EDITORIAL

This issue of *Food Chemistry* is devoted to papers presented at a Chemical Society symposium entitled 'Chemistry of Natural Food Colours', held at the Scientific Societies Lecture Theatre, Savile Row, London, on 24 October, 1978. This is the second of such symposium proceedings to be published in the journal, the first having appeared in *Food Chemistry*, Vol. 4, No. 1, January, 1979. It is relatively only a few years ago that coal-tar products were, apart from a few minor exceptions, the only food colourings available. However, condemnation of successive individual colourings in this group, because of possible health hazards, has led to only a few remaining—and even these seem likely to be withdrawn in the foreseeable future. It is not surprising, therefore, that considerable interest has developed in colourings from natural sources and the papers which make up this issue of *Food Chemistry* are not only indicative of that interest, but illustrative of the complex chemistry involved.

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NATURE AND DISTRIBUTION OF CAROTENOIDS

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ABSTRACT

There is a wide distribution of carotenoids (naturally occurring tetraterpenes) in higher plants and animal tissues. They are formed from the specific precursor, melavonic acid, through the basic pigment, lycopene, and thence by a number of pathways.

Carotenoids in higher plants are found in photosynthetic tissues and non-photosynthetic tissues, where many structural variations are found. Reproductive tissues are discussed and the carotenogenic fruit have been divided into eight main groups. Root carotenoids, especially in the carrot, are considered. A section deals with carotenoids in algae and photosynthetic bacteria. A final section deals with carotenoproteins, especially in marine invertebrates.

INTRODUCTION

Carotenoids are, as far as is known, the only naturally occurring tetraterpenes and are very widely distributed throughout the living world. However, they appear to be synthesised *de novo* only by higher plants, mosses, liverworts, etc., algae, photosynthetic and non-photosynthetic bacteria and fungi. Carotenoids which have been isolated only from animal tissues are the result of metabolic changes, generally oxidative, in the ingested carotenoids, brought about by the animals themselves. All photosynthetic tissues contain carotenoids, although the colour is masked by the chlorophylls also present in the chloroplasts (higher plants and algae) or photosynthetic membranes (photosynthetic bacteria). The reason for the co-existence of the two pigment groups is that the carotenoids prevent the

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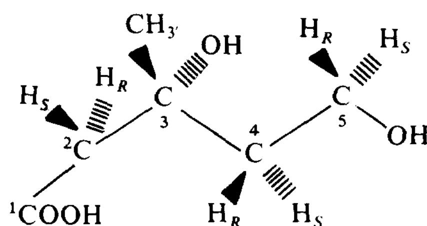
photodynamic sensitisation of chlorophylls which takes place in their absence and leads to destruction of the chloroplast. Light and oxygen are necessary for this effect so photosynthesis in higher plants and algae, as it is known today, could not exist had not carotenoid and chlorophyll synthesis evolved together (Goodwin, 1979*a*).

The distribution of carotenoids in the plant kingdom will be better appreciated if the way in which they are biosynthesised is understood. An outline of this process is given in the next section; for full details see Britton (1976) and Goodwin (1979*b*). Full details of the chemistry of the carotenoids are given by Isler (1971) and Moss & Weedon (1976).

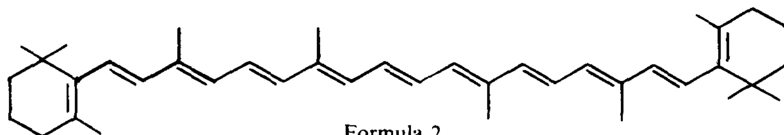
FORMATION

The first specific precursor of all terpenoids is mevalonic acid, a branched C_6 compound (Formula 1) formed from three molecules of acetyl-CoA:



Formula 1

It is converted into the branched C_5 compound, isopentenyl pyrophosphate (IPP), four molecules of which form the C_{20} geranylgeranyl pyrophosphate (GGPP) (Fig. 1). Two of these condense tail to tail with the elimination of two molecules of pyrophosphate to yield the first C_{40} carotenoid precursor, phytoene; this is then desaturated stepwise to yield the red pigment lycopene (Fig. 2). This can be considered the basic pigment from which branch a number of pathways leading to various cyclic carotenes (hydrocarbons) and xanthophylls (oxygen-containing carotenoids). The three cyclising enzymes acting on lycopene yield carotenes with β-, ε- and γ-rings (Fig. 3), for example: β-carotene (two β-rings) (Formula 2), α-carotene (one β- and one ε-ring) (Formula 3) and γ-carotene (one β-ring and one acyclic or ψ-end group) (Formula 4). γ-Rings are rare and, up to now, have been



Formula 2.

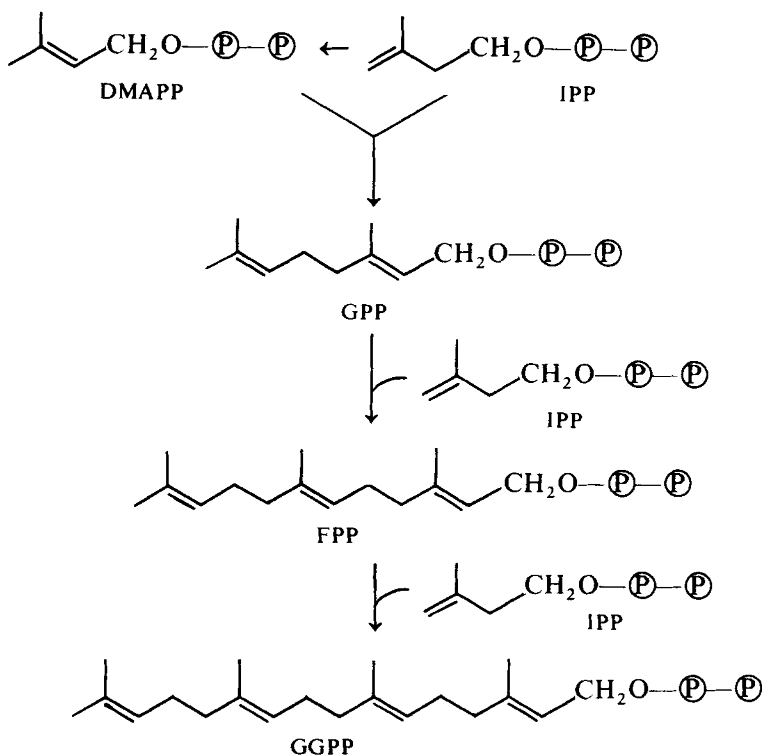


Fig. 1. The conversion of isopentenyl pyrophosphate (IPP) into geranylgeranyl pyrophosphate (GGPP).

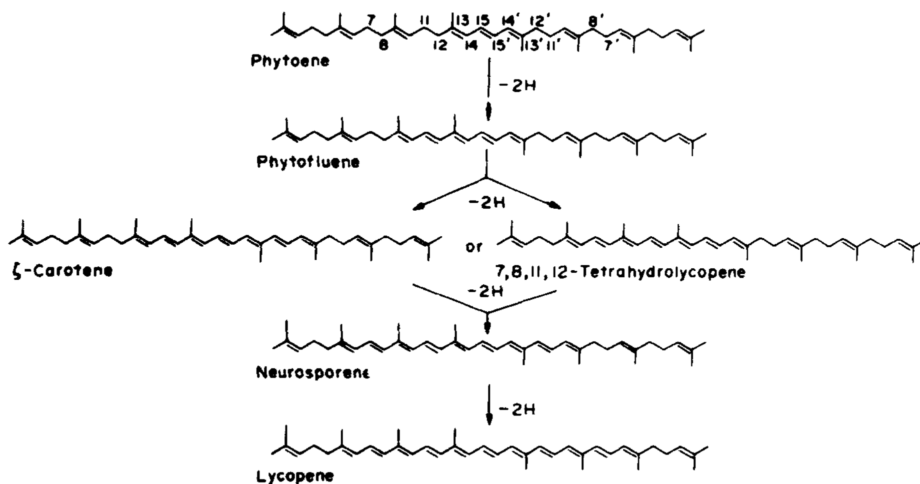


Fig. 2. The desaturation of phytoene to lycopene. The pathway is via ζ-carotene in plants and algae and via 7,8,11,12-tetrahydrolycopene in some fungi and bacteria.

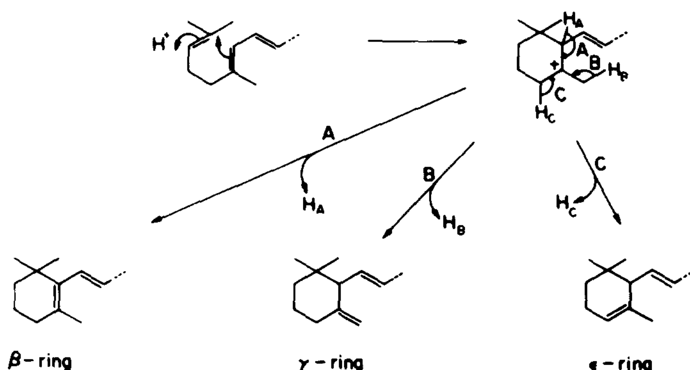
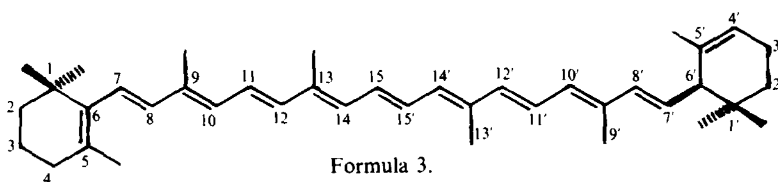
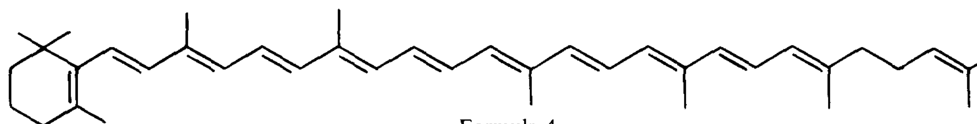


Fig. 3. The mechanism for biosynthesis of β-, γ- and ε-rings of carotenoids.

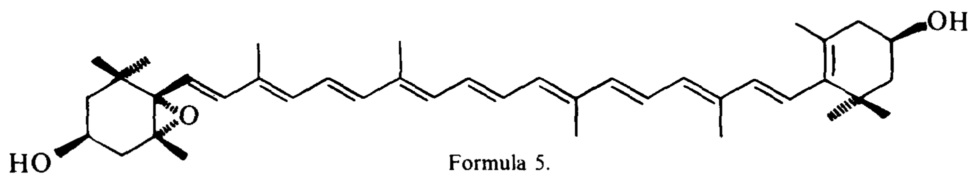
found only in some fungi. Hydroxylation of the hydrocarbons yields such compounds as zeaxanthin (3,3'-dihydroxy-β-carotene) whereas epoxidation leads to compounds such as antheraxanthin (5,6-epoxyzeaxanthin) (Formula 5). The basic numbering of the carotenoid molecule is given in α-carotene (Formula 3).



Formula 3.



Formula 4.

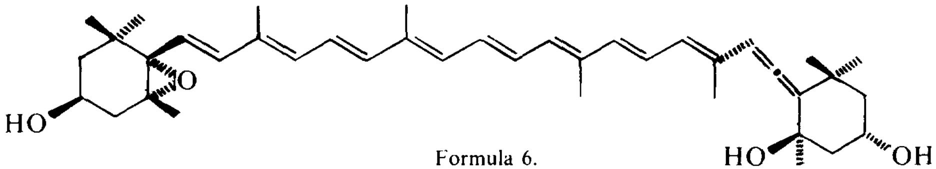


Formula 5.

DISTRIBUTION IN HIGHER PLANTS

Photosynthetic tissues

All carotenoids in photosynthetic tissues are located in the grana of the chloroplast and consist of the same major group of pigments, whatever the source. These are β-carotene (Formula 2), lutein (3,3'-dihydroxy-α-carotene), violaxanthin



Formula 6.

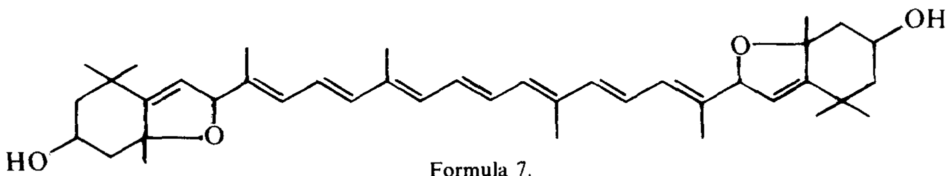
(5,6,5′,6′-diepoxyzeaxanthin) and neoxanthin (Formula 6). Smaller amounts of α-carotene (Formula 3), β-cryptoxanthin (3-hydroxy-β-carotene), zeaxanthin and antheraxanthin are also found. The partly saturated precursors (Fig. 2) are found in green tissues only at a concentration 1/200 that of β-carotene. The xanthophylls occur unesterified, but during autumn senescene, when the chloroplasts disintegrate, the xanthophylls released into the cytoplasm are frequently esterified before they are oxidatively destroyed (Goodwin, 1979*a*).

From the nutritional point of view it is the level of β-carotene, the most effective naturally occurring vitamin A precursor, which is of most interest here. Maximum levels in leaves vary between 200 and 700 μg/g dry weight according to species. Cultural variations do not make significant differences (Goodwin, 1979*a*).

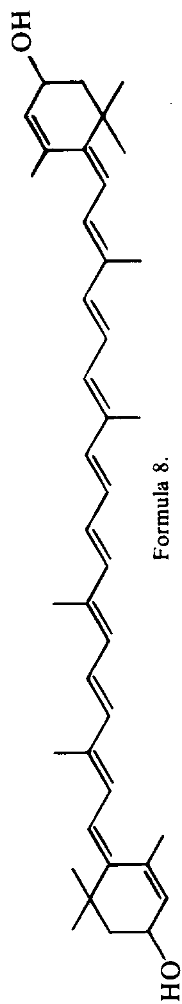
Non-photosynthetic tissues

The distribution of carotenoids in photosynthetic tissue of higher plants is the same throughout the plant kingdom, but when non-photosynthetic tissues are considered, plants exerting their individuality and producing pigments with many structural variations are found. Not all non-photosynthetic tissues produce carotenoids and, at the moment, no explanation for their sporadic distribution has been found. However, in the next sections, those that do make carotenoids will be discussed, particularly insofar as they may produce novel carotenoids or have some importance for food processes.

