/kg) to construct a calibration graph. All determinations were based on peak height, estimations being calculated on the ratio of peak height of AN to the peak height of propionitrile internal standard.

(*b*) *Gas chromatography:* A Perkin-Elmer F42 automated headspace analyser equipped with an AFID for specific nitrogen/phosphorus detection was used. This instrument, which is described elsewhere (Jentzsch *et al.*, 1968), is designed for automated headspace sampling of up to thirty vials contained in a thermostatted water bath. The operating conditions were as follows: column, 1·8 m × 2 mm inside diameter stainless steel tubing (with mid-point T piece to operate with a backflush

facility) packed with 0·2 % Carbowax 1500 on 80/100 mesh Carbopack C; nitrogen carrier gas flow, 40 ml/min; column temperature was kept isothermally at 90 °C. For the AFID the air and hydrogen flow rates were optimised to give the best detector response but were normally approximately hydrogen (69 kN/m^2), air (310 kN/m^2) and a bead setting of 600. The injection time was 9 s, with a sample equilibration temperature of 70°, injector detector 180° and dosing line temperature 180°.

Determination of AN in packaging films

(*a*) *Sample preparation:* A random selection of foods packaged in plastic films (mostly biscuits and snack products) were purchased and identification of the film was carried out by infra-red spectrophotometry using a multiple attenuated total reflectance method. Only those films which showed the presence of AN in the coating (through a strong —C≡N stretch band at 2240 cm^{-1}) were selected for analysis. Measured areas (500 cm^2) of film were wiped with soft tissue to remove grease and visible food residues, and sections were cut into narrow strips, crumpled into 160 ml hypovials and sealed with rubber septa.

(*b*) *Film analysis:* Film samples contained in glass hypovials were heated at 120 °C in an air-circulating fan oven for 30 min prior to analysis. Headspace vapour injections (1 ml) were removed manually using a 2 ml Pressure-Lok gastight syringe (Precision Sampling, Baton Rouge, LA, USA) and were injected into the GC under the conditions described above. For calibration standards a solution of AN in ethyl acetate (0·1 μg/μl) was prepared, microlitre amounts being injected into hypovials containing uncoated film. Estimation of AN in the film samples was based on peak height measurement in direct proportion to a calibration standard chromatographed immediately prior to the sample.

(*c*) *Gas chromatography–Mass spectrometry:* A Du Pont Model 21-490B mass spectrometer interfaced with an all glass jet separator to a Pye 104 chromatograph was employed and was tuned on the molecular ion of authentic AN (m/z = 53). The source was operated in the EI mode (70 eV) at 200 °C. The GC conditions were as follows: glass column (2·7 m × 2 mm inside diameter) packed with 10 % Carbowax 1500 on Diatomite C-AW operated isothermally at 85 °C with a helium carrier gas flow of 30 ml/min.

Determination of AN in foods

(*a*) *Sample preparation:* The total contents of each plastic tub (in most cases, soft margarines) were homogenised under liquid nitrogen in a pestle and mortar, the contents being thoroughly mixed to ensure homogeneity. Samples (2 g) were weighed into glass vials, sealed, capped and then 10 μl was added of a solution of propionitrile (internal standard) in 1,2-dichlorobenzene, of a concentration of 0·02 mg/ml prepared by serial dilution from an initially weighed amount of propionitrile. For the preparation of calibration standards, margarine was obtained packaged in polyethylene which, on analysis, was shown to be free of AN or any

interference peak in the AN or propionitrile position. To the weighed sample, microlitre amounts (0–10 μl) of a solution containing AN and propionitrile in dichlorobenzene were added to give a calibration curve over the required range (0–0·08 mg/kg). Additionally further microlitre (0–10 μl) amounts of a solution of identical strength to the above (containing propionitrile alone in dichlorobenzene) were added to give a combined added volume in each case of 10 μl and thereby a constant added amount of internal standard. Determinations were, as previously, based on the ratio of peak heights of AN to propionitrile internal standard.

(*b*) *Gas chromatography:* GC conditions were identical to those for the plastics except that the nitrogen detector was operated at maximum sensitivity in order to detect AN at the levels present in the foods.

(*c*) *Gas chromatography–Mass spectrometry:* A Perkin-Elmer F42 automated headspace sampler was coupled directly through an all-glass jet separator to a VG 7070H mass spectrometer as described elsewhere (Gilbert & Startin, 1981). The mass spectrometer source was operated in the EI mode (70 eV electron energy; 200 μA trap current) and was maintained at 200 °C. The accelerating voltage was 4 kV. For low resolution selected ion monitoring (SIM) the magnet current was set to monitor the ion current at m/z 53 (molecular ion of acrylonitrile) with source and collector slit width adjusted to give flat top peaks with just sufficient resolution to separate unit masses at m/z = 53. The electron multiplier was operated at 2×10^4 gain. For SIM of m/z = 53·026 at a resolving power of 2000, a peak switching unit was employed using a lock mass in perfluorokerosene at m/z = 51·0046 to correct for drift. The use of a sample and hold facility enabled the amplifier output to be displayed on a 10 mV chart recorder and showed low signal noise with adequate stability during the course of a day's operation.

RESULTS AND DISCUSSION

The use of SIM for detection enabled determinations to be made with high sensitivity combined with good specificity. The headspace technique described for the determination of AN in a variety of packaging films was adequate for quantification down to 0·001 mg/m^2 and although the method employed was not directly evaluated for AN, it has been previously demonstrated (Gilbert *et al.*, 1980) for VDC (of a higher boiling point than AN) to be adequate for release of monomer in terms both of temperature and duration of heating of the film. The survey results given in Table 1 show residual AN in the coatings to be low and, in many cases, below the limit of detection. This was mainly due to the low proportion of AN used in the formulation of the coating and the thin layers of coating applied to the substrate. It was concluded from the data in Table 1 that the migration of AN into foods from these film applications was likely to be immeasurably small and therefore food analyses were not conducted in this instance.

TABLE 1
OBSERVED LEVELS OF ACRYLONITRILE MONOMER IN A VARIETY OF RETAIL PACKAGING FILMS

Product	*Film type (substrate)*	*Level of AN in film*[a] *(mg/m²)*	*(ppm)*
Biscuits:			
Mallow creams	Cellulose	<0·001	<0·01
Currant	Polyolefin	0·013	0·36
Garibaldi	Cellulose	<0·001	<0·01
Bourbons	Polyolefin	0·011	0·34
Nut Cookies	Cellulose	0·003	0·04
Snack products:			
Flavoured crisps	Polyolefin	0·006	0·2
Flavoured crisps	Polyolefin	0·022	0·7
Plain crisps	Cellulose	<0·001	<0·01
Flavoured crisps	Polyolefin	0·014	0·42
Flavoured crisps	Polyolefin	<0·001	<0·01

[a] Mean values from not less than three determinations.

For the analysis of AN in ABS and soft margarines the technique described was used successfully for numerous surveys of packaged foods from retail outlets. The method had a quantification limit of approximately 0·1 mg/kg for AN in ABS and 0·005 mg/kg for AN in soft margarine. An internal standard was used for all measurements with the nitrogen detector to correct for any slight changes in detector response over long periods of operation. For both the plastics and foods, good straight line calibration graphs were readily obtained, with typical standard errors on the mean for determinations in soft margarines ranging from 1·5 to 3·5 % with AN concentrations from 0·079 to 0·023 mg/kg. In Fig. 1 typical chromatograms are illustrated for AN in soft margarine using the nitrogen detector. As shown, control margarines packed in polyethylene or high impact polystyrene gave no response at the retention time of AN. Confirmation of some of these results was carried out by mass spectrometry using single ion monitoring of $m/z = 53$ (the molecular ion of AN).

At low resolution (as shown in Fig. 1) although the AN can be clearly seen, there are a number of other peaks in the chromatogram and $m/z = 53$ is not sufficiently specific to AN for unequivocal confirmation. However, on increasing the resolution to around 2000 (and monitoring $m/z = 53{\cdot}026$) it can be seen that other ions present were lost from this window and a single peak with an AN retention time was obtained. In Table 2, for a few selected survey samples of soft margarines, quantification by both nitrogen detector and SIM was carried out, the good agreement between the two techniques showing the specificity of the nitrogen detector to be generally adequate.

A number of surveys of AN in both soft margarines and ABS tubs purchased from retail outlets over the past 4 years have been carried out using the methodology described in this paper. The detailed results are published elsewhere (Ministry of

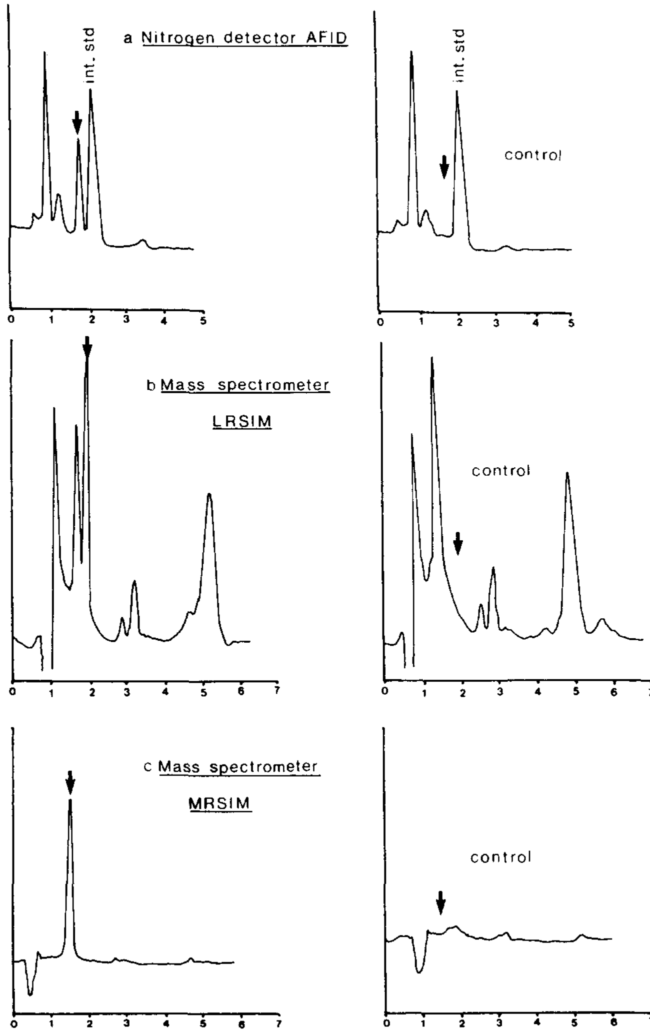

Fig. 1. Headspace chromatograms from above heated soft margarine samples as controls and spiked with AN. (a) Using nitrogen detector, internal standard, propionitrile. 0·032 mg/kg AN. (b) Mass spectrometry (LRSIM) m/z = 53. 0·016 mg/kg AN. (c) Mass spectrometry, medium resolution, m/z = 53·026. 0·008 mg/kg AN. Chromatographic conditions: column 1·8 m × 2 mm inside diameter stainless steel packed with 0·2% Carbowax 1500 on 80/100 mesh Carbopack C. Flow rate, 40 ml/min, operated isothermally at 90 °C.

TABLE 2
OBSERVED LEVELS OF ACRYLONITRILE IN ABS CONTAINERS AND SOFT MARGARINES

Level of AN in tub (mg/kg)	*Level of AN in soft margarine (mg/kg)*[b]		
	AFID	*LRSIM*	*HRSIM*
<0·1[a]	<0·01	<0·005	—
6·7	0·035	0·036	0·038
1·7	0·040	0·048	0·052
3·8	0·020	0·024	—
3·1	<0·01	0·010	—
6·6	0·01	0·009	—

[a] This sample was packaged in polyolefin tub, not ABS.
[b] Mean values of a minimum of three determinations.

Agriculture, Fisheries and Food, 1982) but show a range of AN from 0 to 10 mg/kg in ABS tubs (mean of 6 mg/kg) and a range of 0·01 to 0·02 mg/kg AN in the soft margarine with occasional samples containing up to 0·04 mg/kg. There was generally, and somewhat surprisingly, no obvious correlation between the AN level in the tubs and the level in the margarine. For example, as shown in Table 2, a sample containing 1·7 mg/kg AN in the ABS tubs was found to have a high level (0·04 mg/kg) of AN in the margarine whilst, conversely, another sample (analysed during the course of the survey) containing 9·2 mg/kg AN in the tub contained only 0·01 mg/kg AN in the margarine. However, as nothing is known of the history of the samples, these differences may be a reflection of the different lengths of time the samples had been stored under chilled conditions before purchase. There was no trend towards particular brands having either high or low levels of AN in the soft margarines and therefore no suggestion that the differences in the compositions of these margarines affected the extent of migration. Although, for the surveys, the total contents of the tubs were homogenised, in a limited number of preliminary experiments there was found to be no significant observable difference in the AN levels between the centre of the soft margarine and the layer in contact with the wall of the tub. This suggests that either the major proportion of migration occurred on filling of the tubs with the margarine in a liquid state or that, conversely, migration occurs during storage with subsequent rapid mass transfer of AN throughout the contents of the tub.

An additional but minor food packaging use of ABS is for concentrated butter and shortening and the results of some controlled storage experiments are shown in Table 3. Tubs containing similar residual AN levels, when stored for 8 months with concentrated butter, showed migration levels ranging from 0·01 to 0·04 mg/kg, there being no apparent explanation for these differences. Shortening stored for differing periods in both cases showed no detectable AN in the food.

The results of all these surveys clearly show low, but nevertheless measurable, AN

TABLE 3
OBSERVED LEVELS OF ACRYLONITRILE IN ABS CONTAINERS AND FOOD PRODUCTS

Product	*Storage time at 7°C (months)*	*Residual AN (mg/kg)*[a]	
		Tub	*Food*
Concentrated butter	8	3·7	0·03
Concentrated butter	8	4·5	0·04
Concentrated butter	8	4·5	0·01
Shortening	6	2·0	<0·01
Shortening	3	1·9	<0·01

[a] Mean values from four determinations.

levels in retail foods, but this must be seen in perspective. The packaging applications of ABS are somewhat limited, and the contribution of soft margarine to the total diet is small, so that the average intake of AN is correspondingly minute.

The method of analysis described in this paper was shown to be suitable for routine surveillance of AN in packaging and foods. For the future it could find application for monitoring of foods either for other monomers or for AN in other, so-far unexamined, foods should there be future changes in packaging practices.

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COMPARATIVE BIOCHEMISTRY OF TOMATO FRUITS DURING RIPENING ON THE PLANT OR RETARDED RIPENING

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ABSTRACT

Tomato fruit (Lycopersicon esculentum) *kept in an atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen showed an increase in glucose, fructose and citric acid with a decrease in starch and malic acid after 4 weeks. However, there was no synthesis of lycopene after this time, nor change in multiple form pattern of the following respiratory enzymes: alcohol dehydrogenase EC 1.1.1.1; lactic dehydrogenase EC 1.1.1.27; malic dehydrogenase EC 1.1.1.37; malic enzyme EC 1.1.1.40; isocitrate dehydrogenase EC 1.1.1.42; 6-phosphogluconate dehydrogenase EC 1.1.1.44; glucose-6-phosphate dehydrogenase EC 1.1.1.49; NADH dehydrogenase EC 1.6.99.3; NADPH dehydrogenase EC 1.6.99.1; glutamate oxaloacetate transaminase EC 2.6.1.1; hexokinase EC 2.7.1.1; phosphoglucomutase EC 2.7.5.1 and glucose-6-phosphate isomerase EC 5.3.1.9.*

It is suggested that the modified atmosphere allowed changes relying on enzyme regulation but prevented events which involve de novo *enzyme synthesis.*

INTRODUCTION

The events during ripening of tomato fruit, *Lycopersicon esculentum* Mill, occur synchronously (Hobson & Davies, 1971). These changes include: loss of chlorophyll and development of carotene and lycopene; synthesis of enzymes degrading cell walls; evolution of carbon dioxide and ethylene; breakdown of starch; increase, in equal quantities, of glucose and fructose; concentrations of citric and glutamic acid increase and malic acid decrease. However, different mechanisms of regulation are thought to control the various processes. Whereas the evolution of carbon dioxide in pome fruit can be attributed to an increase in mitochondrial efficiency (Hulme & Rhodes, 1971) and malate decarboxylation at the climacteric rise, the same is not

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true of tomato fruit (Hobson, 1970). However, the control of phosphofructokinase may provide a control point for increased respiration in tomato fruit (Chalmers & Rowan, 1971). Glucose for increased respiration is supplied from starch hydrolysis, although amylase has not been reported as increasing in activity during ripening (Clements, 1970).

It has been shown that new messenger RNA is synthesised at the red stage of tomato fruit and two new protein bands are recognisable (Rattanapanone *et al.*, 1977; Grierson *et al.*, 1980). This may be related to the *de novo* appearance of two isoenzymes of polygalacturonase and a considerable increase in invertase activity (Grierson *et al.*, 1980). Carotenoid biosynthesis can be genetically prevented in mutants deficient in any one of the many enzymes converting mevalonate to isopentenyl pyrophosphate and on to phytoene, lycopene and carotene, but it has not been established whether *de novo* enzyme synthesis is required to complete the sequence in ripening (Goodwin, personal communication).

Colour change and increased respiration during fruit maturation have been separated for a considerable time by gibberellin application (Dostal & Leopold, 1967). There has not been any indication of separation of colour change from respiration by modified gas atmospheres, because, as yet, there have been few reports of any biochemical changes during gas treatment.

The experiments reported here show that modified gas atmosphere of a certain composition could restrict changes in colour but does not retard the regulation of others.

MATERIALS AND METHODS

Plant growth, sampling and initial treatment

Tomato plants were grown in normal commercial glasshouses and fruit taken for analysis at the immature green, mature-green (green skins but fully formed seeds), orange and red stages. A large quantity (300 kg) of mature-green fruit was placed in sealed chambers containing 5% oxygen, 5% carbon dioxide and 90% nitrogen at 12 °C. Four fruits were taken from the plant at each of the above growth stages (any fruit without mature seeds were discarded from the mature-green category), or removed from store at one of several sampling times and extracted for 12 h with boiling 80% aqueous ethanol in a Soxhlet apparatus for assay of substrates. Additional fruits were removed at the same time and protein extracted by macerating with 200 mM phosphate buffer, containing 10 mM ascorbate, 1% PVP and 100 mM EDTA (w/w) at pH 8 and 4 °C (1:1 tissue buffer).

Gas analysis

Respiration changed the concentration of oxygen and carbon dioxide in the sealed chambers and this was adjusted daily by flushing with nitrogen to decrease the

carbon dioxide and with oxygen to increase its concentration. Carbon dioxide was measured using an infra-red gas analyser and oxygen by gas chromatography. The carrier gas was nitrogen at 30 ml/min and a hot wire detector was used for oxygen measurement after passage through a column of molecular sieve 5A. Oven temperature was 60 °C.

Colour formation

Appearance of red colour was estimated using the Gardiner tristimulus colour meter. The three calibration tiles used for the red–green ('a' scale) were: light green −15·0; light red +22·4; strong red +49·4.

Monosaccharide and organic acid analysis

The monosaccharides glucose and fructose were analysed by gas chromatography after 100 μl samples were dried in a rotary film evaporator, dissolved in 1 ml pyridine and the trimethyl silyl derivatives formed. Xylose was used as internal standard and the GC conditions were those of Goodenough & Kempton (1977).

Organic acids in the ethanolic extract were determined by enzymic analysis. Citrate was converted to oxaloacetate and acetate by citrate lyase. Oxaloacetate was further converted to malate by malate dehydrogenase and any pyruvate formed (decarboxylation product of oxaloacetate) was converted to lactate by lactate dehydrogenase (Möllering & Gruber, 1966). Amounts of NADH oxidised in these reactions were used to quantify the citrate present. Malate was measured in a comparable way (Goodenough & Thomas, 1980).

Starch

Starch from the dried residue of ethanol-extracted mature-green and stored fruit was either solubilised by dimethylsulphoxide or boiling with distilled water and then further treated with 6·0N perchloric acid (final conc.). It was hydrolysed with amyloglucosidase at pH 4·6 and glucose determined enzymatically by reaction with hexokinase and glucose-6-phosphate dehydrogenase. The NADPH formation at 340 nm was measured during the reaction and used to calculate the amount of starch (Keppler & Decker, 1974).

Protein

Protein was estimated using the Folin reagent, after TCA precipitation (Lowry *et al.*, 1951) or by the dye-binding method (Bradford, 1976). Bovine serum albumin (fraction versus fatty acid free) was used as a standard protein.

Polyacrylamide gel electrophoresis and staining for protein or enzyme multiple forms

The proteins and enzymes were separated by electrophoresis on rods of polyacrylamide (5 × 80 cm) consisting of a 7 % separation and 2·7 % spacer gel

Maurer No. 1a pH 8·3 (Maurer, 1971). The buffer in both upper and lower reservoirs was Tris/glycine, pH 8·3. Electrophoretic separation was for at least 1 h at 2 °C, 1 mA/tube towards the anode.

Protein was stained using 0·04 % Coomassie blue G.250 in 3 % perchloric acid, making destaining unnecessary (Reisner *et al.*, 1975). In most cases the formation of NADPH or NADH was used to develop the colour of methyl thiazolyl tetrazolium (MTT) using a phenazine methosulphate (PMS) as intermediate catalyst. The intensity of the developed bands was measured using a gel scanning attachment on a double beam spectrophotometer at 550 nm.

The following enzymes were stained using the methods of Harris & Hopkinson (1975): phosphoglucomutase EC 2.7.5.1, hexokinase EC 2.7.1.1, glucose-6-phosphate isomerase EC 5.3.1.9, glucose-6-phosphate dehydrogenase EC 1.1.1.49, 6-phosphogluconate dehydrogenase EC 1.1.1.44; alcohol dehydrogenase EC 1.1.1.1, lactic dehydrogenase EC 1.1.1.27, malic enzyme EC 1.1.1.40, malic dehydrogenase EC 1.1.1.37, isocitrate dehydrogenase EC 1.1.1.42, glutamate oxaloacetate transaminase EC 2.6.1.1, NADH dehydrogenase EC 1.6.99.3 and NADPH dehydrogenase EC 1.6.99.1. Nomenclature was that recommended by the IUB (1978).

RESULTS

Effects of ripening and storage on sugars and organic acids

Figure 1(a) and (b) shows the changes in glucose and fructose during fruit ripening on the plant and after storage of mature-green fruit for 8 weeks. Hydrolysis of starch has taken place in the first 4 weeks of storage and is similar to the amount of hydrolysis found when fruit change from green to orange in that the starch content fell from 11–12 % to 2–3 % of the dry weight in both cases. This means that at least 75 % of the increase in glucose and fructose can be accounted for by starch breakdown. Figure 2(a) and (b) shows that during the first 4 weeks of storage citric acid increased in concentration and malic acid decreased to the same extent as fruit maturing from the green to orange stages.

Effect of ripening and storage on lycopene formation

During normal ripening, colour changes in tomatoes of the cultivar Sonatine occur over a short period of time (5–10 days), depending on the temperature. In the experiments described in this paper synthesis of lycopene was retarded even though the changes in monosaccharides and organic acids had occurred. The mature-green fruit ripened rapidly after removal from storage (Fig. 3). However, after 8 weeks of retarded ripening only slight synthesis of lycopene took place, equivalent to approximately 10 % of the final red colour developed after storage.

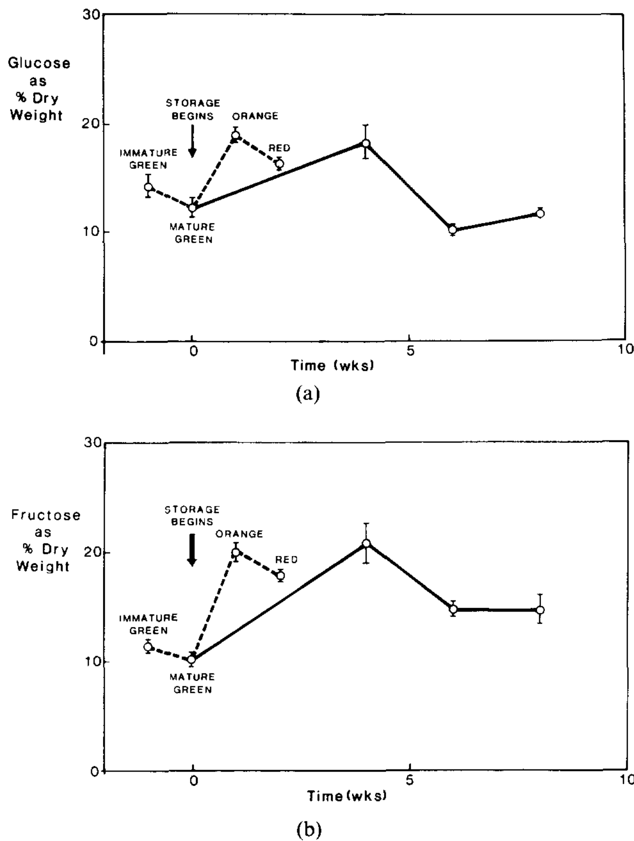
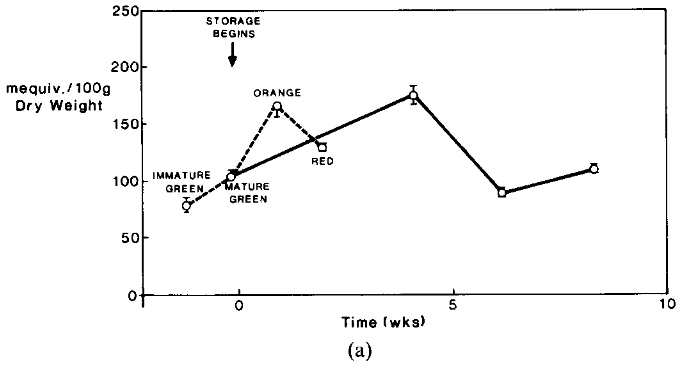


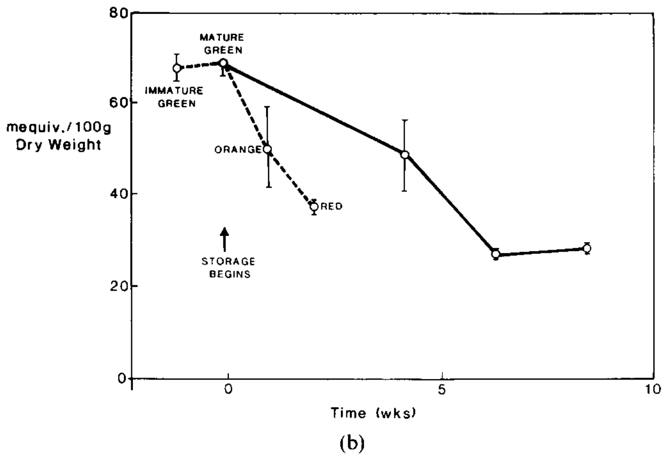
Fig. 1. Change in concentration of glucose (a) or fructose (b) concentrations during either normal ripening (dashed line) or storage of mature-green fruit for 8 weeks in atmospheres of 5% carbon dioxide, 5% oxygen and 90% nitrogen. Bars represent standard error of the mean.

Effect of ripening and storage on electrophoretic distribution of proteins and enzyme multiple forms

Gross proteins: After extraction from the tissue the constituent protein fractions were separated by acrylamide gel electrophoresis. Figure 4 shows the patterns obtained after staining with Coomassie blue G.250. Extract of the immature-green fruit showed one less band than the mature-green, and two bands between R_{BPB} 0·0–0·05 were present in mature-green and orange fruit but not in ripe fruit. Although a band at R_{BPB} 0·85 appeared in mature-green fruit it was absent in the other stages and two bands at R_{BPB} 0·28 and 0·45 were lost after the immature stage. After 6 weeks of retarded ripening the mature-green fruit showed a redistribution of protein with more of the protein at R_{BPB} 0·8–0·95 and less in the area of R_{BPB} 0–0·5 where a number of bands apparent in the mature-green fruit before storage had now



(a)



(b)

Fig. 2. Changes in citric acid (a) or malic acid (b) concentrations. Details as for Fig. 1.

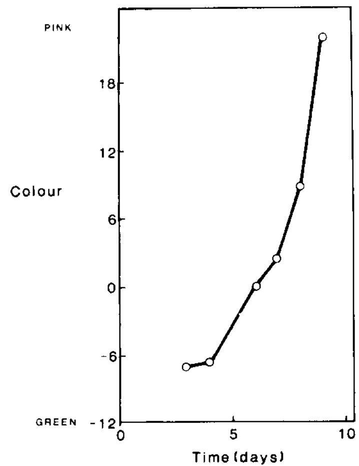


Fig. 3. Change in colour from light-green to red of tomato fruit held at 25°C in normal atmospheres after 20 days of controlled atmosphere storage.

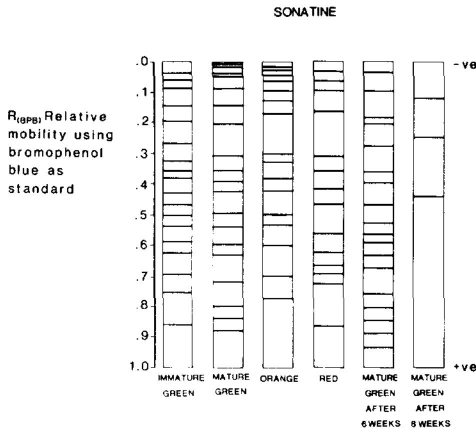


Fig. 4. Electrophoretograms of proteins after separation and staining on rods of polyacrylamide gel.

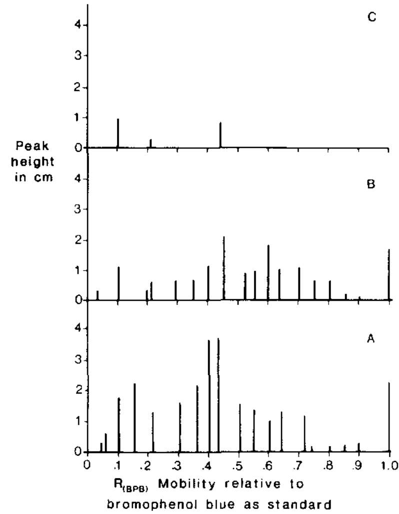


Fig. 5. The quantification of proteins separated by electrophoresis on polyacrylamide gels by scanning the gel with a spectrophotometer attachment at 550 nm. Peak heights are shown versus the distance travelled relative to bromophenol blue. A. Mature-green fruit before storage. B. Mature-green fruit after 6 weeks' storage. C. Mature-green fruit after 8 weeks' storage.

been lost. After 8 weeks of storage there was considerable loss of protein bands. In a previous paper we have shown a histogram of the main protein bands after measurement by a densitometer (Goodenough & Thomas, 1980) and a similar treatment is shown in Fig. 5. The total amount of protein decreases during ripening, as well as during the first 6 weeks of storage (Table 1).

Extractions were repeated to try and allow for artifacts arising from methods of preparation; in most cases the mature-green fruit have similar numbers and mobility

TABLE 1
PROTEIN CONTENT OF SONATINE TOMATOES RIPENED IN AIR OR STORED IN CONTROLLED ATMOSPHERES

Stage of ripeness or storage	*Protein content (mg/g fresh weight)*
Immature green	0·805
Mature-green	0·455
Orange	0·184
Red	0·116
4 weeks' storage of mature-green	0·662
6 weeks' storage of mature-green	0·374
8 weeks' storage of mature-green	0·392

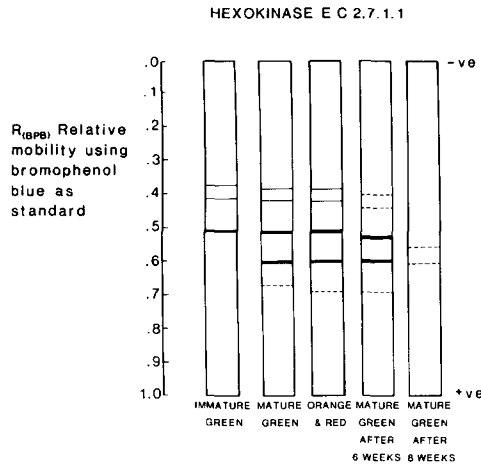


Fig. 6(a). Electrophoretograms of hexokinase isoenzymes. Solid lines indicate bands capable of being quantified by scanning at 560 nm. Dashed lines are bands visible by eye but not detectable by scanning at 560 nm.

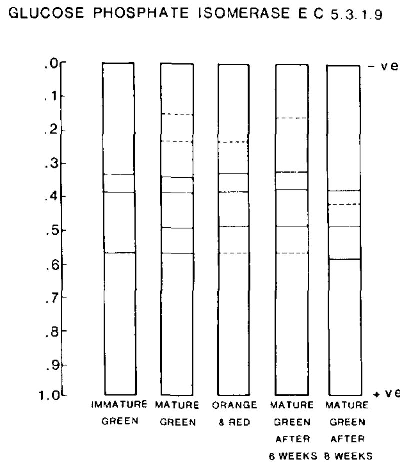


Fig. 6(b). Electrophoretograms of glucose phosphate isomerase isoenzymes. Details as for Fig. 6(a).

of multiple forms of enzymes to that found by Hobson (1974) but differences may be due to cultivar or extraction medium.

Specific enzymes whose activities affect phosphorylated glucose: Hexokinase phosphorylates glucose at the 6 carbon position. The hexokinase (EC 2.7.1.1) from tomatoes has five main multiple forms in mature-green tissue and these decrease slightly in intensity with ripening. After 6 weeks storage, only two main forms are still present and 2 weeks later both of these are very indistinct (Fig. 6(a)).

The enzyme glucose phosphate isomerase (EC 5.3.1.9) is known to have up to ten multiple forms in tomato fruit, six of which stain only lightly (Hobson, 1974). In Sonatine the four heavily stained bands were identified, but only two of the lightly stained bands. During ripening the number of forms and the activity of those remaining decreases slightly and this trend is continued during storage. However, the functioning of this enzyme is probably unimpaired during storage and there is still more than 80 % of activity of unstored fruit present in fruit stored for 8 weeks (Fig. 6(b)).

When enzymes of the pentose phosphate pathway were examined, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) gave three main bands of activity and one minor band. The bands were widely spaced (R_{BPB} 0·4–0·6), as opposed to the four bands of Hobson (1974), which were close together and made up of one main band with three light bands. There was a decrease in activity of glucose-6-phosphate dehydrogenase over 6 weeks of storage and almost complete disappearance of activity by 8 weeks. A similar trend was recorded for 6-phosphogluconate dehydrogenase (EC 1.1.1.44) where enzyme activity decreased over 8 weeks of storage (Fig. 7(a) and (b)).

Enzyme activities in the citric acid cycle: Malic dehydrogenase (EC 1.1.1.37) is active in all extracts of tomato fruit and consists of two multiple forms, probably representing the two forms of enzyme, mitochondrial and extramitochondrial (data not presented). The $NADP^+$ linked decarboxylating malic enzyme (EC 1.1.1.40) has three main bands and three minor bands at the mature-green stage, similar to that described by Hobson (1974), and a loss of the three minor bands was recorded during ripening or storage. The presence of a malic enzyme indicates that malate can be converted to pyruvate but there is no evidence to suggest a greatly increased activity of the enzyme is present during ripening. The mitochondrial ($NADP^+$)

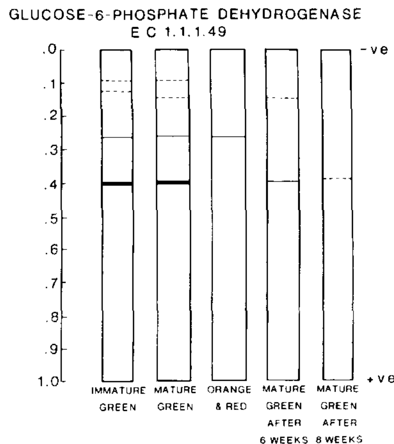


Fig. 7(a). Electrophoretograms of glucose-6-phosphate dehydrogenase. Details as for Fig. 6(a).

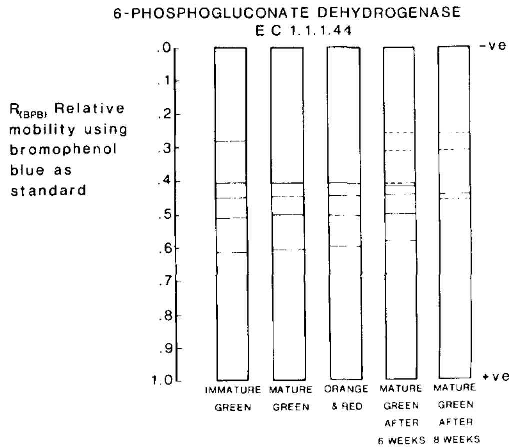


Fig. 7(b). Electrophoretograms of 6-phosphogluconate dehydrogenase. Details as for Fig. 6(a).

isocitrate dehydrogenase (EC 1.1.1.42) is also found throughout storage with two bands (not shown).

Glutamate oxaloacetate transaminase (EC 2.6.1.1) has been reported as having some regulatory function in tomato fruit mitochondria (Besford & Hobson, 1973) and the three main bands found previously (Hobson, 1979) were present throughout ripening and storage (data not illustrated).