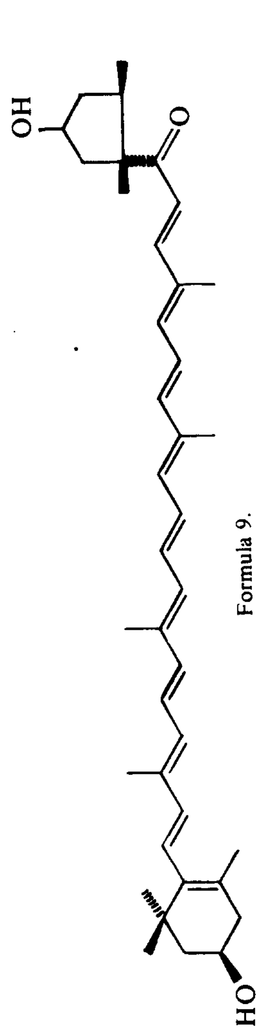
(a) *Reproductive tissues:* The first carotenoid isolated from anthers was antheraxanthin (Formula 5), but it is now known that it is present in traces in all green tissues; it has not yet been detected in pollen, but other epoxides, such as α-carotene 5,6-epoxide, have. In flowers which contain carotenoids, the pigments accumulate in chromoplasts which appear to derive from chloroplasts in the same manner as in carotenogenic fruit. Flowers which produce carotenoids fall into three main groups: (i) those in which are found highly oxidised pigments such as the furanoid oxide auroxanthin (Formula 7); (ii) hydrocarbons such as lycopene or β-carotene (Formula 2) and (iii) highly species-specific carotenoids such as the



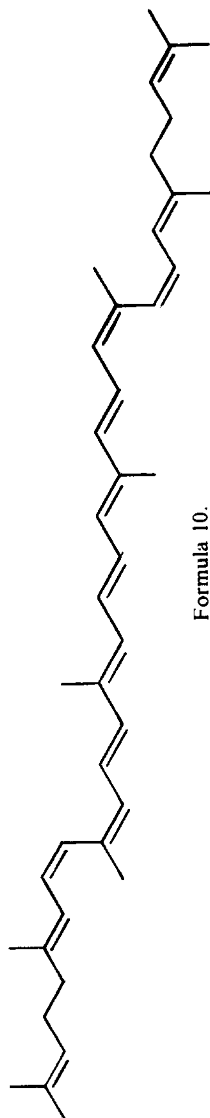
Formula 7.



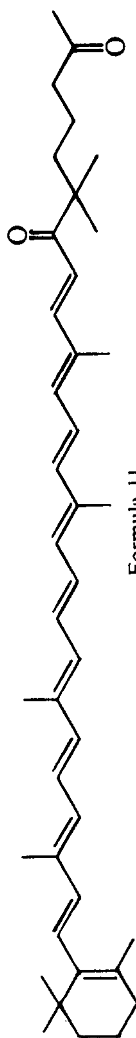
Formula 8.



Formula 9.



Formula 10.



Formula 11.

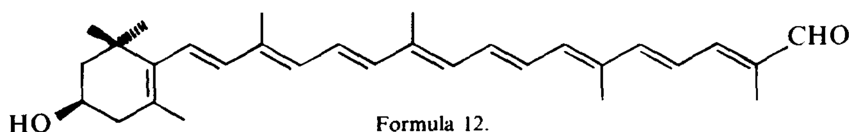
TABLE 1
SOME EXAMPLES OF THE DIFFERENT TYPES OF CAROTENOID DISTRIBUTION IN FRUIT (GOODWIN, 1979*a*; GOAD & GOODWIN, 1970)

Type	*Species*	*Carotenoids*
I	*Pyracantha rogersiana*	Traces
II	*Sambucus nigra*	Chloroplast carotenoids
III	*Hippophae rhamnoides*	Lycopene series
IV	*Mangifera indica*	β-Carotene derivatives
V	*Crataegus pratensis*	Epoxides
VI	*Capsicum annuum*	Species-specific pigments (e.g. capsanthin, Formula 9)
VII	*Pyracantha angustifolia*	Procarotenes (e.g. prolycopene, Formula 10)
VIII	*Murraya exotica*	Secocarotenoids (e.g. semi-β-carotenone, Formula 11)
IX	*Citrus* spp.	Apocarotenoids (e.g. β-citraurin, Formula 12)

retrocarotenoid eschscholtzxanthin (Formula 8) from *Eschscholtzia californica*. In some petals the carotenoid concentration can be very high; in the red fringes of the corona of *Narcissus poeticus recurvis*, for example, β-carotene represents 16·5% of the dry weight of the tissue (see Goodwin, 1979*a*).

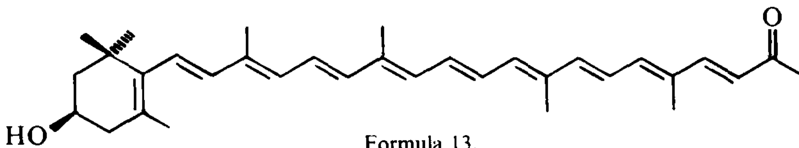
The chloroplasts present in green unripe fruit, in most cases, gradually change into chromoplasts on ripening, and, as the photosynthetic apparatus disintegrates, enzymes are either newly synthesised or derepressed, and carotenoid synthesis, often of novel pigments, is enormously stimulated. Typical examples are the tomato and the red pepper.

Carotenogenic fruit can be divided into several main groups according to whether they produce (i) only traces of carotenoids; (ii) carotenoids similar to those found in chloroplasts; (iii) lycopene and derivatives in considerable amounts; (iv) large amounts of β-carotene and its derivatives; (v) large amounts of epoxides; (vi) pigments which are, to a great extent, species-specific; (vii) considerable amounts of pro-carotenes, (viii) seco-carotenoids, or (ix) apocarotenoids. All these are considered in detail elsewhere (Goodwin, 1979*a*; Goad & Goodwin, 1970). The pigments in some characteristic fruit are listed in Table 1. However, it should be noted that capsanthin has a five membered ring, that some of the double bonds in the polyene chain can exist in the *cis*-configuration, as in prolycopene (Formula 10) (see Zechmeister, 1962 for a review), that seco-carotenoids such as semi-β-carotenone (Formula 11) have had a ring cleaved between C-3 and C-4, and that apocarotenoids, such as β-citraurin (Formula 12) are carotenoids from which fragments have been removed from one or both ends.



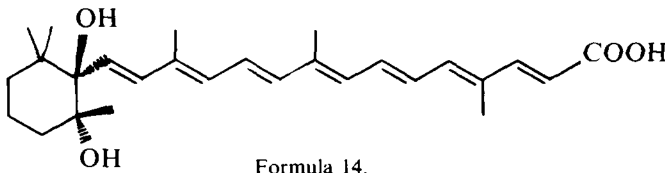
Formula 12.

Classical work on mutant strains of tomatoes has produced those in which, for example, lycopene is replaced by an equivalent amount of β-carotene; in other words, the β-cyclase absent from, or repressed in, commercial varieties has been allowed expression. This is a nutritionally important change in that a vitamin A-inactive carotenoid is replaced by a vitamin A-active carotenoid, but the conservatism of the general public has not made it acceptable. New crosses of citrus fruit have produced fruit with very large amounts of apocarotenoids. For example, the trigeneric hybrid of the oval kumquat (*Fortunella margarita*) with the Rusk citrange (*Poncirus trifoliata* x *Citrus sinensis*) contains very large amounts of β-citraurin (Formula 12) and β-apo-8′-carotenal (Goodwin, 1979*a*; Stewart & Wheaton, 1973). Published work on carotenoids in citrus fruit abounds in descriptions of artefacts, which are mainly due to the acidic environment which converts 5,6-epoxides into 5,8-epoxides, and to the use of acetone and alkali in the extraction procedure which results in the formation of methyl ketones such as reticulataxanthin (Formula 13) (see Goodwin, 1979*a*; Stewart & Wheaton, 1973).



Formula 13.

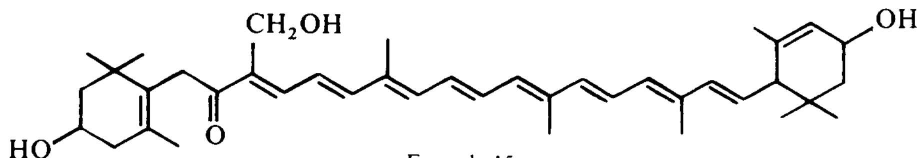
(b) *Roots:* The most important carotenogenic root is the carrot, in which the main pigment in commercial varieties is β-carotene (Formula 2) with xanthophylls representing only about 5 % of the total pigments present (Goodwin, 1973). In wild carrots xanthophylls, at low concentrations, preponderate (Goodwin, 1979*a*). Normal commercial strains contain about 60–120 μg β-carotene per gramme of fresh weight, but strain selection can increase this to 310–370 μg (Goodwin, 1976). One strain of red carrots has been reported in which the cyclising enzymes must have been repressed, because it accumulates lycopene in place of β-carotene (Goodwin, 1976). Some sweet potatoes also contain significant amounts of β-carotene, whereas the unique apocarotenoid azafrin (Formula 14) is the major pigment in the roots of *Escobedia scabrifolia* (Goodwin, 1976).



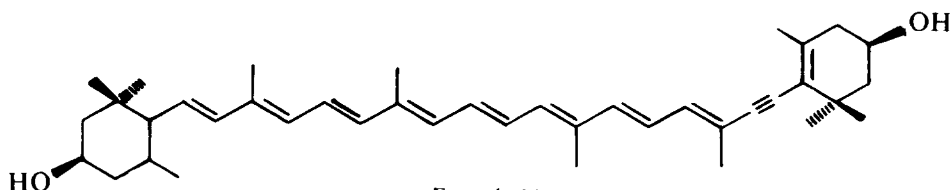
Formula 14.

Algae and photosynthetic bacteria

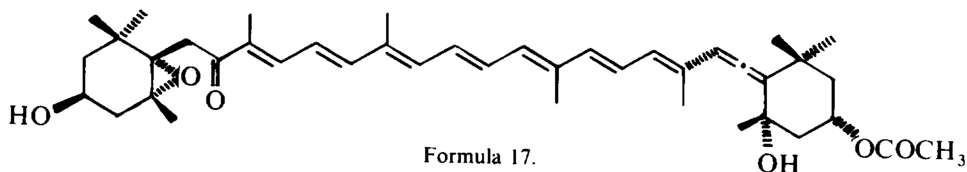
Although, strictly speaking, the carotenoids in algae and photosynthetic bacteria



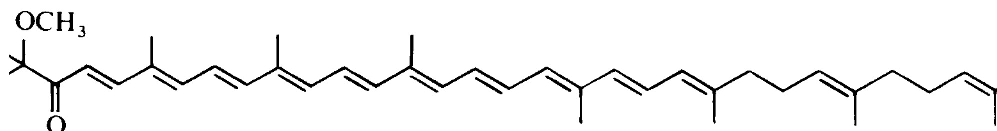
Formula 15.



Formula 16.



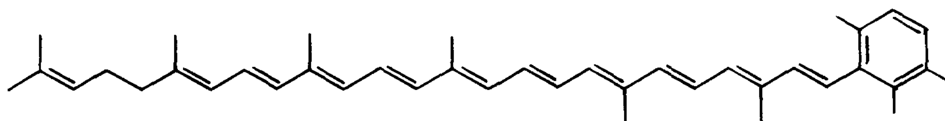
Formula 17.



Formula 18.

are outside the scope of the present paper, they should be considered briefly. As indicated earlier, photosynthetic tissues of higher plants all contain the same limited number of pigments. Algae and photosynthetic bacteria are markedly different in this respect and often produce new and specific pigments. Three examples are taken from each group for illustration. (a) Certain green algae produce carotenoids such as siphonaxanthin (Formula 15), in which a keto group appears in the chain and an in-chain methyl group is oxidised to a primary alcohol. (b) Cryptophyta and many diatoms synthesise acetylenic carotenoids, such as diatoxanthin (Formula 16). (c) Fucoxanthin (Formula 17), with numerous special structural features, is probably the most abundant carotenoid in nature, occurring, as it does, as the major carotenoid of the Phaeophyta, which includes the giant oceanic kelps.

The photosynthetic bacteria differ from other photosynthetic tissues in accumulating acyclic pigments characterised by methoxy groups at position 2, additional double bonds at C-3,4 and keto groups conjugated to the conjugated double bond system. All these features are present in spheroidenone (Formula 18)



Formula 19.

synthesised by *Rhodopseudomonas sphaeroides*. Some green photosynthetic bacteria uniquely produce carotenoids with aromatic rings; for example, chlorobactene (Formula 19) is found in *Chlorobium* spp.

CAROTENOPROTEINS

Although carotenoids probably exist in plants in part as carotenoproteins, the most obvious and spectacular manifestations of these compounds are in marine invertebrates. For example, the green of lobster eggs and the dark-purple of the lobster carapace are both carotenoproteins, as are the deep reds of many starfish and the blues of *Vellella* (Cheeseman *et al.*, 1967; Zagalsky, 1976). The carotenoproteins are generally water-soluble and much more stable to light than the component pigments separated from their apoproteins. Carotenoproteins are now under intense study and their potential importance for the food industry is obvious.

The nature of the binding of pigment and protein which results in such spectacular changes in colour (e.g. astaxanthin, 470–475 nm in light petroleum; crustacyanin, 633 nm in phosphate buffer, pH 7) is not yet fully understood, but it is now possible to dissociate and reassociate carotenoproteins and, in some cases, to replace the naturally occurring pigment by one with a closely related structure. In some cases in which the change in colour is not so marked it is thought that the carotenoid is simply dissolved in the lipid of a lipoprotein.

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SOME RECENT ADVANCES IN THE SYNTHESIS OF NATURAL CAROTENOIDS*

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ABSTRACT

The total syntheses of naturally occurring (3R,3′R)-zeaxanthin, (3S,3′S)-astaxanthin, (3S,3′S)-asterinic acid and of some related xanthophylls are discussed.

Asymmetric hydroboration has been applied to the total synthesis of all configurational isomers of zeaxanthin. The total synthesis of natural actinioerythrin is described and the absolute configuration confirmed by X-ray crystallographic analysis of an intermediate.

The optical resolution of α-hydroxy-carbonyl compounds via (−)-camphanic acid esters appears to be generally applicable and can be used as an analytical method for the determination of the optical purity of α-hydroxy-carbonyl carotenoids.

Some technological applications are indicated.

INTRODUCTION

The carotenoids form one of the most important groups of natural pigments. According to a list by Straub (1976), more than 400 naturally occurring carotenoids are known. They are responsible for many of the brilliant colours in fruit, vegetables, fish, Crustacea, eggs and other plants and animals (Plate 1). It has been estimated that nature produces over 100 million tons of carotenoid pigments a year (Zechmeister, 1958). The vital rôle of carotenoids in plants and animals as attractants or deterrents is obvious. Equally important are other physiological functions: carotenoids may act as photoprotective agents by absorbing the potentially harmful light energy or by quenching singlet oxygen. Carotenoids may

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also function as accessory pigments in photosynthesis as sensitisers and oxygen transporters (Kienzle & Isler, 1978). Some carotenoids possess vitamin A activity. As a matter of fact, β-carotene is the most important natural source of vitamin A for man.