Soluble NADH and NADPH dehydrogenase: The soluble NADH dehydrogenase enzymes react with cytochrome-c or lower homologues of coenzyme Q or artificial electron acceptors such as 2,6-dichlorophenolindophenol. Hobson (1974) has identified the NADH diaphorase enzyme (EC 1.6.2.2) and these are probably difficult to distinguish from NADH and NADPH dehydrogenase (EC 1.6.99.1 and EC 1.6.99.3) using the electrophoretic and histochemical staining technique described in this paper. The use of cytochrome-c as acceptor is the only way of directly identifying soluble NADH dehydrogenase but, by using dichlorophenol-indophenol, it has been possible to do a general survey of the dehydrogenase enzymes during ripening and storage. The greatest intensity of both dehydrogenases was recorded at the mature-green stage in fruit and activities of both dehydrogenases

TABLE 2
PEAK AREA OF NADPH AND NADH DEHYDROGENASE REACTIVE ZONES AFTER SEPARATION ON POLYACRYLAMIDE GEL BY ELECTROPHORESIS

Peak area (cm)	*Weeks of storage*		
	0	*6*	*8*
(a) NADH dehydrogenase	70	10	2
(b) NADPH dehydrogenase	100	20	5

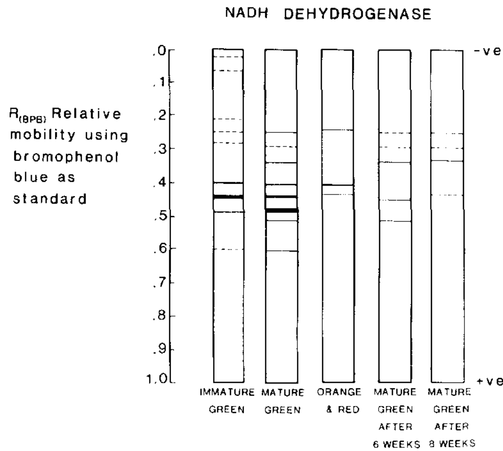


Fig. 8(a). Electrophoretograms of NADH dehydrogenase. Details as for Fig. 6(a).

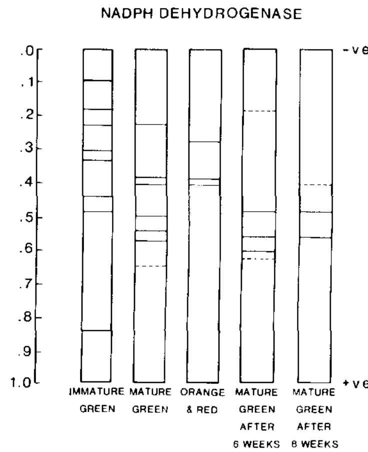


Fig. 8(b). Electrophoretograms of NADPH dehydrogenase. Details as for Fig. 6(a).

declined markedly during storage. After 8 weeks there was only negligible activity of NADH dehydrogenase and reduced activity of NADPH dehydrogenase (Fig. 8(a) and (b) and Table 2).

Effect of ripening and storage on hydrolases: Peroxidase (EC 1.11.1.6) and esterase (EC 3.1.1.1) were detected at all stages of ripening and storage. Esterase in orange tomato fruit showed two main bands of activity, one at R_{BPB} 0·30 and one at R_{BPB} 0·7, only the first of which seemed to be present in mature-green fruit. Hobson (1974) showed two main bands at R_{BPB} 0·35 and 0·85, and six bands staining lightly, three staining moderately. The five main, or moderately staining, bands were usually identified in the work reported here and the main band of activity at R_{BPB} 0·7 seemed

to become more pronounced during storage. Peroxidase remains constant during storage.

DISCUSSION

Recent work of Grierson *et al.* (1980) has shown that, on normal ripening, the two forms of polygalacturonase are formed in orange fruit whereas they cannot be detected in green fruit. Some enzyme changes seem almost certainly to be due to *de novo* synthesis (Grierson *et al.*, 1980). In the experiments reported here, attempts to measure polygalacturonase quantitatively were not successful during storage because of lack of time to extract and measure this enzyme while analysing the other enzymes electrophoretically in as short a time as possible after extraction. However, a further experiment using fruit grown in a subsequent year has shown that polygalacturonase is not found in green fruit during storage (Goodenough *et al.*, in preparation). While there is no evidence that other enzymes are formed *de novo*, the appearance of lycopene in such large quantities may be due to synthesis of new enzymes just before red colour development.

The amount of starch detected in green fruit was in the range of 11–12 % of dry weight but solubilisation and separation of starch from dried residue was difficult using either boiling water or dimethylsulphoxide. When a range of cultivars is examined some have rapid separation of starch but others seem to have starch complexed with the cellular material. In this case the starch only accounted for 75 % of the increase found in glucose and fructose. However, it is possible that there was not complete solubilisation of starch from the extracts as fruit stored in the dark must have developed monosaccharides from the starch. No other polysaccharide was found in either soluble or insoluble tomato residue. In other cultivars examined there is a complete balance between starch degraded and monosaccharides produced. Starch phosphorylase or amylase must degrade the starch initially and Clements (1971) has shown that amylase is present throughout development. Although some change in enzyme activity or regulation must occur to allow starch breakdown during tomato fruit ripening, there have not been any studies on the qualitative or quantitative properties of the enzymes involved. Examination of these enzymes is important before the loss of starch can be explained in the storage period. In potato tubers low temperatures induce an increase in disaccharides from starch but the regulation of this process has not been determined (Isherwood & Rigg, 1977). In arum spadix rapid breakdown of starch has been elucidated and involves hexokinase and phosphoglucomutase, as well as amylase (Bulpin & Rees, 1978). The results described in this paper indicate that the concentrations and regulation of starch phosphorylase, amylase, hexokinase and phosphoglucomutase during fruit storage and ripening should be examined and may lead to development of a suitable storage indicator.

Starch breakdown results in an increased amount of glucose which can, in turn, increase the rate of glycolysis. If high concentrations of pyruvate and acetyl CoA were made available to the mitochondria, then oxaloacetate would be utilised at a faster rate. As the primary control point of the citric acid cycle is generally acknowledged to be the condensation of acetyl CoA with oxaloacetate, increased glucose and acetyl CoA would have the effect of increasing citrate and decreasing malate. Citrate is accumulated in the tissues rather than fully utilised and this is not understood, although Davies and Maw (1972) have shown that C^{14} labelled citrate is not utilised as rapidly in red fruit as in green fruit. As they made no further investigations of enzyme activity there is no proof that the normal regulatory point of citrate metabolism, NAD linked isocitrate dehydrogenase, is controlling citrate use.

The point for increased control of monosaccharides is 6-phosphofructokinase and this enzyme is inhibited by high concentrations of citrate. It is reported that the kinetics of 6-phosphofructokinase control ripening (Chalmers & Rowan, 1971). However, if phosphofructokinase is the rate limiting step in glycolysis in tomato fruit ripening there must be linked control of amylase, isocitrate dehydrogenase and possibly other enzymes involved in key transformations which have not yet been examined. There is obviously some apparent contradiction over activity of key enzymes such as malate dehydrogenase which was reported to show little change in activity during development (Hobson, 1974) and also reported to increase during development (Hobson & Davies, 1976).

The work described here shows a change in substrate concentrations after 4 weeks retarded ripening but no concurrent changes in multiple forms from that found in mature-green fruit. After 8 weeks' storage the colour change (green–red) did not take place until the fruit was removed from its environment and occurred very slowly with more rapid fungal rotting in any physically damaged areas. Scandalios (1974) reported that the resolution achieved by polyacrylamide gel electrophoresis relies mainly on change and size differences which can easily be affected by extraction and storage of extracts. The differences shown in Figs 6(a) to 8(b) between mature-green multiple forms and mature-green fruit after 8 weeks' storage almost certainly do not indicate new molecular species but slight differences in the mobility of the existing multiple forms. Thus, in Fig. 6(a) and (b) hexokinase has four forms at the mature-green stage and only traces of the two most intense bands are found after 8 weeks' storage. Glucose phosphate isomerase has six forms at the mature-green stage and three of these are definitely present after 8 weeks' storage with a trace of a further band in a new position. It would be interesting to co-chromatograph the bands but this would mean storage of extracts for up to 8 weeks, which can lead to equally big differences developing during storage (Scandalios, 1979). In the case of gross protein there was no statistical difference between protein at 6 and 8 weeks (Table 1) but only three main forms were resolved by electrophoresis. This apparent decrease in protein multiple forms has been reported before (Goodenough & Thomas, 1980)

and represents a loss or amalgamation of some multiple forms; presumably while the fruit is still green fraction I protein constitutes a considerable amount of soluble protein. Enzyme activities of NADH and NADPH dehydrogenases were very low, as were the enzymes of the hexose monophosphate shunt and hexokinase. Even if the activities of the NADH and NADPH dehydrogenases were not measured alone, the so-called diaphorase also contributing to the activity, this decrease in activity must be rate limiting in the storage of tomato fruit. However, this would have to be checked using a quantitative assay involving cytochrome-c as acceptor.

If it is considered that completely new protein synthesis produces cell wall degrading enzymes (Grierson *et al.*, 1980) and possibly enzymes forming lycopene during ripening, then the results reported here show a distinct separation of two processes. First there is the change in enzyme regulation which results in starch degradation, i.e. increase in glucose and fructose concentration and the decrease in malic acid and increase in citric acid. Second there is the *de novo* synthesis of polygalacturonase and perhaps lycopene forming enzymes. This is similar to the two-stage process of Tigchelaar & McGlasson (1978) and Tigchelaar *et al.* (1978), who postulated a fall in substrate concentration brought about by cell wall bound enzymes which had been released by cell wall degrading enzymes. The results reported here do not support their theory as substrate concentrations change before any measurable change in softening of fruit. However, the proposal of a two-stage process in ripening depends on further work to show unequivocally that polygalacturonase is not formed during the period of substrate change in concentration. Similarly, this form of retarded ripening may allow examination of the kinetics and properties of enzymes involved in changing substrate concentrations free from *de novo* enzyme synthesis. The regulation and specific activity of amylase and starch phosphorylase, 6-phosphofructokinase, malic enzyme and isocitrate dehydrogenase deserve examination.

Storage in a certain gas atmosphere has led to a separation of two distinct sets of processes in tomato fruit ripening. In the first process starch breakdown is accompanied by monosaccharide increase and organic acid changes in the interconnected sequence of glycolysis and the citric acid cycle. The second process involves lycopene and polygalacturonase formation. Ripening may still be co-ordinated, however, if the second process relied on transcription which took place after 4 weeks' storage but translation only occurred after removal from the storage atmosphere. Further experiments to test this hypothesis will be reported.

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FACTORS INFLUENCING THE CAFFEINE CONTENT OF BLACK TEA: PART 1—THE EFFECT OF FIELD VARIABLES

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ABSTRACT

The caffeine content of made teas was influenced by seasonal, genetic, agronomic and cultural factors. The highest levels of caffeine were produced during the peak harvesting season when shoot growth rate was most rapid. Teas produced in the off-season contained about 50% less caffeine. The caffeine content of four clones varied by about 35% and varied in response to nitrogen fertiliser application, although, generally, caffeine levels increased with increasing N rate. The concentration of caffeine in the different shoot components varied markedly, being much higher in younger tissue and in leaf tissue than in stem tissue. A coarser plucking standard would result in a tea lower in caffeine content than tea produced from small shoots.

INTRODUCTION

Tea is the most widely consumed non-alcoholic beverage in the world and is produced from the shoots of the perennial shrub *Camellia sinensis* (L.) O. Kuntze. Its popularity as a refreshing drink and mild stimulant is principally due to the caffeine content of the tea plant. Caffeine (1,3,5,-trimethylxanthine), along with the two isomeric dimethylxanthines, theobromine and theophylline, which are present in minor amounts in tea, are well known central nervous system stimulants (Stagg & Millin, 1975). Caffeine also contributes to the characteristic taste of a tea infusion, forming a physico-chemical complex with polyphenols, principally of the theaflavin class (Roberts, 1962; Collier *et al.*, 1972). Complex formation modifies the taste characteristics of both the caffeine and theaflavin components (Millin *et al.*, 1969), the complex itself possessing the desirable taste described as 'tangy astringency' (Sanderson *et al.*, 1976). This complex contributes to the formation of the coloured

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precipitate or 'cream' when a tea liquor is allowed to cool (Roberts, 1962; Smith, 1968). The degree of cream formation is largely dependent upon the caffeine content (Smith, 1968) and is used by professional tea tasters as an indication of quality and hence for the evaluation of a tea.

The importance of the caffeine content in determining the character of the beverage and recent literature reports (Stagg & Millin, 1975; Graham, 1978; Greden, 1979) raising the question of the clinical importance of caffeine ingestion prompted the present series of investigations. The influence of genetic constitution, climatic conditions, fertiliser application and harvesting policy on caffeine levels in Central African black tea is reported in this paper. A second paper examines the effect of various manufacturing procedures on the caffeine content of the tea produced (Cloughley, in press).

MATERIALS AND METHODS

Source of materials

All leaf material used throughout this investigation was obtained from tea bush populations grown under defined agronomic conditions at the Nsuwadzi Tea Research Station (latitude 16 °S, altitude 630 m) in the Mulanje district of the tea-growing region of southern Malawi. Samples of four commercially exploited clones of diverse morphological and biochemical characteristics, MT12, SFS371, MFS76, SFS204, were obtained from a 4^3 factorial field trial planted in November, 1968. Each clone was planted at four population densities and four nitrogen fertiliser rates. Full details of the design, treatments and the agronomic management of the experiment have been reported (Dale, 1970; Cloughley, 1982*a*). In mid-February (1978) tea shoots of commercial standard, i.e. consisting of the terminal bud with two or three fully expanded leaves, were manually harvested ('plucked') from the 64 experimental plots. Since it is known that chemical changes occur throughout the day (Hilton *et al.*, 1973) it was necessary to standardise the time of plucking; hence, throughout these experiments the leaf was harvested before 07.00 h. Samples of MT12 and SFS204 from each of the lowest and highest nitrogen plots were also taken over a 12-month period from the beginning of the main production season in December, 1978. The bushes were plucked at regular intervals of 10–11 days during the main season from December to May and thereafter during the off-season on occasions when a sufficient population of harvestable shoots was available.

In mid-March, 1979 a hundred shoots of each clone of the three leaves and a bud size category were plucked. Twenty shoots of each clone were excised and the following components were obtained, bud (B), first leaf (L_1), first stem (S_1), second leaf (L_2), second stem (S_2), third leaf (L_3) and third stem (S_3). The components of each clone were separately dried in an oven, ground and analysed for caffeine. The exercise was carried out in quintuplicate.

The effect of nutrition on the caffeine content of a population of genetically heterogeneous Indian hybrid seedlings was investigated in another agronomy trial. In mid-February, 1978 samples were taken from the harvest from 27 plots comprising a 3^3 factorial experiment. Nitrogen, phosphate and potassium had each been applied at three levels (Grice *et al.*, 1977).

Manufacture

Black tea was prepared under controlled conditions in the tea station pilot production plant using a standard manufacturing procedure representative of that commonly used in tea industry in Central Africa. The details of the standard manufacture used throughout this investigation are fully described elsewhere (Cloughley, in press).

Caffeine determination

Made tea infusions corresponding to the drinking strength used by professional tasters (1:40) were prepared according to a standard procedure as previously described (Hilton & Ellis, 1972). The liquor was filtered through cotton wool to remove the infused leaf particles and through the spiral column of a water-cooled glass condenser to reduce the temperature rapidly. The infusions invariably reached ambient temperature within the 60 s allowed for this operation. It was necessary to bring the infusion to ambient temperature quickly to prevent the complexing of caffeine with the 'cream', the coloured precipitate formed during the natural cooling of a tea liquor (Rutter & Stainsby, 1975). A sample (10 ml) of a 1 in 100 dilution (prepared in triplicate) was transferred to a separating funnel and ammonium hydroxide (1 ml) was added to convert caffeine to the undissociated form of reduced solubility. Redistilled chloroform (10 ml) was added and the contents vigorously shaken for 60 s. Caffeine was removed in the chloroform layer and the concentration determined spectrophotometrically. The optical density was measured at 276 nm in a stoppered cell against a redistilled chloroform blank and the caffeine content was obtained from a calibration graph derived from measurements on standard caffeine solutions and expressed on a dry mass basis. Using the method of Jalal & Collin (1976) it was shown that the concentration of theobromine and theophylline never exceeded 50 and 1·0 mg %, respectively, and therefore the presence of the purine bases made a negligible contribution to the caffeine assay.

RESULTS AND DISCUSSION

Clonal variation and fertiliser application

Since the population effect had no influence on caffeine content this treatment is precluded from further consideration. It can be seen from Table 1 that there were marked differences in the caffeine content of the clonal teas considered. The mean

TABLE 1

THE EFFECT OF NITROGEN APPLICATION ON THE CAFFEINE CONTENT (% DRY WEIGHT) OF BLACK TEA PRODUCED FROM VARIOUS CLONES IN FEBRUARY, 1978

Clone	*Nitrogen application rate (kg/ha)*				*Mean*
	45	*135*	*225*	*315*	
MT12	3·20	3·59	3·80	4·25	3·71
SFS371	2·60	2·99	3·25	3·37	3·05
MFS76	2·46	3·25	3·30	3·36	3·09
SFS204	2·22	2·71	2·93	3·19	2·76
Mean	2·62	3·14	3·32	3·54	

Significant effects		SE
Clones	***	0·28
Nitrogen	***	0·28
C × N	*	0·55

* $P < 0{\cdot}05$; *** $P < 0{\cdot}001$.

caffeine content of the tea produced from MT12 was almost 35 % greater than that contained in SFS204. There was also a progressive increase in mean caffeine content with increasing nitrogen fertiliser application so that the caffeine content of teas produced from plots receiving 315 kg of N per hectare was over 35 % greater than that obtained at the lowest N level. It is, however, evident from the significant interaction among treatment effects that the caffeine content of the clones varied in response to application of nitrogen. In the case of clone MFS76 for example, the response to N at the two highest levels was negligible. This effect was probably related to the unusually poor yield response to N fertiliser observed for this clone since the 1974–75 season.