Bacteria, algae and the higher plants have the ability to produce carotenoids. Animals, however, seem to depend on diet as their source of carotenoids.

Subsequent transformation of these dietary pigments may sometimes lead to characteristic animal carotenoids which are normally not found in organisms capable of carotenogenesis. A typical example is astaxanthin, the characteristic pigment of Crustacea.

Thus, a very wide variety of carotenoidal pigments may be found in nature. The synthetic organic chemist, however, had to follow a long and difficult path. Nevertheless, total synthesis of carotenoids has developed into a field of great importance during the last decades and a very large number of interesting structures has already been synthesised (Mayer & Isler, 1971; Weedon, 1976; Mayer, 1979).

Plate 2 shows the formulae of six synthetically available carotenoids. They cover a wide colour range from yellow to red and even to blue.

β-Carotene (1), UV-maxima at 465 and 492 nm (in chloroform), the naturally occurring pigment in a great variety of foods in our daily diet (carrot, spinach, lettuce), was the first carotenoid made commercially available by industrial synthesis (Roche, 1954).

Zeaxanthin (2), UV-maxima at 463 and 492 nm (in chloroform), is very widely distributed. It is the main carotenoid of yellow maize.

Canthaxanthin (3), with UV-maximum at 486 nm (in chloroform), usually synthesised from β-carotene, became commercially available for food colouring in 1964. It has been isolated from the edible mushroom *Cantharellus cinnabarinus*, from the skin of trout (Thommen & Gloor, 1965), feathers of birds, algae and other sources (Weedon, 1971).

Astaxanthin (4), UV-maximum at 494 nm (in chloroform), is the main pigment of salmon, trout and lobster. Astaxanthin is of special interest since it constitutes the chief prosthetic group in the carotenoproteins (Zagalsky, 1976). The constitution of astaxanthin was elucidated by the fascinating work of Kuhn & Sörensen (1938).

Actinioerythrin (5), UV-maximum at 518 nm (in chloroform), the characteristic violet–red pigment of the sea anemone *Actinia equina* L., is a mixture of fatty acid esters of the parent actinioerythrol which is the only known naturally occurring carotenoid with 2-nor end groups. The change of the ring size results in a striking shift in the colour. Actinioerythrol itself has not yet been reported in nature; nor has violerythrin (6), the first blue carotenoid, with a UV-maximum at 580 nm (in chloroform).

Actinioerythrin (5) has been isolated and its structure has been elucidated (Hertzberg & Liaaen-Jensen, 1968). These authors also showed that (6) was the oxidation product of actinioerythrol (7) (Fig. 1). The latter could be reconstituted

Plate 1. Natural sources of carotenoids (photograph by Marcel Beck).

β-Carotene (**1**)

HO (3R,3′R)-Zeaxanthin (**2**) OH

Canthaxanthin (**3**)

HO (3S,3′S)-Astaxanthin (**4**) OH

Acyl-O (3S,3′S)-Actinioerythrin (**5**) O-Acyl

Violerythrin (**6**)

Plate 2. Colour range and formulae of some synthesised carotenoids (photograph by Marcel Beck).

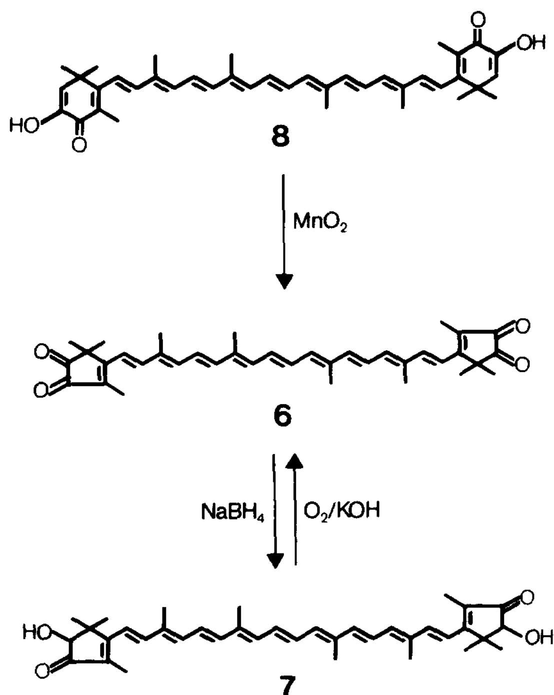


Fig. 1. Synthesis of violerythrin (6) and actinioerythrol (7) from astacene (8).

from (6) by borohydride reduction. Workers were able to obtain (6) through oxidative ring contraction of astacene (8) (Holzel *et al.*, 1969). Total syntheses of violerythrin (6) and optically inactive actinioerythrol (7) were recently reported (Kienzle & Minder, 1976, 1978).

It is often forgotten that more than half of the natural carotenoids possess chiral centres and occur naturally in optically active form. Thus, (2), (4) and (5), due to their chiral centres at C-3 and C-3′, belong to the group of optically active carotenoids.

DISCUSSION

The synthesis of an optically active carotenoid presents a unique challenge to the synthetic organic chemist. The translation of a laboratory-scale total synthesis of such complicated and sensitive molecules as carotenoids into a technical process is an even more ambitious task.

In designing a synthesis of an optically active carotenoid it seems to be best to start with a small chiral component of the desired configuration. The question is then how to obtain the chiral building units.

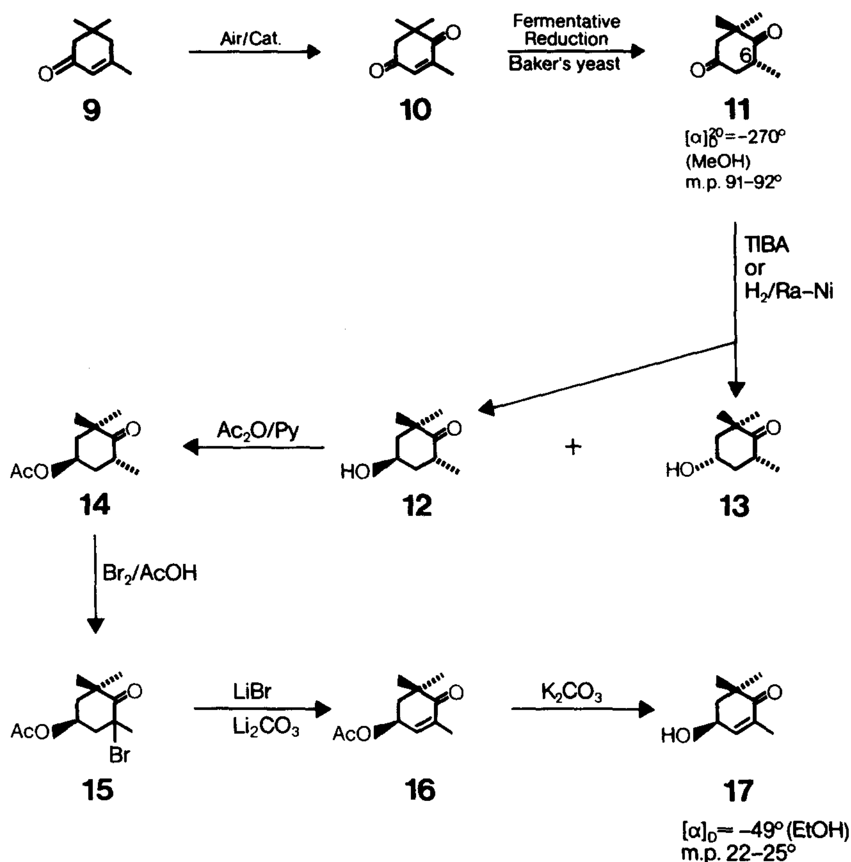


Fig. 2. Synthesis of (4R,6R)4-hydroxy-2,2,6-trimethylcyclohexanone (12) and of (4S)-4-hydroxy-2,6,6-trimethyl-2-cyclohexen-1-one (17).

In principle, they may be obtained by any of the following four methods:

(i) *Enzyme catalysed processes*
A procedure using one common chiral starting material obtained by an enzymic reduction (Leuenberger *et al.*, 1976) for the synthesis of several xanthophylls and related compounds has recently been worked out by chemists of Hoffmann-La Roche (Kienzle & Mayer, 1978; Kienzle *et al.*, 1978; Mayer, 1979).

(ii) *Asymmetric synthesis*

(iii) *Derivatives from natural sources*
For example, (3S,5R,3′S,5′R)-capsorubin was synthesised starting from natural (+)-camphor (Weedon, 1973).

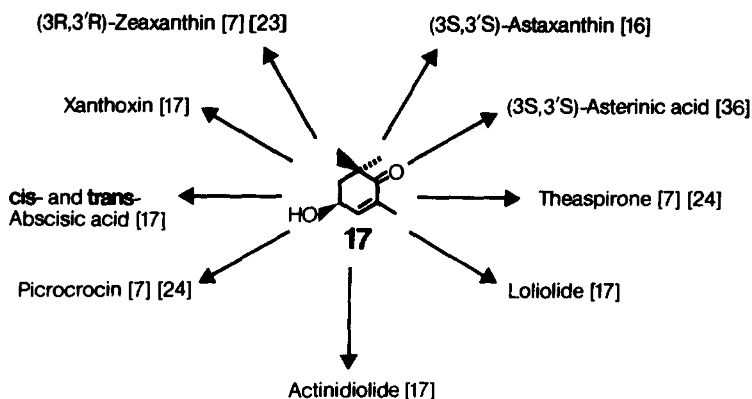


Fig. 3. Synthesis of optically active xanthophylls and other natural products from an enzymically prepared intermediate. Abscisic acid (*cis-* and *trans-*) (Kienzle *et al.*, 1978); actinidiolide (Kienzle *et al.*, 1978); (3S,3'S)-astaxanthin (Kienzle & Mayer, 1978); (3S,3'S)-asterinic acid (Bernhard, 1978; Bernhard *et al.*, 1978); loliolide (Kienzle *et al.*, 1978); picrocrocin (Mayer, 1979; Mayer & Santer, unpublished results); theaspirone (Mayer, 1979; Mayer & Santer, unpublished results); xanthoxin (Kienzle *et al.*, 1978); zeaxanthin (Mayer *et al.*, 1975; Mayer, 1979; Mayer *et al.*, unpublished results).

(iv) *Optical resolution via diastereomeric derivatives*
For example, resolution of racemic α-ionone via 1-menthydrazone (Sobotka *et al.*, 1943; Naves, 1947; Tscharner *et al.*, 1958; Buchecker *et al.*, 1973).

In the following section, successful syntheses of optically active xanthophylls relying on methods (i), (ii) and (iv) will be described.

(a) *The synthesis of optically active xanthophylls from an enzymically prepared starting material*

Isophorone (9) (Fig. 2), a technical product readily available from acetone, was first oxidised to oxo-isophorone (10) by air. Chirality at C-6 was introduced by an enantio-selective fermentative reduction of the double bond using baker's yeast (Leuenberger *et al.*, 1976). Subsequent metal hydride reduction of the resulting diketone (11) gave predominantly the hydroxy ketone (12). Bromination of the corresponding acetate (14) furnished a mixture of epimeric bromides (15) which, on dehydrobromination, yielded the unsaturated ketone (16). Subsequent hydrolysis then led to the unsaturated hydroxy ketone (17) which proved to be a key intermediate for the synthesis of several optically active xanthophylls as well as for other natural products (Fig. 3) (Kienzle & Mayer, 1978; Kienzle *et al.*, 1978; Mayer, 1979).

(3R,3'R)-Zeaxanthin: The absolute configuration of natural zeaxanthin, (3R,3'R) was established (De Ville *et al.*, 1969) by correlation to fucoxanthin whose absolute configuration had been determined by X-ray crystallographic analysis of a degradation product. Samples of zeaxanthin isolated from different natural sources

have been shown to have the same chirality (Aasen *et al.*, 1972). The first total synthesis of (3R,3'R)-zeaxanthin, identical with the natural pigment, was announced in 1975 (Mayer *et al.*, 1975). Since then, various reaction sequences leading to the important C_{13}-intermediate (3R)-3-hydroxy-β-ionone (23) (Fig. 4) have been elaborated (Mayer, 1979). A very efficient route affording (23) and the corresponding acetate (24) in excellent chemical and optical yields has recently been developed

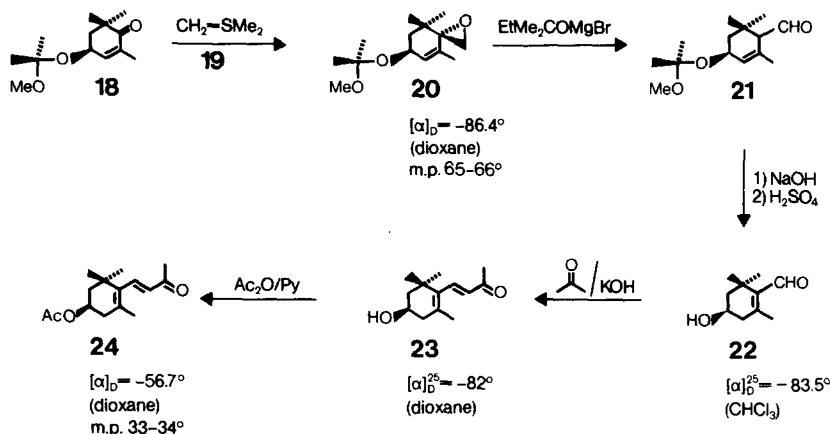


Fig. 4. Technical synthesis of 3(R)-3-acetoxy-β-ionone (24).

(Mayer, 1979). Thus, treatment of the protected hydroxy ketone (18) with the sulphurylide (19) readily gave the epoxide (20) which was transformed into (3R)-3-hydroxy-β-cyclocitral (22) via the intermediate aldehyde (21). Base-catalysed condensation of (22) with acetone furnished (23) and the corresponding acetate (24).

The synthesis of the C_{40}-skeleton was then achieved according to the building scheme $C_{15} + C_{10} + C_{15} = C_{40}$ (Fig. 5), first employed for the synthesis of zeaxanthin as an optically inactive 1:1 mixture of racemate and *meso*-form (Loeber *et al.*, 1971). The C_{15}-*Wittig* salt (25), prepared from (23) by conventional methods (vinylation followed by treatment of the intermediate vinyl carbinol with Ph_3P/HCl) was reacted with the fully conjugated C_{10}-dialdehyde (26) to give, after isomerisation, all-*trans*-(3R,3'R)-zeaxanthin in excellent yield. When the C_{10}-dialdehyde (33) with a central triple bond was used, all-*trans*-(3R,3'R)-15,15'-didehydro-zeaxanthin was obtained which, after partial hydrogenation and isomerisation, was converted into the desired pigment.

(3S,3'S)-Astaxanthin: Astaxanthin has been found in many Crustacea, such as the common lobster, in salmon, trout and numerous other organisms (Weedon, 1971).

The absolute configuration of astaxanthin isolated from the lobster has been determined (3S,3'S) (Andrewes *et al.*, 1974). The same chirality was shown for

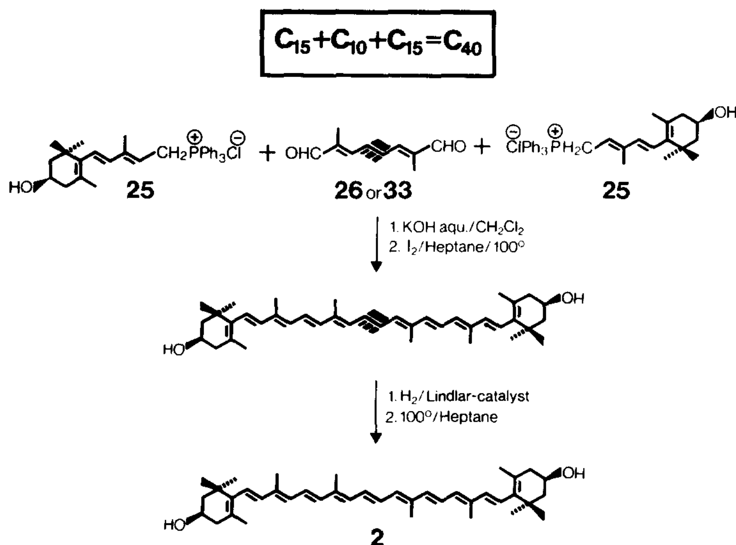


Fig. 5. Technical synthesis of (3R,3'R)-zeaxanthin (2).

astaxanthin produced by the spider mite (Veerman *et al.*, 1975). However, astaxanthin isolated from the yeast *Phaffia rhodozyma* has the 3R,3'R-configuration (Andrewes *et al.*, 1976; Andrewes & Starr, 1976). This is the first example of a naturally occurring carotenoid biosynthesised in different optical forms. Incorporation of this yeast into the diet (15% wt/wt) of rainbow trout induced pigmentation within 43 days (Johnson *et al.*, 1977). Lobsters fed the same diet did not readily accumulate the carotenoid of the yeast, although they became pigmented on a diet of live brine shrimp.

The first synthesis of (3S,3'S)-astaxanthin (4) has recently been accomplished (Kienzle & Mayer, 1978). A key intermediate was again the unsaturated hydroxy ketone (17). The main steps of their synthesis are outlined in Fig. 6.

The chiral hydroxy ketone (17) could be transformed into the protected keto-aldehyde (29) by alkylation with (E)-5-hydroxy-3-methyl-2-pentene-4-yne-1-ol, allylic rearrangement, oxidation and esterification. Catalytic partial reduction of the triple bond in (29) led to the aldehyde (30) with a 7,8-*cis*-double bond (IUPAC, 1974). Reduction with sodium borohydride followed by treatment with phosphorus tribromide furnished the crystalline bromide (31). Although it is known that a *cis*-double bond adjacent to the cyclohexene ring is extremely unstable in carotenoids (Ramamurthy *et al.*, 1975), in this case, compounds (30) and (31) proved to be unexpectedly stable. However, spontaneous isomerisation to the all-*trans* compound (32) occurred on treatment of (31) with triphenylphosphine. *Wittig*-reaction of (32) with the C_{10}-dialdehyde (26) then gave (3S,3'S)-astaxanthin

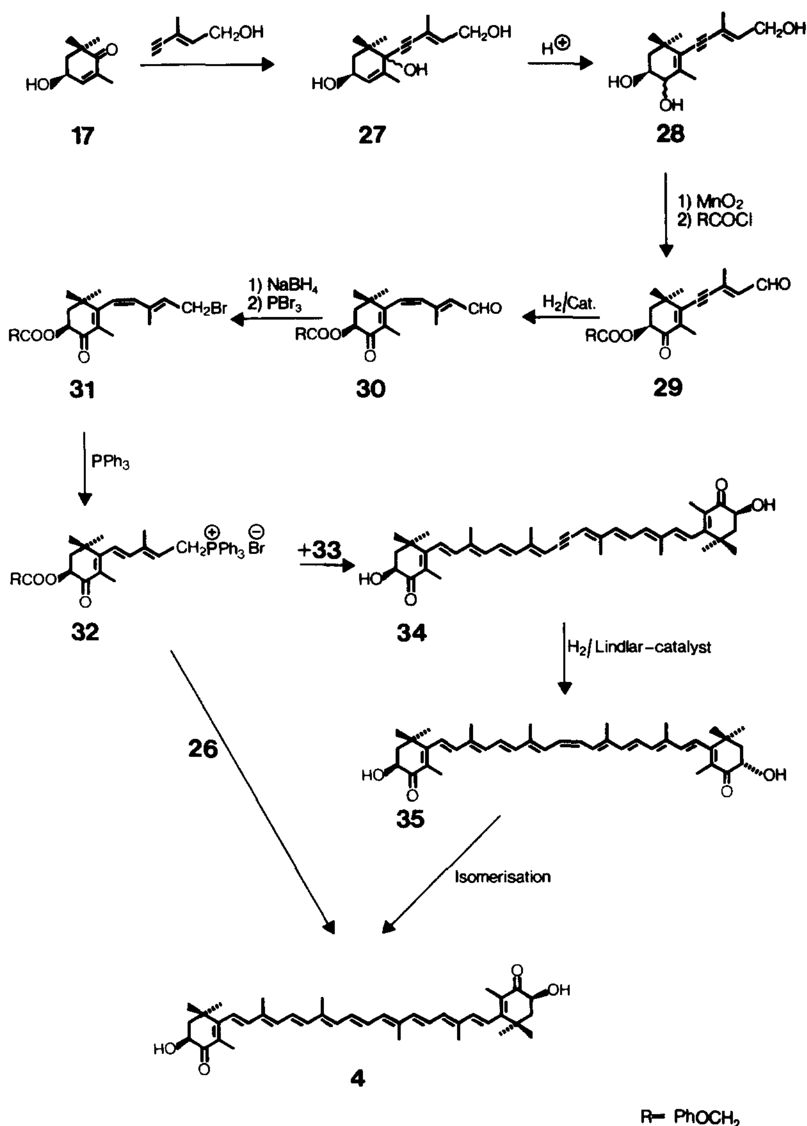


Fig. 6. Synthesis of (3S,3′S)-astaxanthin.

(4). On the other hand, reaction of (32) with the C_{10}-dialdehyde (33) led to the 15,15′-dehydro compound (34). Hydrogenation over *Lindlar* catalyst furnished (3S,3′S)-15-*cis*-astaxanthin (35). The CD curve of (35) was found to be the near mirror image of that of its all-*trans*-isomer (4) (Englert *et al.*, 1977). Isomerisation of (35) to (4) occurred easily on exposure to light or heat. The (3S,3′S)-astaxanthin

obtained in this way proved to be identical in all respects to the natural product isolated from the lobster *Hommarus gammarus* (Englert *et al.*, 1977).

(3*S*,3′*S*)-*Asterinic acid:* Asterinic acid is the main pigment of the starfish *Asterias rubens*. Together with astaxanthin, it occurs as a blue protein complex (Euler & Hellström, 1934). Initially, it was assumed that asterinic acid consisted of a single compound or that it might even be identical with astaxanthin. In 1970, the separation into two compounds, namely 7,8,7′,8′-tetradehydro-astaxanthin (36)

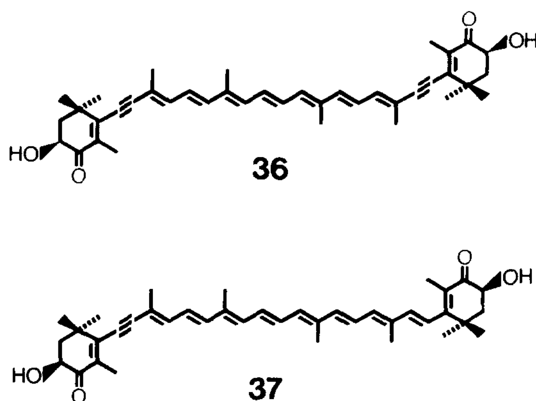


Fig. 7. Asterinic acid.

and 7,8′-didehydro-astaxanthin (37) (Fig. 7), became possible (Francis *et al.*, 1970). Recently, the absolute configuration of the two compounds has been shown to be (3S,3′S) (Berger *et al.*, 1977).

The first total synthesis of these two compounds confirming the assignment of the absolute configuration has recently been reported (Bernhard, 1978; Bernhard *et al.*, 1978).

Sodium borohydride reduction of (29) (Fig. 6), followed by treatment with phosphorus tribromide, gave the corresponding bromide which, on reaction with triphenylphosphine, produced the crystalline 9-*trans*-C_{15}-phosphonium bromide (38) (Fig. 8). Subsequent *Wittig* reaction with the C_{10}-dialdehyde (26) yielded (39) as a violet crystalline product. Although the CD curve of its diacetate showed similar Cotton effects at the same wavelengths as for the diacetate of the natural product (Francis *et al.*, 1970), spectroscopic properties proved the 9,9′-di-*cis*-structure for (39). Attempts to isomerise (39) to (36) failed.

The exceptional stability of 9-*cis*-isomers in the case of carotenoids with a 7,8-triple bond has been noted in a few other cases, e.g. for alloxanthin (7,8,7′,8′-tetradehydro-zeaxanthin) (Weedon, 1971a, b; Cheng *et al.*, 1974).

To avoid isomerisation in position 9, the reverse *Wittig* reaction (Surmatis & Ofner, 1961) utilising the C_{10}-diphosphonium salt (40) as central component was tried (Fig. 9).

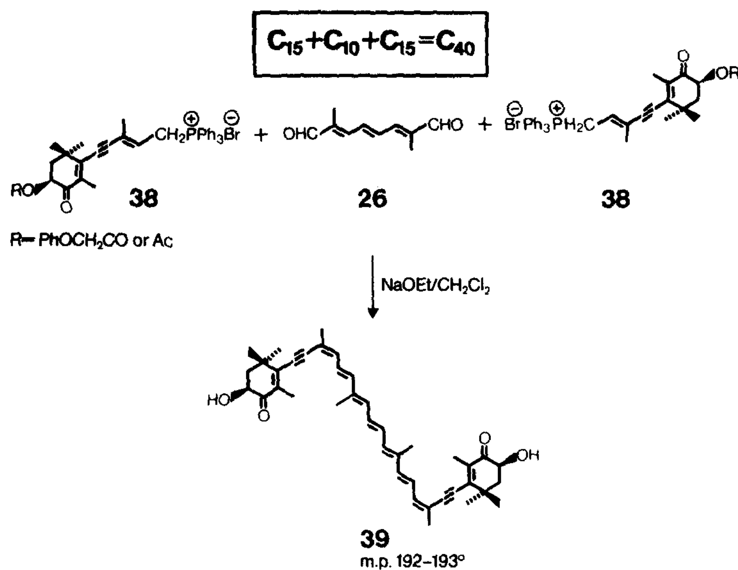


Fig. 8. Synthesis of 9,9′-di*cis*-(3S,3′S)-7,8,7′,8′-tetradehydro-astaxanthin (39).

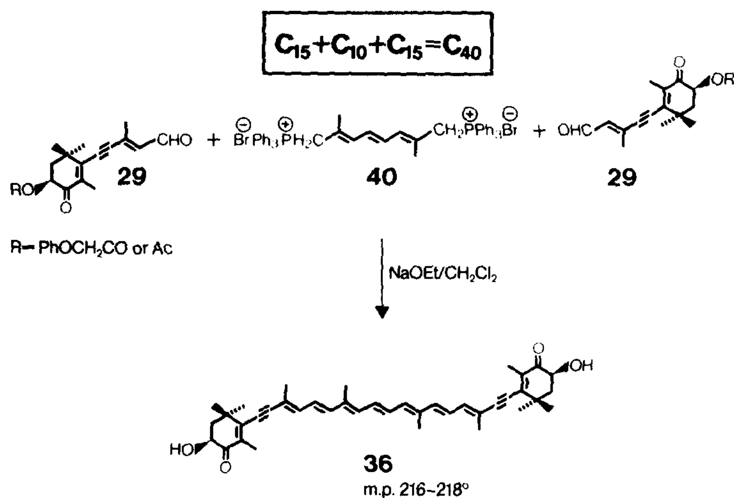


Fig. 9. Synthesis of all-*trans*-(3S,3′S)-7,8,7′,8′-tetradehydro-astaxanthin (36).

This time, treatment of the 9-*trans*-C_{15}-aldehyde (29) with the crystalline C_{10}-diphosphonium bromide (40) under *Wittig* conditions furnished, after chromatographic purification, all-*trans*-(3S,3'S)-7,8,7',8'-tetradehydro-astaxanthin (36) as fine violet crystals. All physical properties of its diacetate were identical to those of the diacetate of the natural product isolated from the starfish *Asterias rubens*, thus confirming the absolute configuration proposed (Francis *et al.*, 1970; Berger *et al.*, 1977).

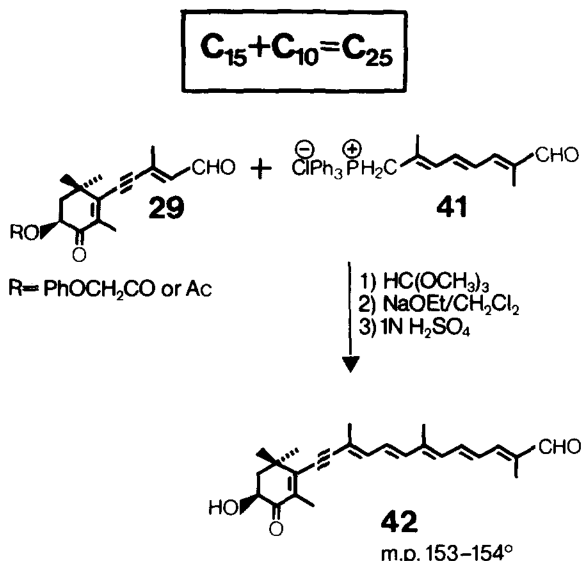


Fig. 10. Synthesis of all-*trans*-(3S)-7,8-didehydro-12'-apo-astaxanthinal (42).

For the synthesis of the monoacetylenic component (37) (Fig. 7) of asterinic acid, the scheme $C_{15} + C_{10} = C_{25}$ (Fig. 10) followed by $C_{25} + C_{15} = C_{40}$ (Fig. 11) was used.