It can be seen from Table 2 that nitrogen application level also had a highly significant effect on the caffeine content of Indian hybrid tea. Caffeine levels in the teas produced from the plots receiving the highest N rates were over 25 % greater than those in low N rate teas. Since similar Indian hybrid material is established on

TABLE 2

THE EFFECT OF N AND K ON THE CAFFEINE CONTENT (% DRY WEIGHT) OF BLACK TEA MANUFACTURED FROM INDIAN HYBRID SEEDLINGS IN FEBRUARY, 1978

Nitrogen (kg/ha)	*Potassium application (kg/ha)*			*Mean*
	0	*35*	*70*	
45	1·84	2·18	2·25	2·09
180	2·23	2·46	2·52	2·40
315	2·38	2·78	2·75	2·64
Mean	2·15	2·47	2·51	

Significant effects		SE
N	***	0·30
K	*	0·30

* $P < 0{\cdot}05$; *** $P < 0{\cdot}001$.

more than 60 % of existing plantation land, this result is of general importance to the tea industry. Of the other two major nutrients only K has a significant effect on caffeine levels. It is noteworthy that in the Mulanje district there are also strong yield responses to both N and K. A comparison of Tables 1 and 2 shows clearly that the four commercial clones had higher levels of caffeine than the unselected hybrid material. This was really to be expected since the selection programme which gave rise to these clones was based on quality considerations, and it has been demonstrated that caffeine contributes to tea quality (Wood & Roberts, 1964; Millin *et al.*, 1969; Sanderson *et al.*, 1976). The two clones, MT12 and SFS204, can be considered as extreme representatives of the *Camellia assamica–sinensis* hybrid spectrum with MT12 illustrating the more pronounced *assamica* traits. Several previous studies have demonstrated some important differences between these divergent clonal types (Hilton, 1974; Cloughley, 1980*a*; Cloughley & Ellis, 1980; Cloughley, 1982*b*). The present study indicates differences in purine metabolism between the two clones, since caffeine in the tea plant is formed by nucleic acid catabolism (Bhattacharyya & Gosh, 1968; Ogutunga & Northcote, 1976).

Seasonal variation

The difference in caffeine content between MT12 and SFS204 was evident throughout the year (Fig. 1). The nitrogen effect also persisted over the entire period of study, although the magnitude of the difference between the two levels of N was considerably reduced during the off-season. The caffeine content was highest during the Central African main production season, lasting usually from December to the

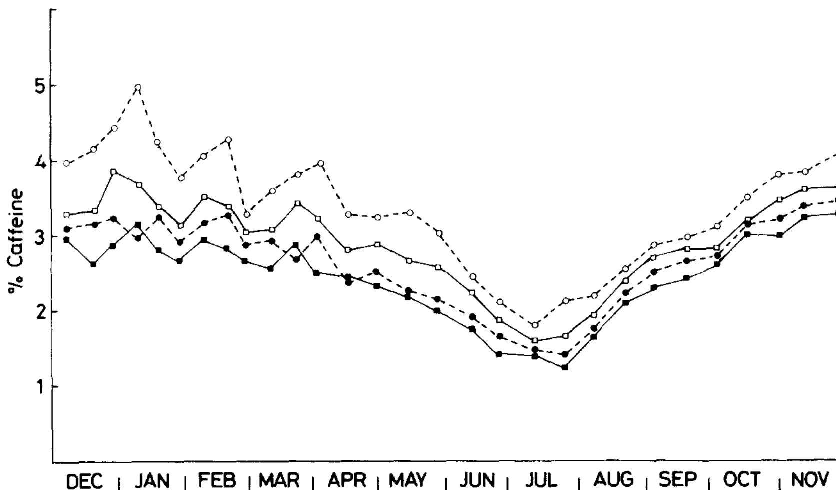

Fig. 1. Seasonal variation in caffeine content (% dry weight) of black teas ○, MT12 at 315 kg N per hectare; ●, MT12 at 45 kg N per hectare; □, SFS204 at 315 kg N per hectare; ■, SFS204 at 45 kg N per hectare.

end of April (Fig. 1). During this period, climatic conditions are favourable for rapid shoot growth and development and typically some 75 % of the annual crop is harvested. Conversely, caffeine levels were lowest during the cold winter months of June and July, when the low night temperatures and short day lengths severely limit growth rate and crop production (Squire, 1979). The effect of the unfavourable growth conditions was to reduce the caffeine levels in the four teas considered by more than 50 % compared with the levels obtained in the teas produced in January, during the peak harvesting period. The data presented have indicated that high caffeine levels are associated with rapid shoot growth rate. This suggestion would agree with the results from the nitrogen trials, since it is known that nitrogen application increases growth rate (Cloughley, 1980*b*). These results raise the interesting possibility that the determination of caffeine levels may indicate growth rates and aid in the prediction of yields.

Caffeine distribution in the harvested shoot

It can be seen from Table 3 that the caffeine content of the apical bud and the leaves was much greater than in the stem components of the plucked shoot for all

TABLE 3
CAFFEINE CONTENT (%) AND DRY WEIGHT (mg) OF THE PLUCKED SHOOT COMPONENTS OF FOUR CLONES HARVESTED IN MARCH, 1979

Shoot component	*Clone*							
	MT12		*SFS371*		*MFS76*		*SFS204*	
	Caffeine	*Dry weight*	*Caffeine*	*Dry weight*	*Caffeine*	*Dry weight*	*Caffeine*	*Dry weight*
B	5·90 (0·431)	21 (2·7)	5·13 (0·361)	10 (1·1)	5·60 (0·371)	16 (2·1)	5·17 (0·347)	12 (1·4)
L_1	5·86 (0·418)	33 (4·1)	5·06 (0·324)	22 (2·1)	4·67 (0·304)	38 (4·7)	5·13 (0·318)	27 (2·6)
S_1	2·45 (0·128)	11 (0·9)	2·76 (0·144)	7 (0·5)	2·54 (0·127)	14 (1·3)	2·64 (0·134)	9 (0·75)
L_2	5·76 (0·401)	65 (7·5)	4·50 (0·264)	46 (5·5)	4·15 (0·221)	86 (9·7)	4·98 (0·235)	58 (3·9)
S_2	1·46 (0·067)	39 (3·3)	2·00 (0·101)	16 (1·2)	1·61 (0·058)	30 (2·5)	1·92 (0·083)	20 (1·1)
L_3	5·12 (0·282)	85 (6·1)	4·50 (0·263)	64 (6·0)	3·87 (0·176)	139 (11·6)	4·00 (0·139)	80 (5·3)
S_3	0·84 (0·034)	33 (1·7)	1·58 (0·054)	15 (0·9)	0·88 (0·095)	24 (1·9)	1·46 (0·067)	21 (1·3)

The numerals in parentheses denote standard deviations.

the clones considered. It is also evident that caffeine levels decreased markedly in successively more mature tissue so that, for example in the case of MT12, the caffeine content of the third leaf was only 50 % of that in the bud.

The variation in caffeine content between the different shoot components will influence the levels of caffeine in tea made from leaf harvested according to different plucking policies. This is evident from Table 4, where the effect of plucking standard is calculated from the data presented in Table 3. Harvesting policy has a profound influence on yield—coarser plucking standards (i.e. longer shoots) producing more crop. The economics of tea production, however, are also affected by the quality and unit price of the product (Cloughley, 1979). Since caffeine contributes to the

TABLE 4
ESTIMATED EFFECT OF PLUCKING STANDARD ON CAFFEINE CONTENT

Plucking standard	*Clone*			
	MT12	*SFS371*	*MFS76*	*SFS204*
1L + B	5·28	4·64	4·43	4·58
2L + B	4·57	4·14	3·84	4·37
3L + B	4·31	4·06	3·64	3·97

character and value of the beverage, harvesting large shoots will have an adverse effect on tea quality.

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NUTRITIONAL AND OIL CHARACTERISTICS OF THE SEEDS OF ANGLED LUFFA *Luffa acutangula*

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ABSTRACT

Luffa seeds (L. acutangula), *a member of the Cucurbitaceae family, were investigated for their potential nutritional and oil characteristics. The yield of seeds from the fruit was 33·5%, while the kernel was found to possess 50% of the seed weight. The protein and fat contents of the kernel were 39% and 44%, respectively, and on a moisture and fat-free basis the kernel's protein content was 74·6%. The fatty acid profile indicates that the glycerides of oleic and linoleic acid constitute 68% of the total kernel oil. Iodine value, saponification value and acid value were 99·5, 190·8 and 10·5, respectively. The maximum melting and freezing points were −3 and −10°C, respectively. The seeds were also found to be a good source of certain amino acids, phosphorus, iron and magnesium.*

INTRODUCTION

With the increased demand for food, feed and raw materials for industrial use, more investigation into available resources is necessary, especially in developing countries where feed and food shortages are more dramatic. Many agricultural products are available but not utilised efficiently. Therefore, investigation of the potential of such products is essential to find new outlets for the utilisation of the world's resources.

The Luffa (sponge gourd) is a vegetable plant of the Cucurbitaceae family from the genus Luffa. The two commonly cultivated species are the angled Luffa (*L. acutangula*) and the smooth Luffa (*L. cylindrica*) (Martin, 1979).

The historical cultivation of Luffa plants has been reviewed by Richardson (1972). They are vegetables that are pleasant to eat and readily accepted by most people of the tropics and countries with warmer climates. The mature Luffas can

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also be used as diesel filters, bath sponges and for medicinal purposes (Porterfield, 1955; Watt & Breyer-Brandwijk, 1962).

The seeds from other curcurbits are often eaten and contribute protein, as well as oil, to the diet (Girgis & Said, 1968). The edible qualities of the Luffa seeds were investigated in Brazil (Martin, 1979), indicating that the pure seed oil can be used as a substitute for olive oil. The oil cake, due to bitterness and probable toxicity, cannot be used for feed but can be employed as a useful fertiliser due to its high content of proteins and phosphorus (Porterfield, 1955). The bitterness in Luffa seeds was reported to be bitter steroids (cucurbitacin B) which has been observed at levels as high as 0·67%. The evidence for and against the toxic effect of Luffa has been summarised by Watt & Breyer-Brandwijk (1962) and Varshney & Beg (1977) found that the poisonous materials in Luffa were due to saponins which yield sapogenins.

The present investigation was undertaken to examine the yield, nutritional value and oil characteristics of *Luffa acutangula* seeds and to determine their potential use on a large-scale basis.

MATERIALS AND METHODS

Materials

Luffa seeds, *L. acutangula*, were obtained from Dr A. Zitnak, Horticulture, and Dr T. Bates, Land Resource Science, University of Guelph, Ontario, Canada.

Methods

The testa from the seeds were removed manually. The kernels were ground in a Wiley mill to pass through a US standard 20 mesh sieve. Moisture was determined directly using the drying method at 102 °C. The rest of the sample was stored in a glass container, placed in a desiccator and stored at 4 °C until used. Proximate analysis was performed in duplicate in accordance with AOAC (1975) procedures: Soxhlet ether extraction for crude fat, macro-Kjeldahl for crude protein (%N × 6·25), ash by heating at 550 °C and crude fibre by digestion with acid and alkaline. The food energy value was calculated from the proximate analysis data by multiplying the fat by 9 and the protein and carbohydrate by 4 kcal/g.

The wet ashing procedure (David, 1962) was used for mineral analysis. An atomic absorption spectrophotometer was employed to detect Mg, Zn, Fe, Cu and Ca; in the case of calcium, a 1 % lanthanum solution was added. The spectrophotometric Molybdovanadate method described by the AOAC (1975) was used for phosphorus determination.

Oil characteristics and fatty acid analysis

The whole seeds were placed in a Sorvall Omni mixer and a solvent mixture of 2:1 chloroform/methanol, *ca.* 20 times of seed weight, was added. The seed–solvent

mixture was homogenised for 3 min and a procedure described by Folch *et al.* (1957) was followed to obtain the lipid extract. The methyl esters of the fatty acid were prepared from the petroleum ether extracts of the lipid residues according to the procedure described by Shehata *et al.* (1970). The fatty acid methyl esters (FAME) were analysed by a Varian 3700 gas chromatograph equipped with a Varian CDS 111 integrator on a 125 cm stainless steel (inside diameter, 3·2 mm) column packed with 15 % DEGS on Chromosorb R2-60/80 mesh operated at 180 °C.

The *cis, cis* methylene interrupted polyunsaturated fatty acids (PUFA) were determined by the lipoxidase method described by the Canadian Food and Drug Directorate (1967). The weight in grams of total *cis, cis* PUFA per 100 g of sample was calculated from the absorbance measured at 234 nm. Total *trans* fatty acids were determined with a Beckman IR 4230 infrared spectrophotometer using the method described in the AOCS cd 14–61. The refractive index was measured at 40 °C with a Zeiss refractometer, the iodine value was determined by the Wijs method (AOCS cd 1–25), saponification value (AOCS cd 3–25), unsaponifiable matter (AOCS cd 60–40), acid value (AOCS cd 3a–63) and hydroxyl value (AOCS cd 13–60), were all determined in triplicate. The freezing and melting curves were determined using a 900-Du Pont thermoanalyser.

Amino acid analysis

A 100 mg dried and fat-extracted sample was weighed into modified Knotes hydrolysis tubes; internal standard and 10 ml of nitrogen-saturated 6N hydrochloric acid was added to hydrolyse the sample for 24 h at 110 °C. The sample was then centrifuged to remove humin, and the hydrolysates were evaporated to dryness using a rotary evaporator under vacuum at 40 °C. A sodium citrate buffer (pH 2) was used to collect the residue. A Technicon amino acid analyser NC-2 system with a 75 cm column (0·5 cm inside diameter) packed with type A ion exchange resin was used. The optical densities of the reaction of the ninhydrin and the individual amino acids were measured at 570 nm and 440 nm.

RESULTS AND DISCUSSION

The yield per hectare of Luffa plants is approximately 75 tonnes. Under normal cultivation conditions, 20 to 30 mature gourds weighing about 35 kg can be harvested per vine (Porterfield, 1955; Martin, 1979).

The results of the present investigation suggest that the seeds of *Luffa acutangula* are a good source for the production of oil and protein concentrates, especially in developing countries. The yield of seeds from the dry mature Luffa fruit was 33·6 %. The seed itself contains 50·4 % kernel and 49·6 % testa. The oil yields from the whole seed (kernel and testa) and kernel were approximately 22·2 % and 44·3 %, respectively.

TABLE 1
PROXIMATE AND MINERAL COMPOSITIONS OF LUFFA SEED KERNEL

Proximate composition (g/100 g)	
Moisture	5·56
Crude protein	39·3
Crude protein (moisture and fat-free)	74·6
Crude lipid (ether extract)	44·3
Ash	4·13
Crude fibre	2·82
Carbohydrate (by difference)	3·91
Food energy (kcal/100 g)	571
Mineral content (mg/100 g)*	
Fe	10·7
Ca	62·0
Zn	5·80
Cu	2·18
P	1050
Mg	330

* Dry weight basis.

The proximate and mineral compositions of Luffa seed kernels are presented in Table 1. Moisture and carbohydrate content were low. The crude protein and oil contents were 39 and 44 %, respectively, and the protein content of the moisture and fat-free sample was 74·6 %. The calculated food metabolisable energy of 571 kcal/100 g is high due to the high oil content. The kernels were also found to be a good source of Fe, Mg and P, with contents of 10·7, 330 and 1050 mg/100 g, respectively.

TABLE 2
AMINO ACID COMPOSITION OF THE LUFFA SEED KERNEL (DRY AND FAT-FREE BASIS)

Amino acid	%*
Aspartic acid	3·75
Threonine	1·38
Serine	2·31
Glutamic acid	9·27
Proline	2·54
Glycine	2·28
Alanine	2·11
Valine	1·55
Cystine	0·55
Methionine	0·54
Isoleucine	1·83
Leucine	3·20
Tyrosine	1·52
Phenylalanine	2·70
Lysine	2·25
Histidine	1·12
Arginine	9·82

* Average of duplicate run.

TABLE 3
OIL CHARACTERISTICS OF LUFFA SEED OIL

Luffa seed oil	*Characteristics*
Oil (% in seed)	44·3
Weight per ml at 20°C	0·9213
Refractive index n_D^{40}	1·4671
Iodine value	99·6
Saponification value	189·6–191·9
Unsaponification matter	1·02
Acid value	9·88–11·12
Hydroxyl value	1·85
Freezing range	−2 to −25°C (max −10)
Melting range	−4 to −18°C (max −3)

The amino acid profile of hydrolysed Luffa protein indicated that essential amino acids, such as lysine, are present in good quantities. Fair amounts of cystine and methionine are also present, as shown in Table 2.

The solvent extracted Luffa oil has a pale green colour, a high acid value of 10·5 and unsaponifiable matter of 1·02. The oil remains liquid at room temperature with maximum melting and freezing temperatures of −3 and −10°C, respectively. The iodine value and saponification value were 99·6 and 190·8, respectively, as shown in Table 3.

The fatty acid composition of Luffa oil is shown in Table 4. Linoleic acid was the major constituent at 43·7%. The total saturated fatty acid content was 32·1%, while unsaturated fatty acids represents 67·9%. The *trans* isomers were present at less than 1% and *cis,cis* PUFA at 21·6%.

These results suggest that Luffa oil is of a semi-drying type, and is comparable with other vegetable oils with respect to its unsaturated fatty acid composition and similar in characteristics to other cucurbitaceae oils such as melon oil (Girgis &

TABLE 4
FATTY ACID COMPOSITION (WEIGHT, %) OF LUFFA SEED OIL

Fatty acid	*Weight, %*
Myristic 14:0	0·45
Palmitic 16:0	20·9
Stearic 18:0	10·8
Oleic 18:1	24·1
Linoleic 18:2	43·7
Total saturated	32·1
Total unsaturated	67·9
cis,cis PUFA*	21·6
Total *trans* isomers	0·20

* *cis,cis* methylene interrupted poly unsaturated fatty acid.

Said, 1968). As a consequence of its fatty acid content, Luffa oil could be used as a substitute for other vegetable oils such as olive, sunflower and rapeseed oils.

The extracted oil from angled Luffa is tasteless, but the cake is bitter—a fact which can limit its utilisation in poultry and animal feeds. Apparently, bitterness and poisonous substances occur in some varieties but not in others, of both cultivated species and it seems that non-bitter seeds are edible (Watt and Breyer-Brandwijk, 1962).

The results of this investigation showed that Luffa seeds are an excellent agricultural product with high nutritional value and can be used as raw material for oil extraction and protein concentrates. However, due to the bitterness and possible toxicity of the seeds of certain varieties, more research is needed to find varieties with edible seeds, free from bitter and toxic substances, and more work on the nature of the glucosides must be conducted. For full utilisation of the seeds, more work is also needed to establish other possible uses.

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DEGRADATION OF SORBIC ACID IN MODEL FOOD SYSTEMS

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ABSTRACT

Solutions of sorbic acid are unstable in the presence of sulphur dioxide and light. At pH 2·1 in the presence of excess sulphur dioxide, sorbic acid was totally degraded in 8 days. Sorbic acid was also unstable in solutions of dilute sulphuric acid. Two volatile products from the degradation in sulphur dioxide solution and in dilute sulphuric acid were identified as α-angelica lactone and 2-methyl-5-acetylfuran.