Thus, *Wittig* reaction of the acetal of the C_{10}-phosphonium salt (41) with the *trans*-C_{15}-aldehyde (29) furnished, after hydrolysis, all-*trans*-(3S)-7,8-didehydro-12'-apo-astaxanthinal (42) which—in its protected form (43)—was condensed with the *Wittig* salt (32) to give all-*trans*-(3S,3'S)-7,8-didehydro-astaxanthin (37).

The spectroscopic properties of its diacetate (44) were in good agreement with those available for the diacetate of the natural product (Francis *et al.*, 1970; Berger *et al.*, 1977).

(b) *The synthesis of some optically active xanthophylls by asymmetric synthesis*

Another efficient entry into the field of optically active carotenoids has recently been found to be the application of asymmetric hydroboration (Brown & Zweifel,

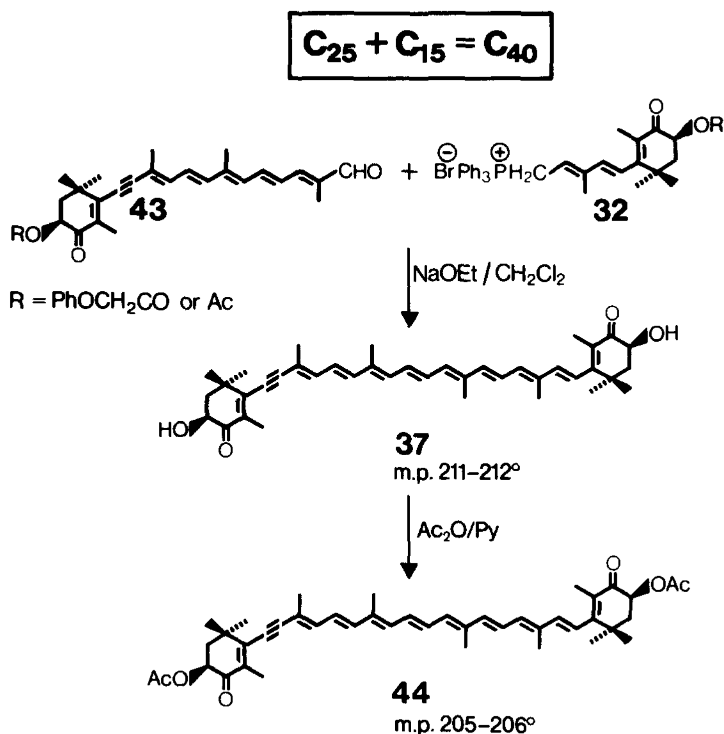


Fig. 11. Synthesis of all-*trans*-(3S,3′S)-7,8-didehydro-astaxanthin (37) and the corresponding diacetate (44).

1961; Brown *et al.*, 1964a; Brown *et al.*, 1964b; Varma & Caspi, 1968; Brown & Yoon, 1976/77; Brown *et al.*, 1977).

Thus, safranal (45) (Fig. 12) was reduced with diisobutyl aluminium hydride to give safranol which was subsequently protected as the acetonide (46). (+)-Diisopinocampheylborane ((+)-$(IPC)_2BH$), which was prepared *in situ* from (−)-α-pinene and borane methylsulphide, attacked this diene system regio-selectively at position 3 to give, after oxidative work-up with sodium hydroxide/hydrogen peroxide followed by acid catalysed removal of the protecting group, the crystalline (3R)-3-hydroxy-β-cyclogeraniol (47). Obviously, the much more sterically hindered 5,6-tetra substituted double bond was not attacked at all. Similar treatment of (46) with (−)-$(IPC)_2BH$, obtained from the enantiomeric (+)-α-pinene and borane methyl sulphide, gave the enantiomeric diol (48).

The absolute configuration and the optical purity of (47) and (48) were determined by correlation with the protected (3R)-3-hydroxy-β-cyclocitral (49), an intermediate in the synthesis of (3R,3′R)-zeaxanthin (2) (Mayer *et al.*, 1975; Mayer, 1979). Thus, reduction of (49) with diisobutyl aluminium hydride yielded the crystalline diol (47)

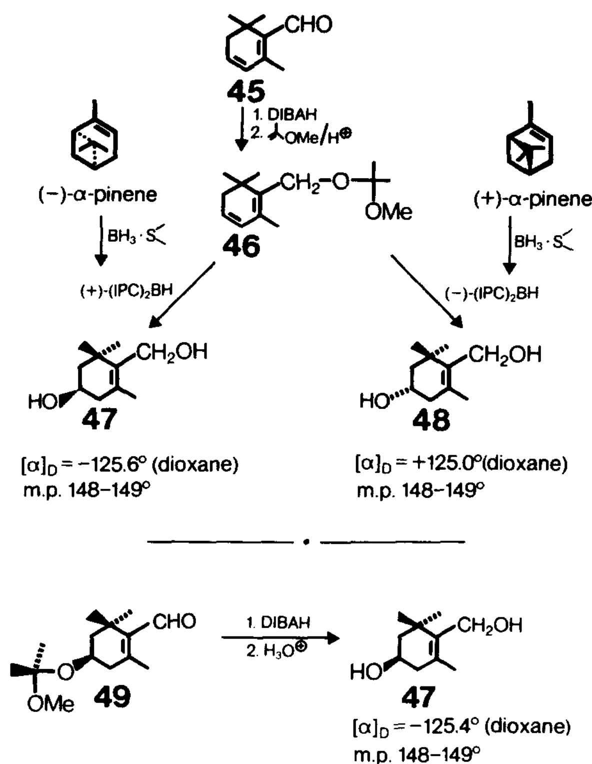


Fig. 12. Synthesis of (3R)-3-hydroxy-β-cyclogeraniol (47) and of (3S)-3-hydroxy-β-cyclogeraniol (48).

which proved to be identical in every respect with the same compound obtained by hydroboration. ^{1}H-NMR studies of the corresponding diacetates of (47) and (48) employing the optically active shift reagent Eu(hfc)$_3$ confirmed the optical purity.

Subsequent selective oxidation of the allylic hydroxyl group of (47) and (48) was achieved with pyridinium chlorochromate (Corey & Suggs, 1975) to give the enantiomeric hydroxy-β-cyclocitrals (50) and (51) (Fig. 13). Transformation of these two compounds into the two enantiomeric phosphonium salts (52) and (53) was accomplished by known methods (Mayer, 1979).

A *Wittig* reaction of the C_{15}-phosphonium salt (53) with the C_{10}-dialdehyde (26) yielded, after isomerisation, all-*trans*-(3S,3′S)-zeaxanthin (54) (Fig. 14). The CD spectrum of (54) was, as expected, an exact mirror image of the CD spectrum of the all-*trans*-(3R,3′R)-enantiomer (2).

By reacting the C_{25}-apo-aldehyde (55) with the C_{15}-phosphonium salt (53) under *Wittig* conditions, all-*trans*-(3R,3′S)-zeaxanthin (56) was formed; which constitutes the internally compensated, optically inactive *meso*-form (Fig. 15).

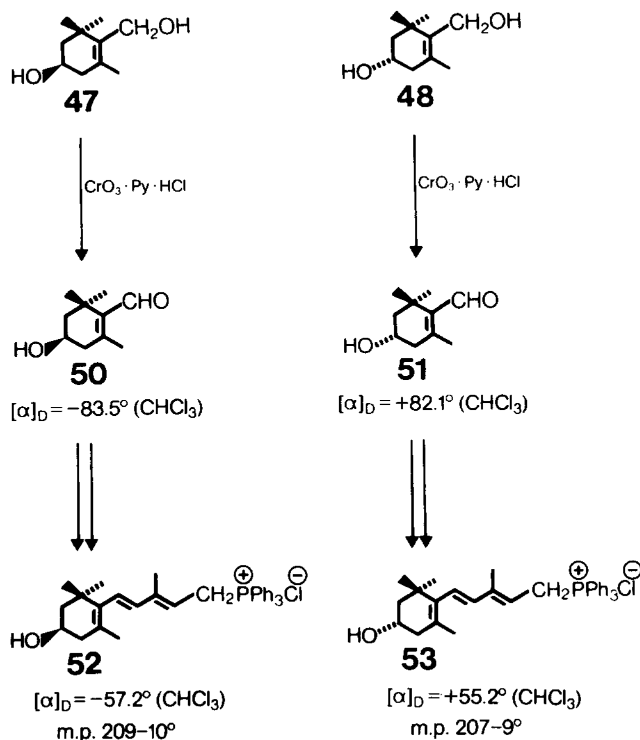


Fig. 13. Synthesis of the enantiomeric C_{15}-phosphonium salts (52) and (53).

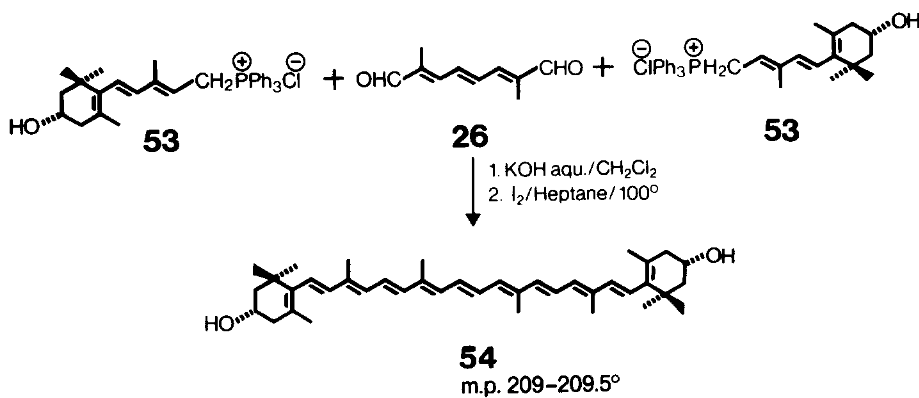


Fig. 14. Synthesis of (3S,3′S)-zeaxanthin (54).

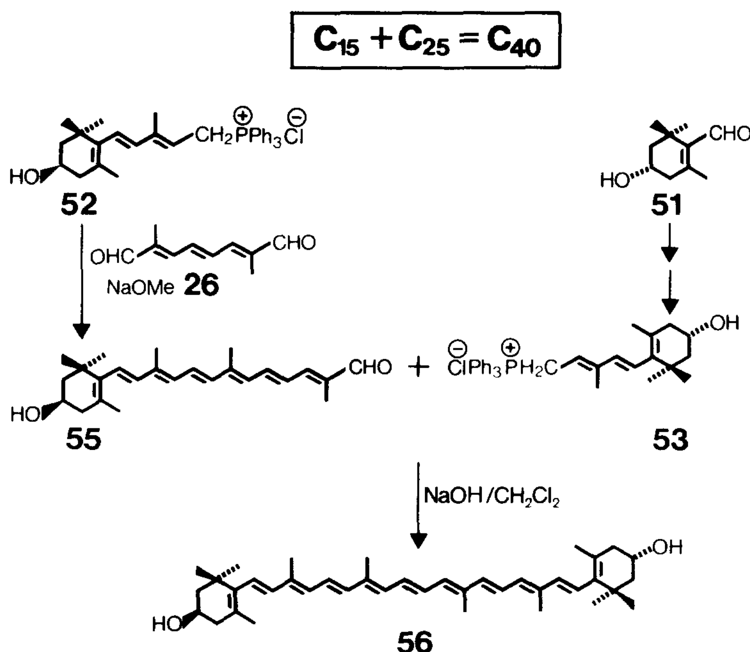
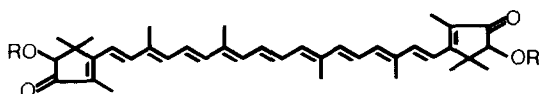


Fig. 15. Synthesis of (3R,3′S)-zeaxanthin (56) (*meso*-zeaxanthin).

(c) *Synthesis of xanthophylls starting from intermediates prepared by optical resolution via diasteromeric derivatives*

Absolute configuration and total synthesis of natural actinioerythrin (Müller *et al.*, 1978; Müller *et al.*, in press): The red variety of the sea anemone *Actinia equina* L. has attracted scientists for a long time. Lederer and Fabre (1933) isolated from this sea anemone a pigment of deep violet-red colour and called it actinioerythrin. They were able to crystallise it (mp 85 °C) and recorded a UV spectrum (Fabre & Lederer, 1934). Moreover, they realised that actinioerythrin was accompanied by traces of α- and β-carotene. The investigation was continued by de Nicola & Goodwin, in 1954.

The constitution 2,2′-dinor-3,3′-dihydroxy-β,β-carotene-4,4′-dione (Fig. 16) has



R = Acyl

Fig. 16. Constitution of actinioerythrin.

been assigned to actinioerythrin from spectroscopic and chemical evidence (Hertzberg *et al.*, 1969).

According to these authors, *Actinia equina* had an average carotenoid content of *ca.* 0·12% of the dry weight, actinioerythrin being the dominant carotenoid. Astaxanthin diester and 2-nor-astaxanthin diester were present in small amounts. A CD spectrum was taken from actinioerythrin (Andrewes *et al.*, 1974), proving optical activity, but no stereochemical conclusions could be drawn.

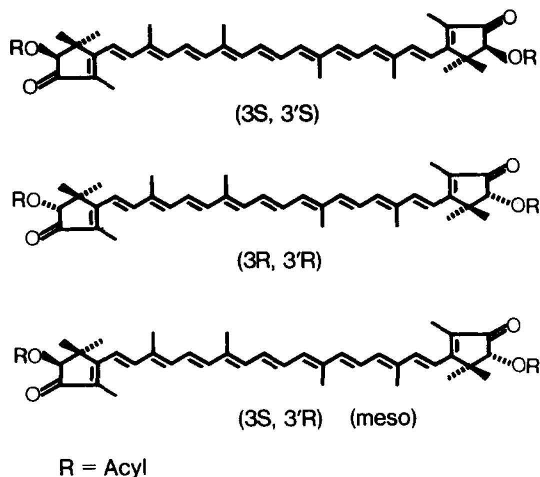


Fig. 17. Optical isomers of actinioerythrin.

Actinioerythrin possesses—in analogy with zeaxanthin and astaxanthin—two chiral centres at C-3 and C-3′ making possible three optical isomers (Fig. 17). These are the (3S,3′S)- and the (3R,3′R)-isomer—which together form a racemate—as well as the (3R,3′S)-isomer which, of course, constitutes the internally compensated optically inactive *meso*-form. It is this mixture of isomers which is usually obtained in syntheses of the so-called racemate.

In order to determine the absolute configuration of actinioerythrin, we have synthesised (3S,3′S)-actinioerythrol (57), (3R,3′R)-actinioerythrol (60), and the corresponding diacetates (58) and (61). The synthesis was accomplished by a *Wittig* condensation according to the building principle $C_{14} + C_{10} + C_{14} = C_{38}$, using the C_{10}-dialdehyde (26) as the central component (Fig. 18).