INTRODUCTION

Sorbic acid is permitted as a preservative in a range of foodstuffs, within many Western European countries. It is frequently used as a replacement for sulphur dioxide. In the United Kingdom, the regulations state that for many products both sorbic acid and sulphur dioxide may be used together. Even where only a single additive is used, sorbic acid may be present in the foodstuff with other organic acids. Other EEC countries also allow sorbic acid to be mixed with other preservatives. In Belgium, ice-cream may contain benzoic acid, sorbic acid and sulphur dioxide up to a total of 50 mg/kg. Denmark allows sorbic acid to be used in jams up to 1000 mg/kg, in which a certain level of sulphur dioxide may be encountered from the fruit. In The Netherlands, many products are permitted to contain both sorbic acid and benzoic acid.

Relatively little is known about the reaction of sorbic acid with other food additives, in particular sulphur dioxide. Aqueous solutions of sorbic acid are appreciably degraded in sunlight over a period of a year (Marx & Sabalitschka, 1965). A geranium type off-flavour in wines has been ascribed to the reduction of sorbic acid to sorbyl alcohol by lactic acid bacteria, followed by reaction of sorbyl

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alcohol with ethanol to form the highly odorous 2-ethoxyhexa-3,5-diene (Crowell & Guymon, 1975). It has been shown by Namika & Wada (1975) that sorbic acid reacts with nitrite to give ethylnitrolic acid.

EXPERIMENTAL

Rate of degradation in sulphur dioxide solution

Sulphur dioxide (BDH Chemicals) gas was bubbled into degassed distilled water (300 ml) for 30 s. The solution was made up to 1 litre with further degassed distilled water, and the sulphur dioxide content was determined by titration. One gram of sorbic acid (Ralph N. Emanuel Ltd, Wembley, Great Britain) was dissolved in 600 ml of degassed distilled water to give a stock solution. Solutions for degradation experiments were prepared by mixing the stock sulphur dioxide and sorbic acid solutions in different ratios with distilled water to give a final concentration of 670 mg sorbic acid per litre. Prepared solutions were divided and half was transferred to a clear bottle and the other half to a dark bottle covered with aluminium foil. The two bottles were stored at a distance of 50 cm under a 20 W fluorescent lamp (Atlas Daylight) in a cupboard and analysed at regular intervals for sorbic acid content.

Levels of sorbic acid were determined by gas chromatography on a column (2 m × 2 mm inside diameter) packed with 10% DEGA + 1% H_3PO_4 coated on Chromosorb W- AW DMCS (80–100 mesh) and operated at 160 °C. Nitrogen was used as the carrier gas at 30 ml/min and detection was by flame ionisation.

To overcome variations within the chromatographic system, a fresh sorbic acid solution, identical to the initial concentration, was also analysed on the same day.

Rate of degradation in acid solution

Sorbic acid (750 mg) was dissolved separately in the following solutions (500 ml).

(i) A buffer consisting of 0·5M KH_2PO_4 (333 ml) and 0·5M Na_2HPO_4 (23 ml) diluted to 1 litre with distilled water to give pH 5·4.
(ii) A buffer consisting of 1M acetic acid (145 ml) and 1M NaOH (50 ml) diluted to 1 litre to give pH 4·5.
(iii) A solution of 0·005M sulphuric acid at pH 2·3.
(iv) A solution of 0·1M sulphuric acid at pH 1·2.

Samples were stored and analysed as described above.

Identification of volatile degradation products of sorbic acid in sulphur dioxide solution

A solution of sorbic acid (240 mg) in distilled water (160 ml) was mixed with aqueous sulphur dioxide solution (237 mg in 200 ml water) in a clear glass bottle. The

solution was stored for 3 months as described above. A portion of the solution (100 ml) was extracted in a Likens–Nickerson apparatus against pentane (10 ml). The extract was carefully evaporated to 1 ml and aliquots were analysed by gas chromatography–mass spectrometry (GC–MS). Analysis was undertaken on a Hewlett–Packard 5992A instrument equipped with a glass capillary column (8 m × 0·5 mm inside diameter) coated with Carbowax 20M. Injections were made through a splitless injection port maintained at 250 °C. The column was temperature programmed from 24 °C to 170 °C at 3 °C a minute, with an initial isothermal period of 3 min. Helium was used as the carrier gas at a flow rate of 3 ml a minute measured at 90 °C.

Identification of volatile degradation products of sorbic acid in 0·005 M sulphuric acid

A solution of sorbic acid (750 mg) in 0·005M sulphuric acid (500 ml) was stored for 2 months as described above. An aliquot (100 ml) was extracted in a Likens–Nickerson apparatus against pentane (10 ml). The extract was carefully evaporated to 1 ml and aliquots were analysed by GC–MS as described above.

RESULTS AND DISCUSSION

The stability of sorbic acid in sulphur dioxide solutions is shown in Fig. 1. When the sulphur dioxide is present in slight excess over the sorbic acid, degradation in the light is very fast, being complete in about 8 days. The reaction is somewhat slower, showing about 85 % degradation over 30 days, when the sorbic acid is present in excess over the sulphur dioxide. If this degradation were to occur in beverages, then sorbic acid, added to wine at the maximum permitted EEC level of 200 mg/litre, in which the level of sulphur dioxide is 450 mg/litre (maximum permitted level), would, in all probability, be lost in 7–14 days. In the absence of light, sorbic acid present in a molar excess over sulphur dioxide hardly degrades at all over a 30-day period.

The rate of degradation of sorbic acid is controlled in part by the intrinsic acidity of the solution and partly by the presence of sulphur dioxide. Figures 1 and 2 show that at pH 2·1 in sulphur dioxide, degradation is complete after 8 days whereas, at the lower pH value of 1·2 in the absence of sulphur dioxide, only 80 % of the sorbic acid disappeared in the same period, thus illustrating the additional effect of the sulphur dioxide. Figure 2 illustrates the fact that, at very low pH values, severe degradation of sorbic acid occurs whether or not the solution is exposed to light whereas, at pH 2·6, light plays an important part in the reaction.

Figure 2 also shows that in most foods to which sulphur dioxide has not been added, sorbic acid is fairly stable, degrading slightly over a period of 2 months at pH values above 5·4.

A solution of sorbic acid dissolved in excess sulphur dioxide was examined for the presence of volatile components after storage in the light for 3 months. From the

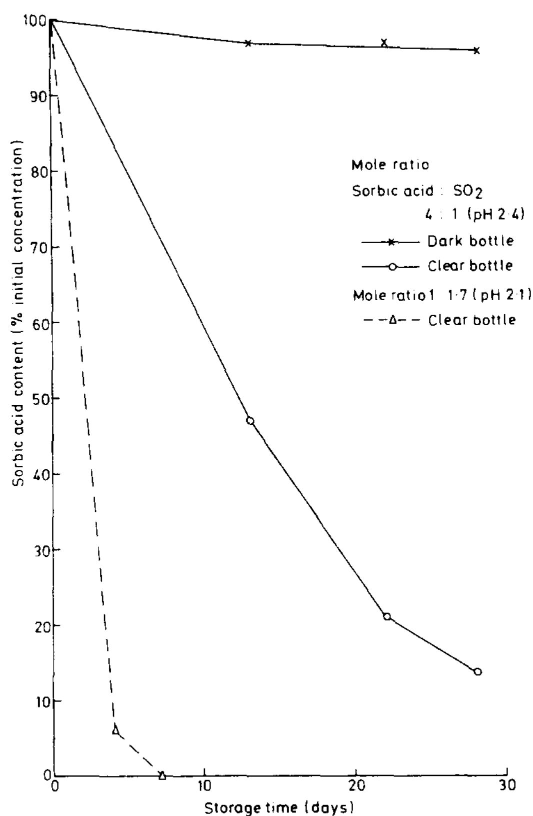

Fig. 1. Stability of sorbic acid in sulphur dioxide solutions under 20 W fluorescent lamp.

mass spectra of the five peaks in the chromatogram the first two were identified as α-angelica lactone and 2-methyl-5-acetylfuran, respectively (Table 1).

The chromatogram resulting from the extract of volatiles from sorbic acid stored in dilute sulphuric acid gave five peaks, two of which were again identified as α-angelica lactone and 2-methyl-acetylfuran, respectively.

The two compounds which have been identified represent five and seven carbon moieties, whereas sorbic acid contains six carbon atoms. Furthermore, two molecules of sorbic acid exactly account for the molecular formulae ($C_5H_6O_2$ and

TABLE 1
MASS SPECTRA OF VOLATILE COMPOUNDS IN DEGRADED SORBIC ACID

Compound	*Mass spectrum* *Relative intensities in parentheses*
α-angelica lactone	55 (100), 68 (42)
	98 (40), 96 (30), 50 (22), 69 (20)
2-Methyl-5-acetylfuran	109 (100), 124 (34)

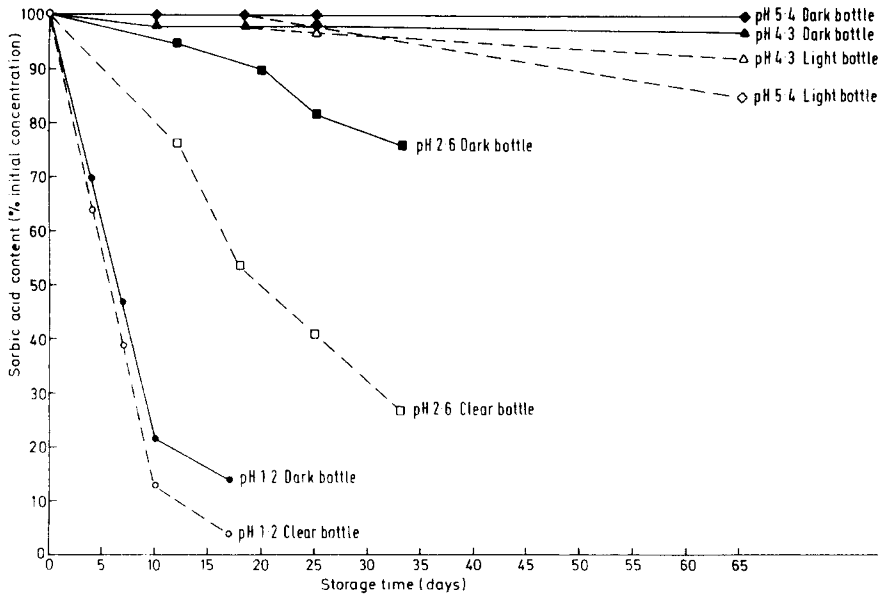

Fig. 2. Degradation of sorbic acid under a 20 W fluorescent lamp at different pH values.

$C_7H_{10}O_2$, respectively) of the two degradation products. The precise mechanism of this transformation is unknown.

The remaining unidentified peaks in both chromatograms yielded definitive mass spectra, but their comparison with both published and private libraries of data failed to lead to any positive identifications.

The volatile compounds which are formed as a result of the degradation of sorbic acid in an acidic medium account for less than 1 % of the products. Experiments are now being undertaken to determine the nature of the bulk of compounds which are formed in the reaction.

ACKNOWLEDGEMENT

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INTERACTION OF LYSINE WITH PECTIN

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ABSTRACT

The interaction of L*-lysine with citrus pectin was studied in a model system to determine under what conditions it would occur and whether it would be of physiological significance. Thirty-nine per cent of the lysine was non-diffusible at pH 7, when the pectin concentration was 0·5 % and the lysine concentration was* 2×10^{-3}M. *Of this, 29 % was restricted due to the Donnan effect and the remainder was specifically bound. Increasing the pH (in the range 6–8) and the pectin concentration both increased the non-diffusible lysine. Increasing the ionic strength and the degree of esterification both decreased the non-diffusible lysine. It was concluded that lysine would not bind to pectin in the intestine because of the high salt concentration there.*

INTRODUCTION

During the last decade there has been a steady accumulation of epidemiological evidence indicating that dietary fibre has a beneficial rôle in the human diet. Various reports have indicated that fibre has a protective effect against colonic disorders (Burkitt, 1973; Walker, 1976), diabetes mellitus (Trowell, 1976) ischaemic heart disease (Trowell, 1973; Kritchevsky *et al.* 1977). This has led to recommendations for increasing fibre intake (Burkitt & Trowell, 1975) which has already manifested itself in the addition of fibre to processed foods such as bread and breakfast cereals.

However, there have also been reports that dietary fibre decreases the utilisation of several nutrients, including minerals, fat and protein (Beyer & Flynn, 1978; Walker, 1975). A number of studies have shown that crude fibre and some purified fibre components decrease the utilisation of protein (Cummings *et al.*, 1976;

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Walker, 1975; Shah, 1981). One possible way in which this could occur is for fibre components to bind amino acids.

The main dietary fibre component containing a substantial number of functional groups at physiological pH is pectin, which can act as a cation exchanger because of its uronic acid component (Branch *et al.*, 1975). Preliminary experiments in our laboratory indicated that L-lysine, an essential amino acid which is positively charged at neutral pH, interacted with pectin. We have therefore investigated the nature and extent of this interaction so as to assess its physiological significance.

MATERIALS AND METHODS

L-Lysine–HCl was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, USA. Trinitrobenzene sulphonic acid (TNBS) and pectin (citrus) were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. It was 72 % esterified as determined by the methods outlined by Kertesz (1951*a*) and a 1 % w/v solution had a carboxylate anion concentration of $1{\cdot}26 \times 10^{-2}$M. Pectin samples from the same source but with different degrees of esterification were a gift from Professor M.T. Atallah, University of Massachusetts, Amherst, Massachusetts, USA.

The interaction of lysine with pectin was measured by the following procedure, with variations as noted below. The reaction mixture contained 50 ml 1 % w/v pectin, 10 ml 10^{-2}M lysine, 2 ml 0·1 M sodium phosphate buffer, pH 7·0, and 1 ml 2 % NaN_3. The pH was brought to 7·0 by adding ~0·40 milliequivalents of NaOH and the ionic strength was adjusted where necessary by adding NaCl, before bringing to volume (100 ml). In experiments to measure the effect of reaction parameters, the concentrations of lysine, pectin, NaCl, phosphate buffer and the pH were all varied. The reaction mixture was shaken on a rotary shaker (100 rpm) at 37 °C for 2 h. The mixture was then filtered using a PM10 ultrafiltration membrane with a 10,000 MW cut-off (Amicon Corp., Lexington, MA, USA) and the lysine in the ultrafiltrate was determined by the TNBS method (Snyder & Sobocinsky, 1975).

Non-diffusible lysine was calculated from the concentration of lysine in the filtrate, as compared with controls which did not contain pectin. Lysine which could be non-diffusible due to the requirements of the Donnan equilibrium (Donnan effect) was calculated according to the formula: $X = 2C_2/(C_1 + 2C_2)$ where X is the proportion of the total lysine in the ultrafiltrate, C_1 is the concentration of the sodium pectate and C_2 is the total concentration of all the salts present, including lysine–HCl, calculated as equivalent monovalent ions.

RESULTS

In the absence of added salt, 39 % of the lysine was non-diffusible when the pectin concentration was 0·5 %, the lysine concentration was 2×10^{-3}M and the pH was 7·0. When the experiment was repeated with ammonium chloride only 28 % of the

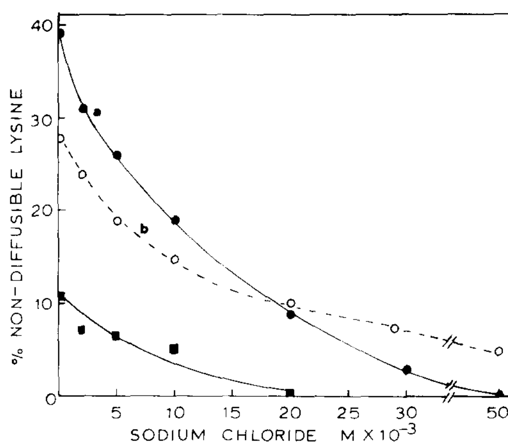

Fig. 1. Effect of NaCl on non-diffusible lysine. The reaction mixture contained 0·5 % pectin, 2×10^{-3}M lysine, 0·02 % NaN_3, 2 mM sodium phosphate buffer, pH 7·0, and sufficient NaOH to bring the pH to 7·0. (a) —●— total non-diffusible lysine; (b) --○-- non diffusible lysine calculated due to Donnan effect, —■— bound lysine ((a) minus (b)).

ammonium ion was non-diffusible. The latter value presumably represents the requirement for electrical neutrality due to the Donnan effect and is in excellent agreement with the calculated value for this system of 29 %. The increased non-diffusible lysine, above that predicted by the Donnan effect, suggests that some of the lysine specifically interacts with the pectin and may be termed 'bound'.

The effect of ionic strength on non-diffusible lysine is shown in Fig. 1. Increasing the ionic strength by adding NaCl caused a progressive decrease in both total non-diffusible lysine and bound lysine. At a concentration of ~20 mM NaCl the total non-diffusible lysine was equal to that predicted by the Donnan effect, and the bound lysine was therefore reduced to zero. At higher salt concentrations, the total non-diffusible lysine was progressively less than predicted and was zero at 50 mM NaCl.

One hundred per cent of the lysine was diffusible when the reaction was carried out in Tyrode buffer, which simulates the intestinal ionic environment, (Livingstone & Livingstone, 1970) and which contains 0·137 M NaCl. The same result was observed when the Tyrode buffer was prepared omitting the divalent cations (2·2 mM Mg^{2+}, 1·8 mM Ca^{2+}), which might compete for binding sites on the pectin.

The effect of pectin concentration on non-diffusible lysine is shown in Fig. 2. At constant ionic strength (Fig. 2A) both the total non-diffusible lysine and the bound lysine (that in excess of the Donnan effect requirement) were directly proportional to the pectin concentration and thereby to the number of carboxylate anions.

A quite different response was obtained when the ionic strength was not equalised at all pectin levels (Fig. 2B). Increasing the pectin concentration caused a

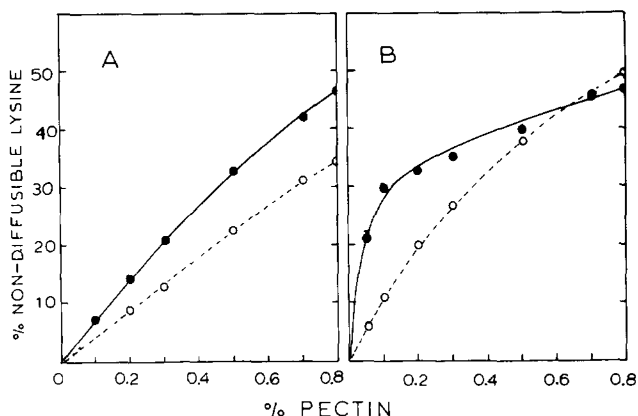

Fig. 2. Effect of pectin concentration on non-diffusible lysine. The reaction mixture contained 2×10^{-3}M lysine, 0·02 % NaN_3, 2 mM sodium phosphate buffer, pH 7·0, and sufficient NaOH to bring the pH to 7·0. A— Adjusted to constant ionic strength with NaCl. B— Not adjusted to constant ionic strength. —●— Total non-diffusible lysine. --○-- Calculated due to Donnan effect.

progressively smaller increase in total non-diffusible lysine, presumably because of the increased sodium ion concentration resulting from neutralisation of the pectin. As a consequence, the bound portion of the non-diffusible lysine was greatest at the lowest pectin concentration and then decreased with increasing pectin level.

The effect of lysine concentration on the non-diffusible lysine is shown in Fig. 3. The percent non-diffusible lysine was greatest at 5×10^{-4}M lysine. At higher lysine levels, more total lysine was non-diffusible, but the percentage of non-diffusible decreased slightly, due presumably to the increasing ionic strength. The percentage of lysine bound also decreased with increasing lysine concentration.

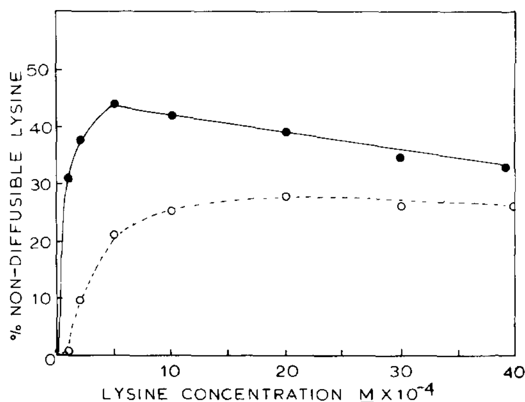

Fig. 3. Effect of lysine concentration on non-diffusible lysine. The reaction mixture contained 0·5 % pectin, 0·02 % NaN_3, 2 mM sodium phosphate, pH 7·0, sufficient NaOH to bring the pH to 7·0 and various concentrations of lysine. —●— Total non-diffusible lysine. --○-- Calculated due to Donnan effect.

The effect of the degree of esterification of the pectin is shown in Fig. 4. At constant ionic strength, the non-diffusible lysine was inversely proportional to the degree of esterification and thus directly proportional to the number of carboxyl groups. Because of the high salt concentration needed to equalise the ionic strength only a small amount of binding, in excess of the Donnan restriction, was observed.

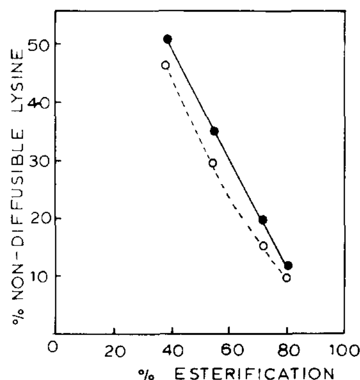

Fig. 4. Effect of degree of esterification of pectin on non-diffusible lysine. The reaction mixture contained 0·5 % pectin, 2×10^{-3} M lysine, 0·02 % NaN_3, 2 mM sodium phosphate buffer, pH 7·0, and sufficient NaOH to bring the pH to 7·0. The ionic strength was equalised with NaCl. —●— Total non-diffusible lysine. --○-- Calculated due to Donnan effect.

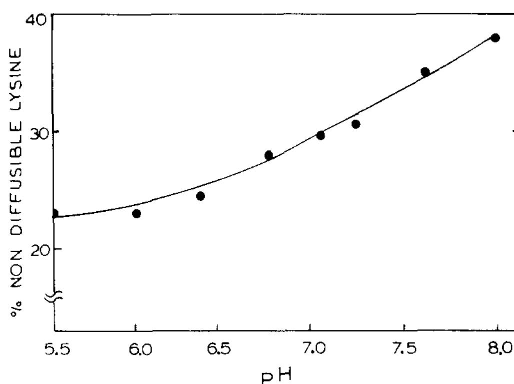

Fig. 5. Effect of pH on non-diffusible lysine. The reaction mixture contained 0·5 % pectin, 2×10^{-3}M lysine and 0·02 % NaN_3. The pH was adjusted with NaOH and the ionic strength equalised with NaCl.

These data, in conjunction with the data from Fig. 3, demonstrate that both total non-diffusible lysine and bound lysine are dependent on the concentration of carboxylate anions. Schweiger (1964) showed that the binding of calcium ions to pectin involved both carboxyl and hydroxyl groups. While our study does not preclude the involvement of hydroxyl groups, it seems clear that the carboxyl group is the primary determinant of the extent of diffusivity, both that due to the Donnan effect and the small amount bound at low ionic strengths.

The effect of pH on non-diffusible lysine is shown in Fig. 5. When the initial pH of the reaction mixture was above 7, we noted a fall in pH during the incubation at 37 °C. The pH reported in Fig. 5 is the final pH of the ultrafiltrate. The rise in non-diffusible lysine from pH 6 to about pH 7 may represent slowly increasing ionisation of the carboxyl groups (pK 3·7, Steddart *et al.*, 1969). The sharper rise in non-diffusible lysine thereafter cannot be attributed to ionisation and more probably reflects an increasing concentration of carboxyl groups due to pectin demethylation at alkaline pH values (Kertesz, 1951*b*).

CONCLUSION

Partially esterified pectin restricts the diffusivity of L-lysine at low ionic strengths. Most of the non-diffusible lysine is due to the Donnan effect, but there is some further binding of lysine at very low ionic strengths; this binding decreases as the ionic strength increases. Total non-diffusible lysine is controlled by the concentration of carboxylate anions on the pectin and increases with pH in the range 6–8. It is unlikely that pectin causes any restriction of lysine diffusivity in the mammalian intestine given the ionic environment prevalent there ($>0{\cdot}1$ M NaCl).

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LEAF PROTEIN ISOLATES FROM SOME EGYPTIAN CROPS

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ABSTRACT

The optimum conditions for the extraction of nitrogen from the leaves of four species of Egyptian crops—bean (Phaseolus vulgaris), *cabbage* (Brassica oleracea), *tomato* (Lycopersicon esculentum) *and sugar cane* (Saccharum officinarum)*—were studied. Nitrogen solubility increased above pH 6 and below pH 3. The isoelectric point of the extracted proteins was in the pH range 3·5–4·5.*

The leaf protein isolates of the four species were prepared and the chemical composition was studied. Leaf protein isolates were incorporated in some ready to eat popular foods—i.e. dehydrated vegetable soup, dehydrated tahmia and fresh butcher's sausage.

INTRODUCTION

There is an acute shortage of protein foods of high biological value among large segments of the world's population, especially in developing countries where protein deficiency, both in quality and quantity, causes widespread malnutrition and undernutrition (Kinsella, 1970).

Numerous investigations have focused on the possibility of utilising proteins obtained from green leaves as dietary supplements to improve the quality, as well as raise the protein level, of deficient diets (Pirie, 1969; Stahmann, 1970).

Extraction and concentration of protein from plant leaves have been under study in several laboratories in the United States, Great Britain, Africa and India.

The present work was aimed at preparing leaf protein isolates from some Egyptian crops in order to study their chemical composition and incorporate them in some local foods. The crops chosen were those cultivated in large areas whose leaves remain green at maturity.

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MATERIALS AND METHODS

Materials

The leaves of four species of Egyptian crops—bean (*Phaseolus vulgaris* var. 'Tender green'), cabbage (*Brassica oleracea* var. 'Capitata'), tomato (*Lycopersicon esculentum* var. 'Rutgers') and sugar cane (*Saccharum officinarum* var. 'Natal Co. 310') were chosen. Only the outer green leaves of cabbage, which are usually trimmed off and discarded in the fields before marketing, were used. Bean and tomato leaves were individually removed from their stems. The green tops of sugar cane, which are ordinarily removed during harvesting and discarded, were used. All samples were collected at the harvested time of the crops. The freshly collected leaves were immediately brought to the laboratory and extracted for their protein isolates.

Analytical methods

Moisture, ether extract, crude protein, total sugars, crude fibre and ash content were determined as described by the AOAC (1965).

Nitrogen extraction at different pH values

Samples of 10 g of fresh leaves were extracted in a Waring Blendor with 80 ml aliquots of distilled water, adjusted to pH values ranging from 1 to 12. Appropriate quantities of 1 N HCl and 1 N NaOH were used to adjust the pH with the final volume being equalised. After centrifugation the supernatants were taken for total nitrogen determination.

Solubility of nitrogen

The method of Smith *et al.* (1959) was used with some modifications. Samples of 25 g of fresh leaves were extracted in a Waring Blendor with a known volume of the solvent (distilled water and 0·01 N NaOH) for 15 min at room temperature. The suspension was centrifuged and total soluble nitrogen was determined in the supernatant by the microkjeldhal method. The results were expressed as percentage of the total nitrogen in the leaves.

Preparation of leaf protein isolates

Crude proteins were isolated by the method of Morrison & Pirie (1961). Leaf protein isolates were dried at 60 °C and ground in an electric mill to pass through a 60–80 mesh sieve and were then packed in tightly closed containers and stored at 4 °C.

Technological methods

The leaf protein isolates of the four crops obtained in this study were used for the supplementation of the following popular foods.

(*a*) *Dehydrated vegetable soup:* The following recipe was used. Green beans powder, 32%; carrot powder, 25%; potato powder, 34%; celery powder, 5%; parsley powder, 1%; sodium chloride, 2% and cumin powder, 1%. The reconstitution of the soup was carried out by mixing 10 g with 120 ml water and 1 g hydrogenated fat and boiling for 10–15 min. Samples were subjected to taste testing.

(*b*) *Dehydrated bean cakes* (Tahmia): Dehydrated *Tahmia* was formulated as follows. Broad bean meal, 40%; rice powder, 25%; powdered toasted Balady bread, 21%; dehydrated onion and garlic powder, 1% each; cumin powder, 2%; coriander powder, 3·5%; sodium bicarbonate, 2%; sodium chloride, 4·5%. Rehydration was carried out by adding about 50 ml of water to 50 g dehydrated *Tahmia* and the mixture was left for 45 min. *Tahmia* cakes were then shaped and fried in oil and examined by taste testing.

(*c*) *Butcher's sausage:* The sausage was prepared as described by El-Zalaki *et al.* (1976). The product was kept overnight under refrigeration at 5 °C before conducting taste testing after frying.

Taste testing: Samples of vegetable soup, bean cakes (*Tahmia*) and sausage were examined for their characteristics—i.e. colour flavour and texture—according to the method of Kramer & Twigg (1962). The results were means of scores of fifteen tasters.

RESULTS AND DISCUSSION

Chemical composition of green leaves

The results in Table 1 show that the highest protein contents (29·7% and 28·0%) were found in bean and tomato leaves, respectively, making them superior to the two other species. Sugar cane leaves had a distinctly low protein content.

Extractability of nitrogen from the leaves

The following experiments were undertaken to determine the optimum conditions for the extraction of protein from the leaves.

TABLE 1
CHEMICAL COMPOSITION OF GREEN LEAVES
(Dry weight basis)

Constituents	*Crop*			
	Bean (%)	*Tomato* (%)	*Cabbage* (%)	*Sugar cane* (%)
Ether extract	9·33	7·50	6·50	6·65
Crude protein	29·7	28·0	20·6	6·04
Total sugars	31·7	32·4	38·4	40·4
Crude fibre	9·53	10·4	15·2	22·4
Ash	19·5	16·3	18·5	19·5

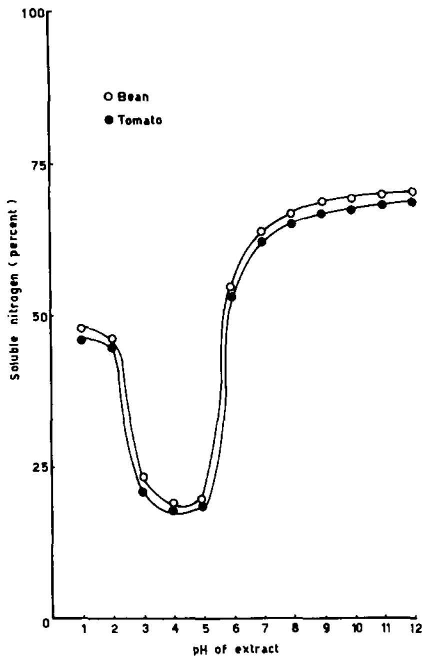

Fig. 1. Effect of pH on extractability of total nitrogen of bean and tomato leaves.

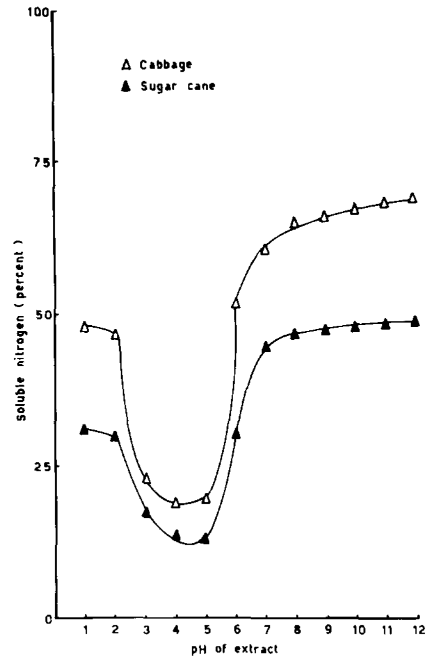

Fig. 2. Effect of pH on extractability of total nitrogen of cabbage and sugar cane leaves.

(*a*) *Solvent to leaf ratio:* Two solvents—distilled water (pH 6·1–6·3) and sodium hydroxide 0·01N (pH 10·3–10·5)—were used at room temperature. The results, expressed as percentages of nitrogen extracted per total nitrogen present in the leaves, showed that six to eight volumes of solvent were needed for near maximum extraction.

(*b*) *pH:* Figures 1 and 2 show that the extractability of nitrogen was higher at pH values above 9 than in the acid pH range with the maximum value being at pH 12 in all cases. The lowest extraction of nitrogen from the four species of leaves occurred between pH 4 and 5. The results are in agreement with those obtained by Betschart & Kinsella (1972) on soybean leaves.

To determine the isoelectric point of extracted leaf proteins, aliquots of the sodium hydroxide-soluble leaf protein extracts were adjusted to pH values from 1 to 12 as previously described and the clear supernatants obtained after centrifugation were tested for nitrogen content. It was found that the minimum solubility of extracted leaf protein occurred in the region of pH 3·5–4·5, which can be considered as comprising the isoelectric point or zone of these proteins. According to Chayen *et al.* (1961), the isoelectric point of leaf proteins is in the pH range of 4·0 to 4·5. Morrison & Pirie (1961) reported that the precipitation of extracted leaf proteins occurred at pH 3–4.

Yield of crude leaf proteins

The results in Table 2 show that the total nitrogen extracted (expressed as a percentage of total leaf nitrogen) varied from 70·2 % in the case of beans to 49·2 % in sugar cane. In spite of the fact that the total nitrogen content of sugar cane leaves was distinctly low, its possibilities are very promising considering the large areas of sugar cane plantations adjacent to sugar factories which permit the economic establishment of a unit for protein isolate preparation from the tops of cane.

TABLE 2
FRACTIONS OF LEAF NITROGEN

Crop	*Leaf*		*Total extractable nitrogen (% of total leaf N)*	*Extracted leaf protein isolate (g/100 g of fresh leaves)*
	Dry matter (%)	*N (% of dry matter)*		
Bean	18·1	4·75	70·2	4·80
Tomato	16·5	4·48	68·9	4·30
Cabbage	10·4	3·29	69·3	2·00
Sugar cane	27·4	0·97	49·2	1·30

Composition of leaf protein isolates

The results in Table 3 show that the protein content of the leaf protein isolates ranged from 48·0 to 69·2 % (on a dry weight basis). The higher level of 69·2 % was found in bean isolates, with tomato and cabbage yielding somewhat lower values (62·6 and 63·9 %, respectively) while sugar cane leaves gave the lowest yield of 48·0 % protein.

Pirie (1969) reported that the leaf protein which he separated contained about 60–70 % protein.

The total sugars content of leaf protein isolates ranged from 10·3 to 25·9 %. The high level of total sugars in the sugar cane leaf protein isolate may be attributed to the special rôle played by the leaves of sugar cane in the synthesis and accumulation of sucrose in the crop.

TABLE 3
CHEMICAL COMPOSITION OF LEAF PROTEIN ISOLATES
(On dry weight basis)

Constituents	*Leaf protein source*			
	Bean (%)	*Tomato* (%)	*Cabbage* (%)	*Sugar cane* (%)
Nitrogen	11·1	10·0	10·2	7·69
Crude protein	69·2	62·6	63·9	48·0
Ether extract	14·2	14·9	17·9	15·5
Total sugars	11·3	14·3	10·3	25·9
Crude fibre	0·85	0·43	0·49	1·43
Ash	3·41	6·75	6·45	8·06

The lipids content (including chlorophyll and other fat soluble pigments) of leaf proteins varied from 14·2 % to 17·9 % which may be regarded as useful components in the isolates.

The bean isolate contained the lowest content of ash (3·4 %) while that of sugar cane contained the highest value of ash (8·1 %). The ash content of the leaf protein concentrates depends on the amount of salts which are co-precipitated with the protein. Concentrate from the dried leaf proteins contained about 2–12 % ash (Aziz, 1970; Hartman *et al.*, 1967). The results in Table 3 indicate that the fibre content of the leaf protein isolates ranged from 0·3 to 1·4 %. These results are lower than those reported by other investigators (Doraiswamy *et al.*, 1969; Tao *et al.*, 1972; Devi *et al.*, 1966). Low fibre content raises the quality of leaf protein isolates.

Supplementation with leaf protein isolates

Kamalanathan & Devadas (1971) showed that leaf protein concentrate was more acceptable in foods which were normally green or dark in colour and when the strong flavours were masked by spices. Pirie (1971) reported that the amount of leaf protein eaten per day should probably not exceed 10 g in adults and less in children.

The following experiments were carried out in order to incorporate leaf proteins in some widely consumed foods using the protein isolate from tomato leaves.

(*a*) *Dehydrated vegetable soup:* The isolate was added to the dehydrated vegetable soup in the proportions 5, 7·5 and 10 %, based on the weight of the recipe, and soups were examined for their characteristics, namely, colour, flavour and texture after reconstitution.

The results indicate that the product containing the isolate in a proportion higher than 7·5 % was judged to be unacceptable, while that containing 7·5 % was acceptable, with scores ranging from good to very good. The sample containing 5 % was accepted, as well as the standard (without leaf protein).

(*b*) *Dehydrated* Tahmia *powder:* The isolate was added to the dehydrated *Tahmia* mix at levels of 5, 7·5 and 10 % and the taste testing panels showed that samples containing leaf protein up to 7·5 % were acceptable.