The key intermediate (62) (Fig. 18) was prepared in the following way (Fig. 19): oxidation of β-ionone (63) to the diketone (64) was accomplished in 55–65% yield with 3·0 equivalents of pyridinium chlorochromate (Corey & Suggs, 1975) in dimethyl sulphoxide at 105 °C for 3 h, a procedure of allylic oxidation which has also been found independently (Moss & Wenger, personal communication). The diketone (64), first synthesised (Henbest, 1951) via 4-bromo-β-ionone in low yield, is

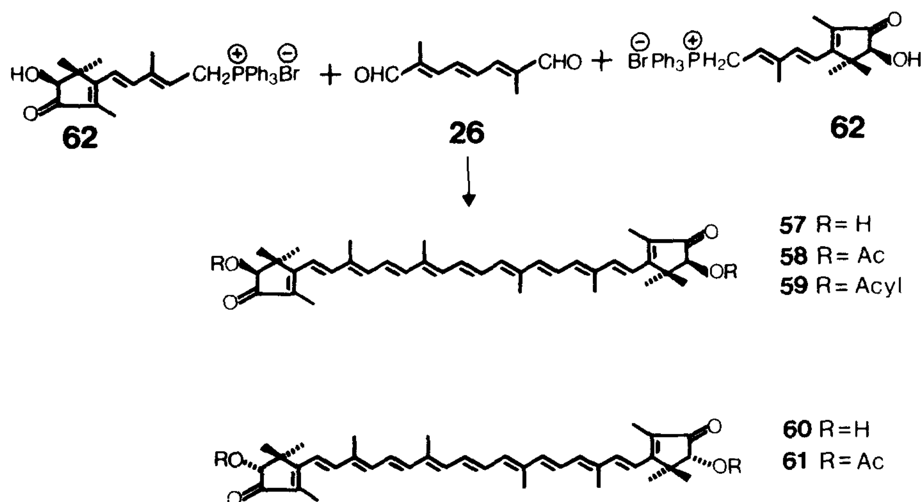


Fig. 18. Synthesis of (3S,3′S)- and (3R,3′R)-actinioerythrol.

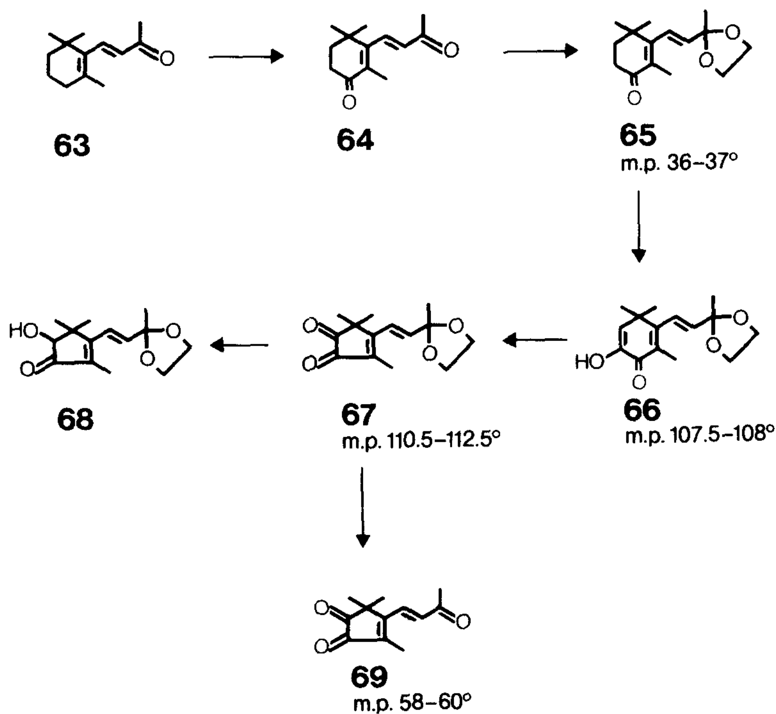


Fig. 19. Synthesis of racemic 2-nor-3-hydroxy-4-keto-β-ionone- 9-ethylene-ketal (68).

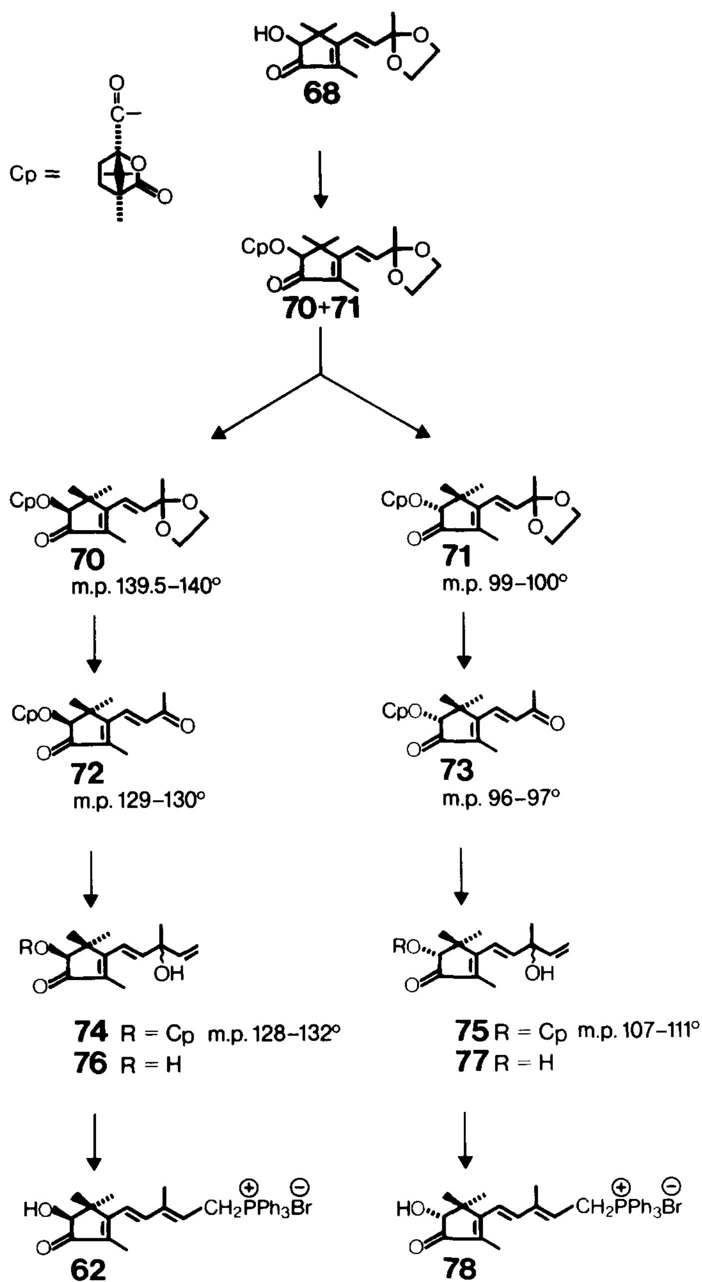


Fig. 20. Optical resolution of 2-nor-3-hydroxy-4-keto-β-ionone-9-ethylene-ketal (68).

Plate 3.

Plate 4.

part of the neutral aroma constituents of tobacco (Leffingwell, 1974; Fujimori *et al.*, 1976); it was also found in the essential oil from black tea (Ina & Eto, 1971). Treatment of (64) with 1·2 equivalents of ethyl *o*-formate and 1·5 equivalents of ethylene glycol in the presence of a catalytic amount of *p*-toluene sulphonic acid gave *ca.* 80% of the monoketal (65), isolated after column chromatography. By direct crystallisation *ca.* 50% was obtained. Subsequent oxidation of (65) by air at room temperature in a tert-butanol/potassium–tert-butanolate/toluene medium (according to Weedon, 1973) provided the enolised diketone (66) in 80% yield. Exposure of (66) to a large excess of manganese dioxide in acetonitrile gave the orange five-membered ketal (67) (mp 110 °C) in 40% yield.

Acid catalysed deketalisation of (67) gave the deep red triketone (69) (mp 68 °C). The ketal (67) was subsequently reduced with 0·26 mol equivalents of sodium borohydride in methanol to produce the pure α-ketol (68) in nearly quantitative yield.

Optical resolution of the hydroxy ketal (68) was accomplished as outlined in Fig. 20. Esterification of (68) with (−)-camphanic acid chloride (CpCl)* of known absolute configuration (Helmchen & Staiger, 1977) gave the mixture of diastereomeric esters (70) plus (71) in more than 90% yield (Gerlach, 1968; Gerlach & Müller, 1972; Gerlach *et al.*, 1976). Separation into (70) and (71) was effected by fractional crystallisation or column chromatography on silica gel. The structure and relative configuration of the ester ketal (70) was determined by X-ray analysis (Müller *et al.*, 1978; Müller *et al.*, in press).

The purity of (70) and (71) used for the X-ray analysis and further synthesis was more than 99·95% according to HPLC analysis.

The ketone (72) was obtained by deketalisation in methanol/aqueous hydrochloric acid. Vinylation of (72) with vinyl magnesium chloride in tetrahydrofurane at −70 °C gave the vinyl carbinol (74) (mp 128–132 °C) as a mixture of C-9-epimers, which was transformed into the C_{14}-phosphonium salt (62) (mp 116–118 °C, not completely pure) via (76). The enantiomeric phosphonium salt (78) was prepared in the same manner from (71) via (73), (75), and (77).

The all-*trans*-configuration of the polyene chain of the diacetates (58) and (61) (Fig. 18) was unequivocally proven by 270 MHz ^{1}H-NMR and 68 MHz ^{13}C-NMR evidence.

Professor S. Liaaen-Jensen and Dr Tauber, Trondheim, re-isolated actinioerythrin from the sea anemone *Actinia equina*, collected at Alnes, Godöy Island, West Norway, by solvent extraction followed by purification on TLC (silica) and crystallisation (Müller *et al.*, 1978).

Identical electronic spectra of the synthetic all-*trans*-diacetates (58) and (61) and actinioerythrin confirmed the all-*trans*-configuration for the natural compound. ^{1}H-NMR evidence supported this assignment.

* Commercially available from FLUKA AG, Ch-9470 Buchs, Switzerland.

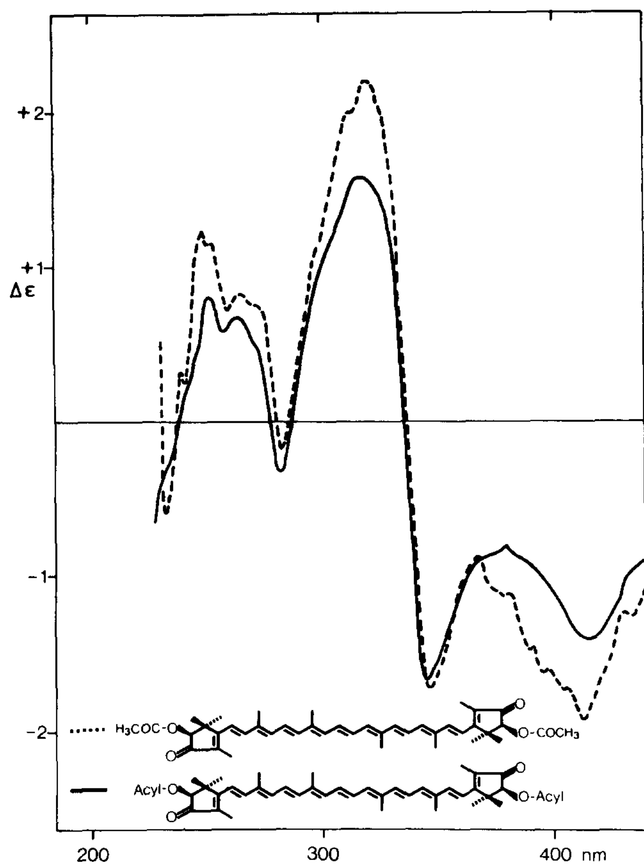


Fig. 21. CD comparison of (3S,3′S)-actinioerythrol diacetate (58) and actinioerythrin, isolated from *Actinia equina* L.

Figure 21 shows the CD spectra of the synthesised (3S,3′S)-actinioerythrol diacetate (58) and of actinioerythrin isolated from the sea anemone *Actinia equina* L. The comparison proves that the natural compound has the (3S,3′S)-configuration.

Esterification of α-hydroxy-carbonyl compounds with (−)-camphanic acid chloride gave in ten cases a mixture of separable diastereomers, thus providing a generally applicable procedure for the resolution of α-hydroxy-carbonyl compounds (Fig. 22). As a rule of thumb, so far without exception, the diastereomer with S-configuration always showed a shorter retention time on HPLC, a smaller R_F value on TLC and a higher melting point. For good results, the presence of the keto group seems to be essential, since allylic or secondary alcohols could not readily be separated.

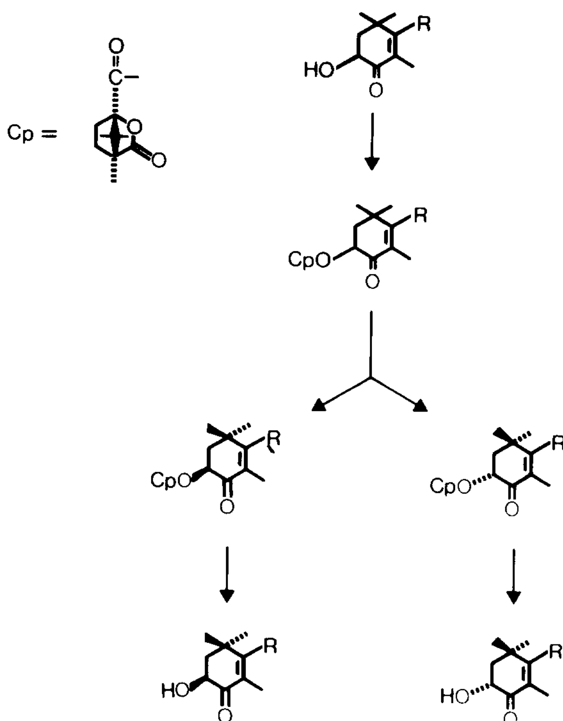


Fig. 22. Optical resolution of α-hydroxy-carbonyl compounds via (−)-camphanic acid esters.

By application of this technique, we were able to synthesise—among other intermediates—the appropriate C_{15} phosphonium salts (79) and (80) (Mayer, 1979) (Fig. 23).

The synthesis of (3S)-3-hydroxy-echinenone (82) and (3R)-3-hydroxy-echinenone (83) was accomplished according to the building principle $C_{15} + C_{25} = C_{40}$ using the appropriate C_{15}-phosphonium salts and the C_{25}-aldehyde (81) (Fig. 24) first synthesised by Rüegg *et al.*, in 1959.