(*c*) *Butcher's sausage:* Sausage in which part of the meat was replaced by broad bean meal and leaf protein isolate (5, 10, 15 and 20 %) was prepared. The results of the taste testing panels carried out on the fried product indicated that that containing the leaf protein isolate in a proportion exceeding 15 % was judged to be unacceptable, while those containing lower levels were acceptable, receiving scores ranging from good to very good.

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NUTRITIONAL VALUE OF SOME CANNED TOMATO JUICE AND CONCENTRATES

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ABSTRACT

Fresh tomatoes, juice and concentrate were analysed chemically. The canned tomato products were not a good source of vitamin C, but their niacin content was higher than that of the fresh tomatoes. Thiamin was not affected by the canning process, but riboflavin was, and vitamin A was slightly affected. Nine elements: Ca, Cu, Fe, Mg, Mn, Ni, K, Na and Zn, were determined in the fresh tomato and its products, but Pb was found only in the final products.

INTRODUCTION

Tomatoes are grown in many regions of the world. In the USA and Europe more than 18 % of the total tomatoes produced are canned. It is very desirable from the point of view of nutritive value to maintain high levels of vitamins in food, as this has received considerable emphasis by both food processors and consumers (Sherkat & Luh, 1976). In the manufacturing process, ascorbic acid may be oxidised to dehydroascorbic acid which can be further oxidised to degradation products with less or no vitamin C activity. It is generally known that the nutrient content of raw fresh vegetables varies from region to region, due to genetic differences, climate conditions and maturity. Similarly, the methods and treatments employed during processing affect the nutritional content of the processed food. Nelson (1972) and Saldana *et al.* (1978) have already discussed the overall losses due to processing of ascorbic acid, thiamine, riboflavin and niacin in some vegetables. Elkins (1974) and Thomas *et al.* (1973, 1974) discussed the presence of metals in the canned fruits and vegetables. The purpose of this paper was to study the effect of processing on the nutritional value of tomatoes.

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MATERIALS AND METHODS

Materials

Fifteen kilograms of the Roma variety tomato (*Lycopersicum esculentum*) were hand harvested at canning ripeness from the University Farm, Pisa, Italy. Tomato juice and concentrate were canned according to the methods of Sherkat & Luh (1976).

Method

A Metrohn Herisau glass pH meter was used to determine the pH of the tomato juice and the concentrate after dilution.

Moisture, protein, ash, crude fibre, fat, vitamin C, riboflavin and niacin were determined according to the methods of the Association of Official Analytical Chemists (1965). Vitamin A was determined according to the procedures of the Association of Vitamin Chemists, Inc. (1965). Thiamine and calculated calories were determined according to the technique of Osborne & Vogt (1978). Carbohydrate was calculated by difference.

An Atomspek HI 170 (Hilger & Watts, 1971) atomic absorption spectrophotometer was used for mineral determination. The instrument setting and other experimental conditions were in accordance with the manufacturer's specifications.

RESULTS AND DISCUSSION

The contents of each can were examined. The physical condition of the lacquer was found to be good.

Table 1 shows the nutrient analysis of fresh tomato, juice and concentrate. The data are averages of triplicate determinations on a wet basis. It appears that there is

TABLE 1
NUTRIENT ANALYSIS OF FRESH TOMATO, JUICE AND CONCENTRATE

Component	*Fresh tomato*	*Tomato juice*	*Tomato concentrate*
pH	4·2	4·2	4·1
Moisture (%)	94·6	93·1	74·3
Protein (%)	0·91	0·86	2·81
Ash (%)	0·54	0·56	1·68
Fat (%)	0·15	0·16	0·48
Crude fibre (%)	0·56	0·45	0·98
Carbohydrate (%)	3·20	4·89	19·74
Calories/100 g	17·8	22·4	94·5
	Vitamins/100 g fresh weight		
Vitamin A (IU)	610	575	512
Vitamin C (mg)	16·5	8·2	—
Thiamin (mg)	0·042	0·042	0·041
Riboflavin (mg)	0·040	0·031	0·024
Niacin (mg)	0·570	0·720	0·780

no considerable difference between the moisture of fresh tomato and tomato juice, but the expected difference is shown in the tomato concentrate. Tomato juice is lower in protein and crude fibre and higher in carbohydrate in comparison with fresh tomato. The difference is due to processing. Tomato juice is heated and treated to remove skin, seeds and fibre.

The vitamin contents of fresh tomato, juice and concentrate are shown in Table 1. There are significant differences in the results. It appears that fresh tomato is the best, except for niacin which was slightly higher in tomato concentrate. During processing, niacin is partially hydrolysed by acid, but the hydrolysate has the same biological activity. Also, niacin is generally stable to heat, light, oxygen and alkali. In fact, niacin is one of the most stable vitamins (Nelson, 1972). Generally, tomato concentrate is a relatively poor source of vitamins except for niacin.

The metals determined in this study showed that fresh tomatoes and their processed products were rich in calcium and magnesium (Table 2), but all the metals determined showed higher concentrations in tomato concentrate as would be expected.

TABLE 2
MINERAL ELEMENTS OF FRESH TOMATO, JUICE AND CONCENTRATE

Element	*Fresh tomato*	*Tomato juice*	*Tomato concentrate*
		mg/100 g dry sample	
Calcium	6·01	6·84	25·2
Copper	0·04	0·05	0·18
Iron	0·04	0·52	1·42
Lead	—	0·02	0·04
Magnesium	5·08	5·11	6·60
Manganese	0·06	0·12	0·41
Nickel	0·06	0·16	0·50
Potassium	0·08	0·09	0·34
Sodium	0·08	0·09	0·31
Zinc	0·11	0·26	0·38

Lead was absent in fresh tomato, but was found in tomato juice and concentrate. The presence of higher levels of metals in tomato products is probably due to processing and canning. These data are in agreement with those of Thomas *et al.* (1973, 1974), Saldana *et al.* (1978) and Elkins (1979).

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CHANGES IN THE PROTEIN COMPLEX OF WHEAT DOUGH AFFECTED BY SOYBEAN 11 S GLOBULIN: PART 1—THE EFFECTS OF SOYBEAN 11 S GLOBULIN ADDITION ON THE TECHNOLOGICAL PROPERTIES OF WHEAT DOUGH

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ABSTRACT

The preparation of soybean 11 S globulin was added to wheaten doughs representing varieties differentiated in their baking qualities, and the changes induced in the protein complex were determined; the multistep extraction method and baking test were applied.

The gluten proteins in the dough of wheat varieties of low breadmaking potential were most affected by soy globulin. A decrease in the contents of the high molecular weight fractions was observed. The disaggregation effect was accompanied by an increase in the contents of proteins dispersible in acetic acid and pyrophosphate buffer.

One of the results of recent progress in breadmaking is the production of enriched bread with improved nutritional and sensoric values. The use of protein preparations based on milk (Jankiewicz *et al.*, 1976) or soybean (Ambroziak, 1976; Chabrowski *et al.*, 1973; Jakubczyk *et al.*, 1973; Pomeranz, 1966) is popular in baking. The general principles of the technological usage of such preparations have been elaborated and their effects on the quality of bread determined. There is, however, only limited information on the rôle played by proteinaceous components in the formation of a gluten matrix of the enriched doughs as well as on the modifications of rheological and physico-chemical properties of the protein complex.

In this study the effect of the addition of the soybean 11 S globulin preparation to the wheat dough has been examined to demonstrate the changes of the farinographic characteristics of enriched dough, its baking properties and the fractional

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composition of the protein complex. The experiments are similar to those undertaken in an analogous study of the effects of β-lactoglobulin addition on the protein complex of wheat dough (Kawka & Jankiewicz, 1977).

MATERIALS AND METHODS

In the experiments described in this paper two laboratory flours, from Grana and Mironowska wheat varieties, with different technological properties, were used.

The preparation of soybean 11 S globulin was isolated according to the method of Wolf *et al.* (1962) and purified by a modified method of Catsimpoolas *et al.* (1967).

The control doughs and the doughs containing the preparation of soybean 11 S globulin were prepared in 0·01M pyrophosphate buffer, pH 7, using a Brabender farinograph. The globulin preparation was added to flour in the form of a protein dispersion (0·03M phosphate buffer, pH 7·6, adjusted to ionic strength $\mu = 0{\cdot}3$ with sodium chloride) in quantities of 2·7 %, 4·1 % and 8·2 % of the protein content in Grana flour and 2·5 %, 3·8 % and 7·5 % in Mironowska flour at levels I, II and III, respectively. The dough consistency was adjusted to 500 Brabender units by subsequent additions of necessary aliquots of pyrophosphate buffer. The ionic strength of the doughs (approximately $\mu = 0{\cdot}1$) was similar to that in the bread doughs containing 1 % sodium chloride.

The fractional composition of the protein complex in the doughs was determined using the multistep extraction procedure according to Coates & Simmonds (1961) with modifications by Jankiewicz (1965) and calculated on the basis of Kjeldahl nitrogen analyses of protein dispersions (Jankowski & Jankiewicz, 1960).

The changes of baking properties of the doughs affected by the soybean 11 S globulin preparation were characterised using a laboratory baking test (Jakubczyk, 1971).

RESULTS AND DISCUSSION

The technological characteristics of the laboratory flours used in the experiments are presented in Table 1.

The flours corresponding to wheat varieties Grana and Mironowska had different extractions, water and mineral, as well as protein, contents. Higher protein content was found in Grana flour. Mironowska flour, however, was better for baking purposes. The water uptake, development of dough and stability of dough (parameters determining farinographic characteristics of flours) were much better in the case of Mironowska flour; this was similar to the results of the laboratory baking test. They corresponded to those obtained by Jankiewicz & Czyż (1974) and by

TABLE 1
THE TECHNOLOGICAL PROPERTIES OF LABORATORY FLOURS

Quality factors	*Wheat variety*	
	Grana	*Mironowska*
Flours		
Extraction (%)	65·0	72·0
Water content (%)	12·5	13·7
Protein (N × 5·7) (%)	13·5	11·6
Mineral substances (%)	0·49	0·59
Farinographic characteristics of doughs		
Water uptake (cm³)	60·2	65·0
Development (min)	3·5	4·5
Stability (min)	5·0	11·0
Laboratory baking test		
Volume of bread/100 g flour (cm³)	400·0	548·0
Porosity (scores)	8·0	7·0
Baking value (scores)	105·0	167·0

Mucha (1972) who classified Grana and Mironowska as the varieties of medium and high baking qualities, respectively.

The soybean 11 S globulin preparation was added to doughs at three levels. Level II was comparable with the 4% of soybean flour addition recommended as optimal by baking technologists (Lockmiller, 1973; Matthews, 1972; Pomeranz, 1966, 1969).

The doughs (Table 2, Fig. 1) showing the lowest water uptakes and the highest stabilities contained soybean 11 S globulin preparation at level II. The non-linear changes of the rheological properties accompanying increasing additions of the

TABLE 2
THE FARINOGRAPHIC CHARACTERISATION OF WHEAT DOUGHS CONTAINING SOYBEAN 11 S GLOBULIN PREPARATION

Dough wheat variety	*Levels* of 11 S globulin addition*	*Water** uptake (cm³)*	*Dough properties Development (min)*	*Stability (min)*
Grana	0	60·2	3·5	5·0
	I	59·6	1·5	3·0
	II	57·4	2·0	3·5
	III	59·6	2·0	2·5
Mironowska	0	65·0	4·5	11·0
	I	63·0	3·5	6·5
	II	61·4	3·5	7·5
	III	63·6	4·0	6·0

* The soybean 11 S globulin preparation was added to the doughs in amounts of 2·7 %, 4·1 % and 8·2 % of the protein content in Grana flour and 2·5 %, 3·8 % and 7·5 % in Mironowska flour at levels I, II and III, respectively.
** The doughs were prepared in 0·01 M pyrophosphate buffer, pH 7.

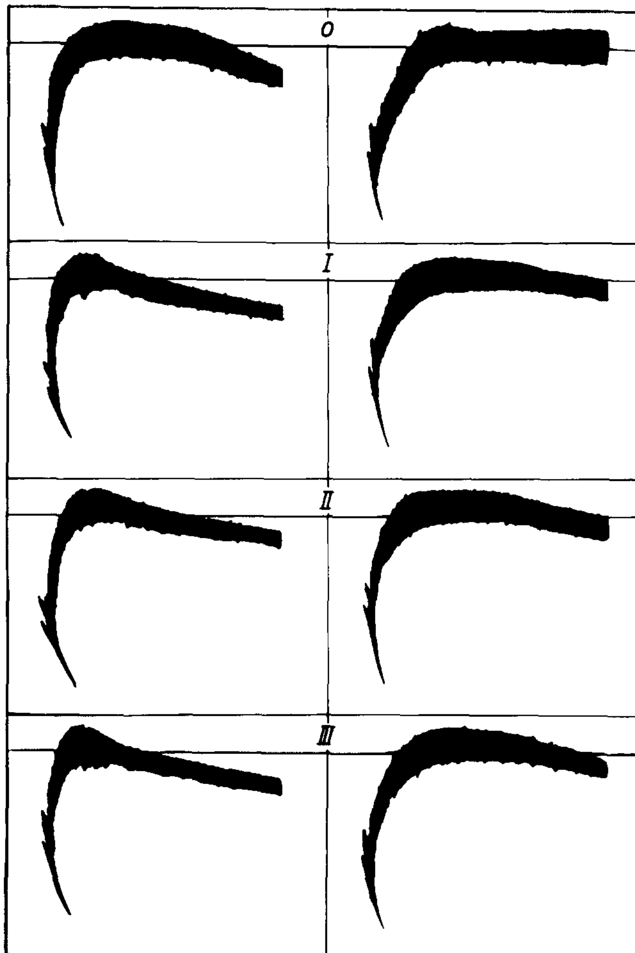

Fig. 1. The farinograms. Left: Grana, right: Mironowska. (For details see Table 2.)

preparation suggest that the soybean 11 S globulin interacts with dough components in different ways and affects not only the physical properties of the protein complex, but other components of the dough as well.

The mode of action of the soybean 11 S globulin preparation was similar for both flours, corresponding to wheat varieties of medium and good baking qualities. The use of sodium chloride for the stabilisation of ionic strength in the model doughs induces some additional changes (Gasiorowski & Jankowski, 1967). Neglecting this fact is one of the reasons for the considerable discrepancy between the results obtained for enriched doughs prepared with any salt additions and bread doughs of increased ionic strength (Gasiorowski & Jankowski, 1967).

Therefore, the results obtained in this study differ in some points from those published by other authors characterising the effects of soybean flour on bread quality (Ambroziak, 1976; Fink, 1972; Finney & Shogren, 1971; Kissell & Yamazaki, 1975; Mizrahi *et al.*, 1967; Pomeranz, 1966, 1969; Wolf, 1969, 1970). In most of the investigations cited above flour of high breadmaking potential, obtained from continental hard red winter wheat, was used and the soybean protein was introduced into the dough in the form of flour or protein isolates and concentrates.

The soybean 11 S globulin aggregates rapidly while the ionic (Wolf, 1969, 1970) strength of the medium is decreased to $\mu = 1{\cdot}0$. To avoid addition to the doughs of highly aggregated preparations showing varying degrees of polymerisation, the protein was used in the form of a dispersion in phosphate buffer ($\mu = 0{\cdot}3$). Although the volume of the solution so prepared did not exceed about a quarter of the total volume of buffer solution added, the effect of the increased sodium chloride content was to cause a decrease in the water uptake measured in the experiments.

To explain the changes induced by the soybean 11 S globulin preparation in the protein complex of enriched doughs, the fractional composition of that complex was determined (Table 3). The results obtained demonstrated an increase in the contents of pyrophosphate buffer dispersible albumins and globulins for addition to the preparation at level I followed by a slight decrease for levels II and III in the case of both wheat varieties. For gluten-type proteins dispersible in acetic acid, the increasing amounts of preparation added caused a remarkable increase in protein contents in the extracts, higher for the Grana variety. The changes occurring in sodium hydroxide extracts were opposite to those found for acetic acid extracts. The decrease in protein contents, however, was greater for Mironowska variety. The total contents of dispersible proteins changed less regularly than those determined

TABLE 3
EFFECTS OF ADDITION OF SOYBEAN 11 S GLOBULIN PREPARATION TO WHEAT DOUGHS ON THE FRACTIONAL COMPOSITION OF THEIR PROTEIN COMPLEXES DETERMINED BY MULTISTEP EXTRACTION METHOD

Dough wheat variety	*Levels* of 11 S globulin addition*	*Nitrogen content in sample, (%)*	*Nitrogen contents in extracts (per cent of total nitrogen content in sample)*			
			0·01M pyrophosphate buffer pH 7	*0·05M acetic acid pH 3·1*	*0·10M sodium hydroxide*	*Total*
Grana	0	2·38	12·5	51·5	10·8	74·8
	I	2·44	13·6	58·3	7·6	79·5
	II	2·47	12·7	60·4	7·3	80·4
	III	2·56	12·7	62·7	6·4	81·8
Mironowska	0	2·04	12·6	50·2	16·7	79·5
	I	2·09	14·8	50·2	9·6	74·6
	II	2·11	14·4	55·5	8·4	77·3
	III	2·18	14·4	60·5	7·7	82·6

* As in Table 2.

for the separate extracts. The total contents of proteins in the three extracts increased for the Grana doughs containing the globulin preparation at levels I to III.

In the case of Mironowska doughs a decrease in the contents of total dispersible protein was observed for additions of the preparation at levels I and II. The addition of soybean 11 S globulin at level III gave some increase in the amount of dispersible proteins, but not so distinct, however, as in the case of the Grana variety. The results obtained in this study indicate that the effect of soybean 11 S globulin is dependent on the proportions and the character of interacting protein complexes and cannot be treated uniformly for wheat varieties of different technological qualities.

The addition, for example, of the globulin preparation to Grana flour showing low tolerability to modifying agents causes a decrease in the contents of insoluble protein fractions (the aggregates of the highest molecular weights) and glutenin-like proteins dispersible in sodium hydroxide. In place of these two groups of proteins increased quantities of the fractions dispersible in acetic acid (prolamin and glutenin-like proteins) were found. Limited fluctuations in the albumin and globulin proteins dispersible in pyrophosphate buffer were observed for all three levels of globulin addition.

The effect of soybean globulin on the baking properties of the enriched flour is shown in Table 4.

The volumes of the loaves were slightly increased for globulin addition at level II. Other quality factors, such as porosity and baking value, or sensory value scores, were almost unaffected as compared with those determined for the control loaves. A difference in the behaviour of the two wheat varieties was found. In this aspect the results obtained differ slightly from those described by other authors (Ambroziak,

TABLE 4

EFFECTS OF ADDITION OF SOYBEAN 11 S GLOBULIN PREPARATION TO WHEAT DOUGHS ON THE RESULTS OF LABORATORY BAKING TEST

Dough wheat variety	*Levels* of 11 S globulin addition*	*Evaluation of bread*				
		Volume (100 g flour) (cm³)	*Volume factor (scores)*	*Porosity factor (Dallman scores)*	*Baking value (scores)*	*Sensory** value (scores)*
Grana	0	400	100	80	85·0	30
	I	390	90	80	77·0	28
	II	430	115	80	97·0	30
	III	400	100	80	85·0	28
Mironowska	0	470	135	90	136·5	34
	I	480	140	90	141·0	34
	II	510	155	90	154·5	35
	III	450	125	90	127·5	34

* As in Table 2.
** According to the instruction of Polish Baking Institute.

1976; Matthews, 1972; Mizrahi *et al.*, 1967; Mucha, 1972; Pomeranz, 1969; Rainey, 1961; Wolf, 1969) who reported some decrease in the bread volume, dependent, however, on the amount of soybean preparation added and the technology applied.

In our study the importance of the variety factor has been demonstrated, especially when the rheological and physico-chemical properties of enriched dough are considered.

CONCLUSIONS

(1) The changes caused by the introduction of soybean 11 S globulin into wheat dough are related to the potential baking quality of the flour used. For soft wheat varieties (e.g. Grana) or lower tolerance to modifying agents, the damage to the dough structure is greater than in the case of hard varieties (e.g. Mironowska).

(2) The changes of rheological properties of the enriched doughs can be accounted for by the modification of the protein complex depending on the shifts between the gluten and albumin and globulin groups of proteins. In most cases a decrease in the amount of high molecular weight fractions (insoluble or dispersible in sodium hydroxide solution) is obtained. The disaggregation effect is accompanied by an increase in the contents of proteins dispersible in acetic acid (part of the gluten proteins) and in pyrophosphate buffer (albumins and globulins).

(3) The addition to the dough of the soybean 11 S globulin in amounts up to 8 % of the protein content in flour does not affect the results of the baking test in the case of wheat varieties representing medium and good breadmaking potential. Higher additions of the preparation should not be recommended.

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Technical Note

The Chemical Constituents of Tomato Seeds

INTRODUCTION

Tomato seeds are a waste product from the food canning industry and find use for animal feeding. Gad *et al.* (1968) determined the chemical constituents of tomato seeds; they found seventeen amino acids in their proteins. Tsatsaronis & Boskou (1975) stated that the amino acid content and the nutritive value of tomato seed proteins were similar to those of soybean and sunflower proteins.

Amelotti *et al.* (1967) and Gad *et al.* (1968) studied the fatty acid constituents of tomato seeds.

The present study was carried out to determine the nutritional value of tomato seeds for food and feed.

MATERIALS AND METHODS

Samples of tomato seeds were taken from fifteen kilogrammes of Roma variety tomatoes (*Lycopersicum esculentum*) at canning ripeness from the University Farm, Pisa, Italy. After juice extraction, the pomace was partially dried and the seeds were removed manually and dried by lyophilisation. Moisture, ether extract, ash, crude fibre, sugars and nitrogen were determined according to the methods of the Association of Official Analytical Chemists (1965) and nitrogen-free extract was obtained by difference. All determinations were made in triplicate.

Amino acid analysis was carried out by using the Aminolyzer automatic amino acids analyser (Optica, Milan, Italy). Tryptophane was determined colorimetrically in the seeds according to Sodek *et al.* (1975).

The following method was used for preparing the mineral solution. A dry ashing procedure was used for atomic absorption and spectrophotometric analyses of all elements. Triplicate $1{\cdot}00 \pm 0{\cdot}05$ g dried samples were weighed into clean, dry platinum crucibles. Each sample was pre-ashed and ashing completed by placing the sample in an electric muffle furnace at 500 °C for 4 h. The ash was dissolved in 5 ml

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TABLE 1
THE CHEMICAL CONSTITUENTS OF TOMATO SEEDS

Constituents	*grammes per 100 g of dry sample*	*Elements*	*milligrammes per 100 g of dry sample*	*Amino acids*	*grammes per 100 g of protein*
Ash	5·5	Calcium	166	Lysine	4·91
Crude fibre	20·1	Copper	2·22	Histidine	2·26
Total sugars	3·1	Iron	17·5	Arginine	8·81
(glucose)		Magnesium	289	Aspartic acid	9·68
Protein	26·2	Manganese	5·12	Threonine	3·10
(N × 6·25)		Nickel	4·16	Serine	4·88
Ether extract	30·4	Potassium	778	Glutamic acid	18·5
Nitrogen free	14·7	Sodium	111	Proline	15·4
extract		Zinc	4·64	Glycine	4·62
				Alanine	3·70
				Half cystine	0·81
				Valine	3·81
				Methionine	0·80
				Isoleucine	3·56
				Leucine	5·81
				Tyrosine	3·40
				Phenylalanine	3·61
				Tryptophane	1·01

of 20 % HCl. The solutions were warmed to effect complete solution and filtered through acid washed filter paper (Whatman No. 42) into 100 ml volumetric flasks. After diluting to volume, the solutions were ready for analysis by atomic absorption spectrophotometry.

An Atomspek HI 170 (Hilger & Watts, 1971), atomic absorption spectrophotometer (AAS) was used for mineral analysis. The instrument setting and other experimental conditions were in accordance with the manufacturer's specifications.

A Hewlett-Packard F & M 5750 gas chromatograph with a dual flame ionisation detector and (6 ft × 3 mm inside diameter) stainless steel columns packed with 10 % diethylene glycol succinate on 80–100 mesh Chromosorb W AW-DMCS were used for the determination of fatty acid methyl esters. Fatty acid methyl esters were prepared with methanol containing 3 % concentrated sulphuric acid (AOCS, 1966). Peak areas and retention times were determined by a Hewlett-Packard Model 3370 A integrator.

RESULTS AND DISCUSSION

The results in Table 1 show the constituent percentages of the tomato seeds as follows: 5·5 ash; 20·1 crude fibre; 3·1 glucose; 26·2 protein; 30·4 ether extract and 14·7 nitrogen free extract. All results are expressed on a dry weight basis.

Tsatsaronis & Boskou (1975), in their study on tomato seed constituents, reported slightly lower constituents than this finding. They also found lower amounts of minerals in tomato seeds grown in Greece.

There are eighteen amino acids in the tomato seed protein (Table 1). Gad *et al.* (1968) found seventeen amino acids in the tomato seed protein grown in Egypt, and Tsatsaronis & Bosku (1975) found eighteen amino acids but in different amounts.

The fatty acid composition of the tomato seed oil is shown in Table 2. There are four saturated fatty acids and three unsaturated fatty acids. Gad *et al.* (1968) found two saturated fatty acids, palmitic (21·9 %) and stearic (3·2 %) and two unsaturated fatty acids, oleic (35·8 %) and linoleic (39·1 %). In addition, Amelotti *et al.* (1967)

TABLE 2
THE FATTY ACID CONSTITUENTS OF TOMATO SEED OIL (AREA PER CENT OF TOTAL METHYL ESTERS)

Saturated fatty acids	%	*Unsaturated fatty acids*	%
Myristic	0·5	Oleic	25·5
Palmitic	16·1	Linoleic	50·5
Stearic	5·5	Linolenic	1·4
Arachidic	0·5		

found that tomato seed oil was composed of three major saturated fatty acids—palmitic (14·8 %), stearic (4·7 %) and arachidic (2 %)—and three major unsaturated fatty acids—oleic (20·3 %), linoleic (54·3 %) and linolenic (2·9 %).

The differences in results between the present investigation and the previous studies may be attributed to the varietal differences, region of cultivation and environmental factors.

The data show that tomato seeds are rich in nutrients. The fatty acid constituents of the tomato seed oil are similar to those of cottonseed oil (Abdel-Rahman and Youssef, 1978). The oil contains more than 75 % unsaturated fatty acids and is consequently a good salad oil. Its meal is a potentially useful animal feedstuff.

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BOOK REVIEWS

Developments in Food Science: 3A Food Flavours. Part A. Introduction. Edited by I. D. Morton and A. J. MacLeod, Elsevier, 1982. vii + 472 pp. Price: US$127·75.

This important book has chapters on Sensory Basis and Perception of Flavour (D. G. Moulton); Techniques of Analysis of Flavours: Chemical Methods Including Sample Preparation (D. A. Cronin); Techniques of Analysis of Flavours: Gas Chromatography and Mass Spectrometry (C. Merritt Jr.); Techniques of Analysis of Flavours: Sensory Methods (R. Harper); Techniques of Analysis of Flavours: Integration of Sensory and Instrumental Methods (J. J. Powers); Sulphur Compounds on Flavours (M. L. Shankaranaray and B. Raghaven, K. O. Abraham and C. P. Natarajan); Pyrazines in Flavour (J. A. Maga); Lipid Degradation Products and Flavours (W. Grosch); Maillard Reaction in Flavour (R. F. Hurrell) and Taints and Off Flavours in Foods (M. J. Saxby).

With such a distinguished set of authors there can be no doubt that the book brings the reader to the very forefront of knowledge in this area and the Editors must be congratulated on such a comprehensive coverage. Bibliographies are thorough and up to date. Some chapters are illustrated and others are not. Obvious errors are few, but the absence of bold settings and running titles detracts from readability.

The price is high but the contents of the book make it difficult to be without.

G. G. Birch

Nitrogen Metabolism in Man. Edited by J. C. Waterlow and J. M. L. Stephen. Applied Science Publishers Ltd., London. 1982. x + 555 pp. Price: £49·00.

A problem associated with books based on symposia is that they become a collection of multi-author articles which do not form a cohesive whole. *Nitrogen*

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Metabolism in Man could easily have suffered from this problem but careful editing, uniformity of style and logical sectioning of the book have given rise to an acceptable volume even though there are over forty chapters in five-hundred-and-fifty pages. The writers are well established leading researchers in the field. Most chapters present concise summaries and reviews of present knowledge of a topic coupled with recent data obtained by the author. However, there are some very short contributions of only two or three pages confined to the description of perhaps only one or two experiments.

The book does not limit itself entirely to nitrogen metabolism in man. Indeed, it would be difficult to do so without resort to data from animal studies, even if only for comparative purposes. There are frequent references to studies on rats and, in the section on amino acid metabolism, an account of nitrogen metabolism in plants and animals is included and there are odd references to microbial metabolism as well. With information available from a variety of species it is important to ensure that there is no doubt about the origin of the data. It is not always clear which species is being considered in the chapters on amino acid metabolism.

This book is concerned with the dynamic aspects of nitrogen metabolism. It attempts to review knowledge of the rates at which various reactions and interconversions occur. This objective is neatly summarised by Professor Waterlow, in his introduction, by the statement: 'We have a reasonably good map but we do not know much about the flow of traffic along the different routes'. The difficult concepts of flux through a metabolic pathway, turnover rates in metabolic pools and tissues and the controlling factors are the major subjects. The reader needs to be familiar with these concepts and their terminology.

The first half of the book deals with amino acid metabolism. Included here are some concise accounts of the rôle of amino acid pools, amino acid transport into cells, inter-organ amino acid exchange, branched chained amino acid metabolism, ammonia, urea and creatine. The second half of the book is devoted to the assessment of protein turnover. The chapters on the techniques involved in such studies deserve particular mention. Anyone embarking on this work would be well advised to consult this section for a balanced review of the validity of the assumptions made with these techniques. It is vital that the interpretation of data from these studies is made with full appreciation of the limitations of the techniques used to obtain the information.

The book is completed by several chapters on protein turnover related to individual tissues, growth, nutritional status and pathological states in man. These chapters illustrate the beginnings of rapidly growing areas of research in the field of nitrogen metabolism and the enormous complexities of such studies.

It is to those involved in these areas of research that this book is primarily directed. It is particularly valuable at pointing out the pitfalls awaiting the unwary researcher in the use of well established techniques. However, there is much to be gained from this book by the teacher and student of biochemistry and it would be a

valuable addition to any biochemist's bookshelf. Regrettably, its price of £49·00 reflects its specialised content and presumably the limited sales expected. It will be in high demand by a limited number of biochemists. It is destined to become a much borrowed book.

A. G. STEVENS

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This book consists of seventeen papers presented at a symposium organised by the Association of Consulting Scientists held in Norwich, Great Britain in November, 1980.

The authors, whose expertise is drawn from across the water and waste treatment field—Water Authorities, consultancy services, equipment manufacturers and industrial concerns with disposal problems—give a most interesting and valuable insight into a number of important areas.

The opening papers are concerned with legislation in the water treatment field. Various EEC directives concerned with water quality and pollution control and the implications for industry of the trend towards tightening legislative control on water discharge to the environment are discussed. Trade effluent charging systems and methods for reducing charges on discharges to sewers are outlined and the economics of aerobic versus anaerobic effluent treatment are examined.

Papers on the increasing presence of micro-pollutants (xenobiotics) and their identification, in a particular catchment area and on the discharge of an odorous chemical from a sewage works seem a little out of place in a symposium concerned with the disposal of food industry wastes but, nevertheless, this topic is of importance to food processors using such surface waters for their raw water supply.

Two papers concerned with the development of anerobic digestion systems for treating food wastes will be of particular interest to many; a paper on an anerobic system for a wheat starch effluent stream should be 'required reading' for all who profess an interest in waste treatment.

Other papers examining waste disposal in specific areas of processing—dairy, malting, brewing and distilling, meat and animal products etc.—make interesting reading and one, focused specifically on the problem of aqueous wastes which contain significant quantities of fatty material, is very useful.

The remaining contributions include recovery and utilisation of materials from food waste streams, concluding with an account of developments in screening, an important, but somewhat neglected, area.

All in all, this is a very readable collection of papers containing much of use to those interested in waste treatment.

J. R. BUTTERS

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All in all, this is a very readable collection of papers containing much of use to those interested in waste treatment.

J. R. BUTTERS

Developments in Food Carbohydrates—3. Disaccharidases. Edited by C. K. Lee and M. G. Lindley, Applied Science Publishers Ltd, London, 1980. xii + 217 pp. Price: £33·00.

The disaccharidase enzymes are of considerable interest to workers in a range of disciplines. The intestinal disaccharidases are essential for the digestion and absorption of the disaccharides in foods which make up a substantial part of the total food carbohydrate and absence of the enzymes or loss of activity is associated with a number of metabolic diseases which are of medical importance. These enzymes are also of growing industrial interest because they provide specific methods for the conversion of food components to products of enhanced value.

In the third volume of the series, Developments in Food Carbohydrate, Drs Lee and Lindley have assembled contributions from several of the workers in the field with the aim of providing a unified picture of the disaccharides from the biochemical, physiological and industrial standpoints. The first chapter covers the biochemistry of invertase (Woodman and Wiseman) and describes the properties of the enzyme, its isolation, properties and structure. Lactases are discussed by Nijpels who focuses attention on microbial lactases and their industrial applications. Bucke reviews the range of enzymes that can hydrolyse maltose. The structure of the α-glucosidase from various sources is compared and the chapter draws attention to the need for much more careful experimentation in this area.

Trehalases are discussed by Labat-Robert with particular emphasis on their molecular structure and the genetic implications of the distributions of the enzyme.

The human sucrase–isomaltase complex is covered by Cheetham who focuses attention on the physiological aspects of this enzyme system and the related nutritional and medical aspects. Lindley reviews the cellobiase, melibiase and other disaccharidases, drawing attention to the potential applications of these enzymes. The biosynthesis and metabolism of food disaccharides are reviewed by Hansen and Brown who provide a good account of the substrates used by the disaccharidases.

In the final chapter, Lutkić and Votava discuss enzyme deficiency and the malabsorption of food disaccharides. This chapter draws together the various conditions that arise when an enzyme is absent or has low activity. Lactose intolerance is of special interest and the various inherited metabolic disorders which result in specific disaccharide intolerances are well reviewed.

The volume provides a good overview of disaccharidases; in many cases, the material shows that there are profound gaps in the experimental evidence. The chapters are well referenced and the reader who wishes to develop a more detailed understanding should find the book a useful entreé into the field. The treatment in the various chapters is not wholly consistent and there are some unexpected placings of chapters. All in all, this is a useful addition to this series and can be recommended to those interested in food carbohydrates from many disciplines.

The opportunities for using these enzymes industrially are very exciting and this

volume will be of especial interest to food scientists who can see industrial applications for these enzymes.

DAVID A. T. SOUTHGATE

Developments in Food Microbiology—1. Edited by R. Davies. Applied Science Publishers Ltd, London. 1982. 219 pp. Price: £24·00.

The developments dealt with cover wide aspects of food microbiology, from the harmful effect of spoilage organisms, their evaluation and control, to the beneficial applications of streptococci in the dairy industry.

Flesh products stored in chill conditions are subject to spoilage through the action of psychotropic organisms. Recent work is described showing that small molecular weight compounds, rather than intact proteins, are the preferred substrate for aerobic psychtrophs. Extensive studies to characterise organisms responsible for off-odours are reported. Although there is no precise definition of spoilage, methods attempting to evaluate it are reviewed and a predictive method based on the temperature history of the stored product is proposed.

The somewhat controversial 'Nurmi concept' of the control of salmonellosis in poultry is discussed. By the introduction of cultures of gut contents into newly hatched chicks the indigenous flora of the bird is rapidly established and resistance to invasion by salmonellae is thereby increased. Evidence for the efficacy of the method is presented.

In the chapter on fish handling, spoilage organisms are considered and attention is also given to organisms responsible for food poisoning. Two of these, which are present in marine environments—*Vibrio parahaemolyticus* and *Clostridium botulinum*—are considered in some detail.

Ultra-high-temperature sterilisation is a comparatively recent process to be applied to foods. There is a well-documented chapter which describes its advantages and applications to the food industry. Problems of heat resistance of bacterial spores and methods of determining spore resistance are discussed.

The need for more rapid methods of estimating microbial populations in foods is recognised. Improvements in rapid counting methods are described and non-counting methods based on metabolic activities of microorganisms are considered in some detail. The value of physical separation of microorganisms from food, to facilitate rapid estimation, is pointed out and available methods reviewed. Ways of evaluating new techniques are critically described.

A more positively beneficial aspect of food microbiology is considered in a chapter on the application of genetic regulation to the streptococci used in the dairy industry. The biochemical pathways in the metabolism of lactose—and hence of lactic acid production—are described and evidence is presented for an association of lactose-fermenting ability with plasmid DNA carrying the appropriate genes.

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Models are proposed for the investigation of the mechanisms whereby lactose-fermenting ability is lost in some cultural conditions.

The book has such a wide range of topics that specialists are likely to find only a small part of it relevant to their particular field. It does, however, offer excellent material with which to update one's knowledge of the wider aspects of food microbiology.

M. E. COATES

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