According to the same building principle, we have synthesised both enantiomers

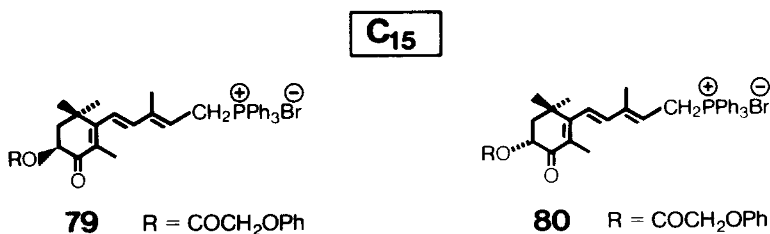


Fig. 23. Enantiomers of C_{15}-phosphonium salts.

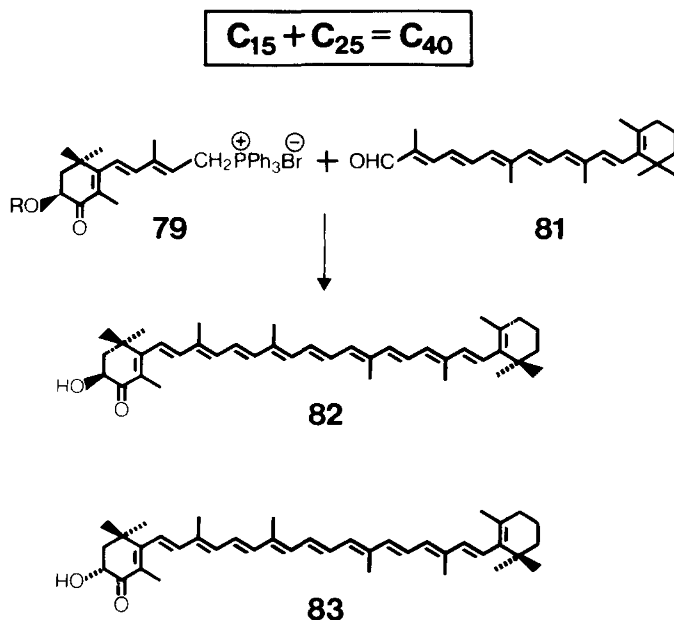


Fig. 24. Synthesis of (3S)-3-hydroxy-echinenone (82) and (3R)-3-hydroxy-echinenone (83).

of adonirubin (Fig. 26). As only one *Wittig* condensation is needed in these cases for the final step, in contrast to the building principle $C_{15} + C_{10} + C_{15} = C_{40}$, high yields up to 80 % were realised. Adonirubin was isolated as dark violet crystals, mp 216–217 °C, UV maximum 471 nm in hexane (astaxanthin = 472 nm in hexane). Hydroxyechinenone and adonirubin have been found in the red flowers of *Adonis annua* L. (Seybold & Goodwin, 1959; Egger, 1965; Egger & Kleinig, 1967a; 1967b). To our knowledge, no chiroptical properties have yet been reported.

Racemic 3-hydroxy-echinenone and adonirubin have been synthesised (Cooper *et al.*, 1975).

Separation of configurational isomers of astaxanthin and some related xanthophylls by HPLC analysis of the corresponding (−)-camphanic acid esters

As mentioned above (Fig. 22), the diastereomeric (−)-camphanic acid esters of racemic α-hydroxy-carbonyl compounds have been found to be readily separable.

This method has recently been applied to the analysis of configurational isomers (4), (88), (90) (Fig. 27) of astaxanthin (Vecchi & Müller, 1979). (3RS, 3′R)-Astaxanthin (92) was transformed into the corresponding mixture of diastereomeric esters (93) which could be separated by HPLC (Fig. 28). The identification of the peaks was achieved by means of reference samples of (87), (89) and (91), which have been obtained by total synthesis. In addition, electronic and mass spectra were

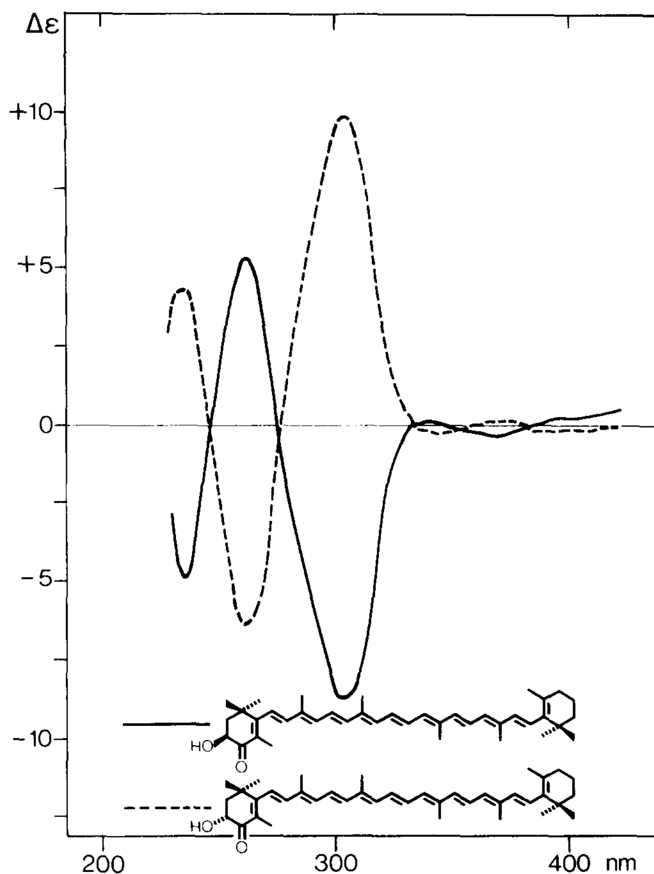


Fig. 25. CD comparison of (3S)-3-hydroxy-echinenone (82) and (3R)-3-hydroxy-echinenone (83).

recorded for each eluted compound. The quantitative evaluation of the chromatogram gave a ratio of 1:2:1 for the areas of the peaks 1, 3, and 5 as well as for those of peaks 2, 4, and 6 (Fig. 28).

The (−)-camphanic acid esters of astaxanthin could be transformed into astaxanthin by saponification in sodium hydroxide/methanol under argon.

Similar preparative and analytical results could be obtained with actinioerythrol (Fig. 11: (7)) and adonirubin (Fig. 26: (85), (86)).

TECHNOLOGICAL APPLICATIONS

In many cases, carotenoids are responsible for the colour in plants, animals, or animal products used as human food. For instance, in fruit juices; in broilers; in fish,

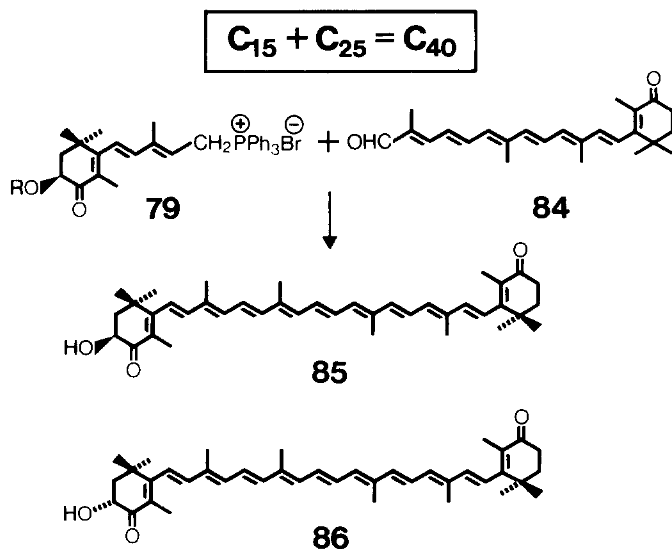


Fig. 26. Synthesis of (3S)-adonirubin (85) and (3R)-adonirubin (86).

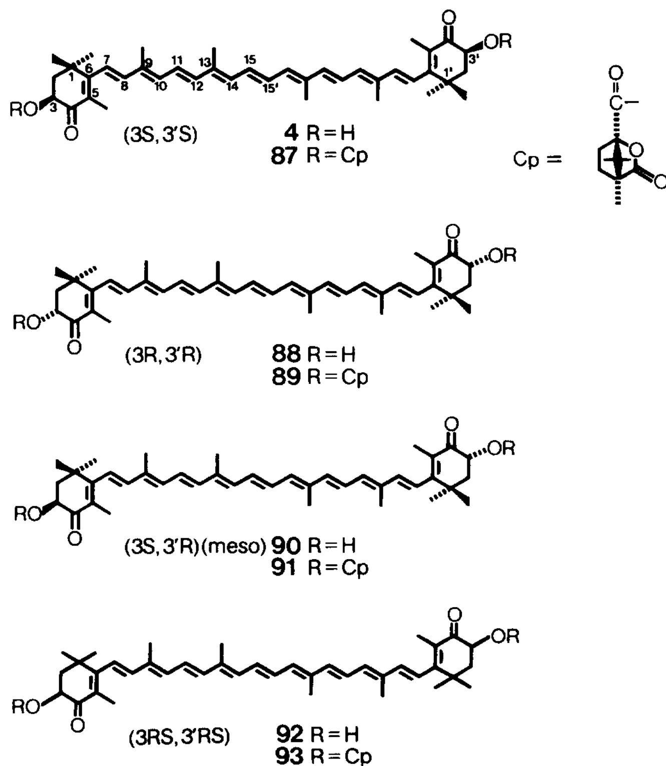


Fig. 27.

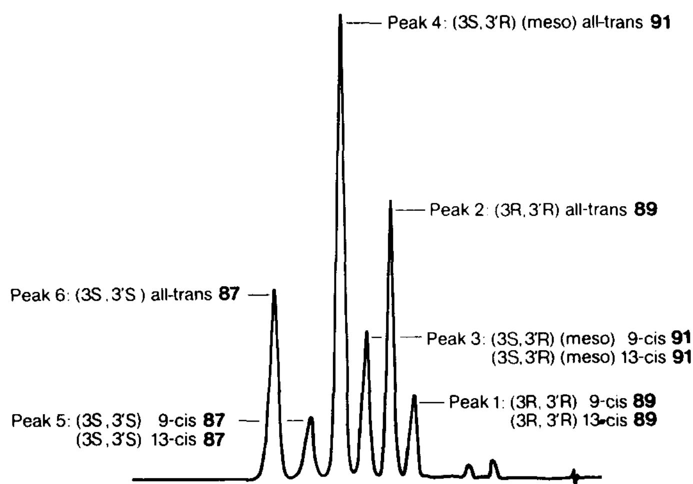


Fig. 28.

such as salmon and trout; in dairy products, such as milk, butter, and cheese and in egg yolk. Since man has been familiar with the visual appearance of these products for thousands of years, their expected characteristic colour is considered as a sign of quality.

In these days of increasing population, there is more and more reliance on fast and economical mass production of food. As a result, however, the natural pigments and vitamins are often not present or only in small amounts. To produce not only quantity but also food of high quality and appealing appearance, lacking pigments and vitamins have to be supplemented. This may be achieved by direct addition of the substance lacking in the finished product (as is done with fruit juices, soft drinks, margarine, oils, fats and shortenings, soups, gravies, desserts, jellies, puddings, candies, bakery products and dressings, see Plates 3 and 4, pages 35 and 36) or by an indirect method, i.e. by addition of a pigment to the feed, as in the production of eggs, broilers, and the breeding of fish.

Crystalline, nature-identical carotenoids produced by chemical synthesis are ideal colorants for food and feed. They offer the advantage of high purity and uniformity and are not suspected of any toxicological effects as are so many artificial food colours.

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EXTRACTION AND CHEMISTRY OF ANNATTO

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ABSTRACT

Annatto extracts contained in the pericarp of the fruit Bixa orellana *are extracted by several methods. Commercial preparations consist of solutions or suspensions of the pigment in vegetable oil or as a water-soluble form in dilute alkaline solution.*

The major pigment of the fruit is cis-*bixin (80%+) with smaller amounts of other carotenoids.* Cis-*bixin, on heating in solution, is partially converted into the* trans *isomer and a yellow degradation product. The extent of the degradation is dependent on the temperature and the duration of the heating process and governs the red/yellow balance.*

The dicarboxylic acid salts of cis-*norbixin and* trans-*norbixin occur in heated alkaline solutions.*

The current toxicological situation of annatto describes recent results of studies at the Unilever Research Laboratory at Colworth House, Bedford.

INTRODUCTION

Annatto extracts have been used for a considerable time, particularly for colouring butter, margarine and cheese. Commercial annatto colours, also known as Orlean or Rocou, are obtained by extraction of the pigments of the pericarp of the fruit of the *Bixa orellana* L, named after Francisco de Orellana, the Conquistador who first explored the Amazon River in 1541.

This large shrub, 2–5 m high, native to tropical America, is now grown in most tropical countries: Bolivia, Brazil, Ceylon, Dominican Republic, Ecuador, Guyana, India, Jamaica, Mexico, Peru and Surinam. The crop is produced on a lesser scale in Africa, e.g. Angola, Kenya, Nigeria, Tanzania and in the Pacific, e.g. Philippines and Hawaii.

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Its occurrence and cultivation were reviewed by Ingram & Francis (1969) and by Ohler (1968). The fruit consists of a burr-like pod, similar to a sweet chestnut, containing between 10 and 50 seeds about the size of grape seeds, covered with a thin layer of soft, slightly sticky, vermilion pulp. A small shrub may be seen in the Royal Botanic Garden at Edinburgh; its flower shows no change from that of the shrub shown in a coloured plate in *Icones Plantarum*, dated 1792, from the Library of the Royal Botanic Gardens, Kew (von Plenck, 1792).

The FAO/WHO (1976) have defined specifications for the methods of production of annatto extracts. Annatto extract in edible oil, as a solution or suspension, may be prepared by pretreatment of the pericarp with hot water or steam, followed by extraction with vegetable oil or mono- and di-glycerides, etc. Solvent extraction is also employed. Aqueous annatto extracts are obtained by heating the pericarp, or the solvent extract thereof, with a solution of sodium or potassium hydroxide at 70 °C or lower, followed by boiling and filtration.

The major carotenoid of the pericarp extract *cis*-bixin, the mono-methyl ester of the dicarboxylic acid *cis*-norbixin (McKeown, 1961), is orange in colour and is insoluble in vegetable oil. It is readily converted on heating into the more stable isomer *trans*-bixin, which is red and soluble in oil, together with degradation products, notably a yellow C_{17} pigment described by Iversen and Lam (1953) and by (McKeown & Mark, 1962; McKeown, 1963, 1965). The *cis*-bixin is required to be in solution before the C_{17} re-arrangement can occur.

PROCESS METHODS FOR ANNATTO EXTRACTS

The following two processes for the production of annatto extracts in edible oil and one for making water-soluble annatto conform to the FAO/WHO Specification and have a favourable legal status.

(*i*) *Oil-soluble bixin*

Annatto seeds, immersed in vegetable oil at a temperature not exceeding 70 °C, are mechanically abraded to remove the pericarp from the waste seed by a process known as 'raspelling'. The slurry of the pigment in oil is heated under vacuum at a temperature which should not exceed 130 °C. The solution is filtered to remove insoluble materials. The major carotenoids include *trans*-bixin, a yellow thermal degradation product and *cis*-bixin.

(*ii*) *Suspension in oil*

Three important methods exist.

(a) The extract of the pericarp in edible oil after 'raspelling' is centrifuged and the finer particles blended with oil to give a suspension in oil. The lack of

thermal treatment is shown by the absence of the yellow degradation product, *cis*- and *trans*-bixins being present.

(b) *Solvent extraction:* The annatto pigment is extracted into a suitable solvent such as acetone, the extracts being washed with hexane, or some other solvent in which the pigment is virtually insoluble, to remove unwanted impurities and odours. Milling in edible oil to a particle size of about 10 microns is then carried out to yield a microcrystalline suspension in oil. The solvents include acetone, dichloromethane, dichloroethane, ethanol, light petroleum and propan-2-ol. The major carotenoids present are *cis*- and *trans*-bixin.

(c) *Mono- and di-glycerides, etc:* Extraction using food grade ingredients such as mono- and di-glycerides, free fatty acids or propylene glycol is also practised.

Barnett & Espoy (1957) pre-soaked the seeds in water before extraction in hot oil with or without free fatty acids such as oleic. Kocher (1958) extracted annatto seeds with alkaline propylene glycol which would yield the alkaline salts of *cis*- and/or *trans*-norbixin. Dendy (1966*a*) reported an alkaline extraction method followed by acid treatment devised for use in East Africa, together with a survey of the patented processes.

The differences between the methods are highlighted by their use as margarine colouring agents (Table 1), which requires a balance of red and yellow pigments. Oil-soluble bixin can be used for margarine colour without further pigmentation, since the degradation pigment provides the yellow contribution. The suspension in oil, however, cannot be used alone and requires a contribution of a yellow pigment such as curcumin which is claimed to impart stability (Todd, 1964).

TABLE 1
ANNATTO CONTRIBUTION TO MARGARINE COLOURING

Extract	*Red*	*Yellow*
Oil-soluble bixin	C_{25}: *trans*-bixin + some *cis*-bixin	C_{17}: degradation product
Suspension in oil	C_{25}: *cis*-bixin + some *trans*-bixin	Yellow pigment required, e.g. curcumin

(*iii*) *Water-soluble annatto*

(a) *From seed:* The pericarp of the annatto seed is extracted by agitation in aqueous alkali at temperatures not exceeding 70 °C to give an aqueous solution of sodium or potassium salts of norbixin, probably in the *cis* and *trans* forms.

(b) *From solvent-extracted bixin:* Bixin, derived from solvent extraction of the pericarp by one or more stages, followed by removal of the solvent, is hydrolysed with aqueous alkali to yield the sodium or potassium salts of norbixin.

CHEMISTRY OF BIXIN

(*i*) *Stereochemistry*

Since bixin was the first *cis* polyene to be recognised in nature, its stereochemistry has been subjected to much investigation (see Table 2).

TABLE 2
CHRONOLOGICAL DEVELOPMENT

Boussingault (1825)	First isolation
Etti (1878)	Crystallisation
Heiduschka & Panzer (1917)	Mol. formula: $C_{25}H_{30}O_4$
Herzig & Faltis (1923)	Isomerisation
Karrer *et al.* (1929)	*cis* → *trans* isomer
Kuhn/Ehmann (1929); Kuhn/L'Orsa (1931, 1932); Kuhn/Winterstein (1928, 1932); Kuhn *et al.* (1929)	Constitution
Karrer & Solmssen (1937)	11-*cis* structure
Zechmeister & Escue (1944)	9-*cis* structure
Karrer & Jucker (1950)	9-*cis* structure
Lunde & Zechmeister (1955)	7-*cis* for Me-bixin (IR)
Barber *et al.* (1961)	pr Me-bixin 9'-*cis* structure
Weedon (1967)	Me-*cis*-(natural) bixin synthesis
Pattenden *et al.* (1970)	Me-*cis*-(natural) bixin synthesis

Therefore, 136 years after bixin was first reported, the structure of *cis*-bixin was shown to be methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate (Fig. 1).

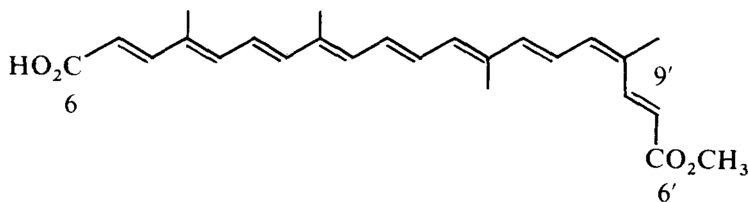

Fig. 1. *Cis*-bixin, $C_{25}H_{30}O_4$.

(*ii*) *Thermal degradation*

Iversen & Lam, 1953, showed that bixin, dissolved in vegetable oil or other solvents, undergoes a complex series of isomerisations and degradations when heated to extraction temperatures. A yellow pigment was isolated from a commercial annatto preparation. Using a paper chromatographic method (McKeown, 1961), a series of pigments from commercial annatto extracts was separated. The effects of thermal treatment of a commercial extract can be seen in comparison with the *Bixa orellana* seed extract.

(iii) Analysis of extracts

Analysis of the extracts from the various commercial processes shows the following major carotenoids:

Oil-soluble bixin	C_{17} Thermal degradation product *trans*-bixin *cis*-bixin
Suspension of bixin in oil	*cis*-bixin + some *trans*-bixin
Solvent-extracted bixin	*cis*-bixin + some *trans*-bixin
Water-soluble extracts (a) Aqueous alkali extract of seed (b) Hydrolysis in aqueous alkali of solvent-extracted bixin	*cis*- and *trans*-norbixin (Na or K salts)

The number and concentration of the minor constituents present may be due to the extractive method, the temperature, the heating period, the origin of seed and the shelf-life of the product. McKeown (1961) isolated seven components in a chloroform extract of *Bixa orellana* seeds (Table 3).

TABLE 3
PAPER CHROMATOGRAPHY OF ANNATTO EXTRACT

Zone	*Identity*	*Colour*	R_f	*Spectra*	*Commercial extract* (%)	*Seed extract* ($CHCl_3$)
1	Orellin?	Yellow	0·01	420, 395	6	7
2	*cis*-norbixin	Orange	0·02	*ca.* 495, 465	4	2
3	Thermal product	Yellow	0·07	423, 401·5	40	1
4	*trans*-bixin	Red	0·11	501,471	17	nil
5	*cis*-bixin	Orange	0·17	494·5, 464·5	12	82
6		Orange	0·22	*ca.* 488, 459	nd	3
7		Pale yellow	0·35	*ca.* 420, 400	9	1
8	Polyene aldehydes?	Orange	0·85	Variable	12	4

nd = not detected.

(iv) Spectral assay

Bixin and its isomers have maxima at about 500 nm and 470 nm in chloroform, whereas the yellow degradation pigments show maxima at about 404 nm and 428 nm (in chloroform) and do not absorb at 500 nm (McKeown & Mark, 1962).

Analysis of the spectra at 500 nm and 404 nm can yield both the total pigment content and the bixin content of the extract (McKeown & Mark, 1962; Reith & Gielen, 1971).

In his final paper, McKeown (1965) proved the structure of the C_{17} yellow pigment produced by thermal degradation of the C_{25} bixin to be as shown in Fig. 2.

(*v*) *Thin-layer chromatography*

Several TLC methods have been developed (Francis, 1965; Dendy, 1966*b*; Hoar, pers. comm.) for the analysis of annatto pigments to replace the earlier paper chromatographic procedures (Van Esch *et al.*, 1959; McKeown, 1961). The method (Hoar, pers. comm.) used at Unilever Research Colworth Laboratory was developed to isolate and identify the main components in commercial annatto preparations and to assist in the studies of the metabolic fate of annatto pigments in the rat and in man.

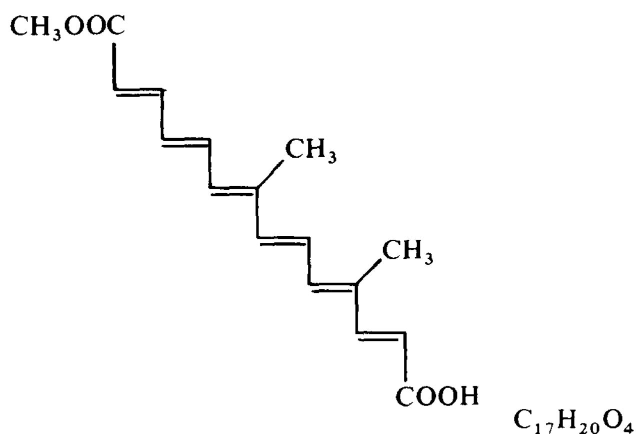

Fig. 2. C_{17} yellow pigment.

Precoated 250 μm silica gel GF plates (Anachem) were developed in petroleum ether (bp 40-60)/diethyl ether/glacial acetic acid (85 + 15 + 2·5). Four developments were used to improve resolution so that the main bands could be removed for subsequent examination by spectrophotometry.

The analytical procedures were sufficiently sensitive to detect oil-soluble bixin and water-soluble annatto at the 1 mg level and suspension of bixin in oil at a level of 0·2 mg in the liver, kidneys or tissue.

TOXICOLOGY

A temporary acceptable daily intake (ADI) of 1·25 mg/kg body weight for annatto extracts has been given (FAO/WHO, 1970*a*, *b*). The monograph from this report dealt extensively with studies by van Esch *et al.* (1959). Metabolic studies on the major carotenoids of annatto were requested. Food Industries Limited commissioned these studies, which were designed to complement the earlier work of

van Esch on what were essentially the same annatto extracts manufactured in accordance with the FAO/WHO specifications without the use of solvents.

This additional work covered the following areas:

(i) acute toxicity in rat and mouse
(ii) mutogenic action
(iii) short-term studies on rat, mouse, dog and pig
(iv) long-term studies on mouse and rat.

This toxicological work was considered by the FAO/WHO (1970*a*,*b*), resulting in an expanded monograph (FAO/WHO, 1976).

The metabolic studies have since been completed and show that annatto carotenoid pigments are satisfactorily metabolised. This included work on acute metabolism in man.

To develop upon this theme, it can be said that in the rat, oil-soluble bixin, suspension of bixin in oil, and water-soluble annatto pigments can be detected in blood within a few hours of administering a single large oral dose. The level of pigments in the blood diminishes during the following 24 h, which suggests that the body is able to metabolise these pigments.

In vitro studies suggest that the principal annatto pigments are metabolised by the liver.

In man, annatto pigments can also be detected in blood after a single oral dose and within a few hours the blood level has fallen to zero: only a trace of these pigments was found in the faeces. Therefore, both in the rat and in man, annatto pigments are absorbed from the intestine into the blood and clearance from the blood is quite rapid.

It is apparent from these studies showing similar absorption from the gut and clearance from the blood of rat and man that the rat is an appropriate test animal and that the results obtained by van Esch and others are relevant to the safety of annatto pigments in food.

This latter information has been submitted to the EEC Scientific Committee for Food which agreed (EEC, in press) that:

> 'the information with which it had been provided on the pharmacokinetics and metabolism of annatto in rats following short- and long-term exposure, and with the results of acute metabolic studies in man satisfied the requirements set out in its Report on Colours (June, 1965).

LEGISLATION

Annatto, assigned E160b under EEC legislation, is permitted for various applications in most countries, as shown in the Appendix.

ACKNOWLEDGEMENTS

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APPENDIX: LEGISLATION SURVEY ON ANNATTO

Country	*Soft drinks*	*Sugar confectionery*	*Flour confectionery*	*Butter*	*Margarine*	*Cheese*	*Ice cream*	*Fish products*
Australia (except Western Australia and New South Wales)	*	*	*	—	*	*	*	*
Austria	—	*	*	*	*	*	*	—
Belgium	*	—	—	< 200 mg/kg‡	< 50 mg/kg‡	—	*	—
Canada	*	*	*	*	*	*	*	*
Denmark	—	—	< 100 ppm†	< 200 ppm†	< 200 ppm†	< 200 ppm†	< 200 ppm†	< 200 ppm†
Finland	*	*	*	*	*	*	*	*
France	*	*	—	—	—	—	*	*
Germany	*	*	—	—	*	*	—	*
Italy	*	*	*	*	*	*	—	—
Japan	*	*	*	*	*	*	*	*
Luxembourg	*	*	*	*	*	*	*	*
The Netherlands	*	*	*	*	*	*	*	*
New Zealand	*	*	*	*	—	*	*	*
Norway	*	*	*	*	*	*	*	*
South Africa	—	*	*	*	*	< 600 ppm†	*	*
Sweden	—	—	—	—	< 200 mg/kg‡	< 150 mg/kg‡	< 25 mg/kg‡	—
Switzerland	*	*	—	*	*	*	*	—
United Kingdom	*	*	*	*	*	*	*	*
USA	*	*	*	*	*	*	*	—

* Permitted.
— Not Permitted.
† Maximum limit.
‡ Maximum limit.
N.B. Not all products falling into the above categories can necessarily be coloured; this applies especially to fish products and flour confectionery.

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CHLOROPHYLL

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ABSTRACT

The distribution of chlorophyll is considered, followed by an historical survey leading to the establishment of the chemical constitution of chlorophylls a *and* b. *Their use as permitted food colours in many countries is discussed. A section gives the commercial production of coppered chlorophyll products for food colouring. Finally, the laboratory preparation, analysis and chromatographic examination of chlorophyll are described in some detail.*

INTRODUCTION

Chlorophyll is the most widely distributed natural pigment and occurs in the leaves and other parts of almost all plants. Its function in the rôle of photosynthesis is universally known and yet its biosynthesis, method of action and ultimate fate in a dying plant are still not clearly understood. Its occurrence in living plant tissue is confined to specialised 'chloroplast' cells, in which it is present in a colloidal suspension, and spectrophotometric studies indicate that a major proportion is in an associated form, possibly with proteins and carbohydrates.

Studies on a wide variety of plants indicate that the chlorophyll pigments are the same in all of them and that apparent differences in colour are due to the presence and variable distribution of other associated plant pigments. In particular, these are the xanthophylls and carotenes which always accompany the chlorophylls. Although the ratio of the yellow pigments to the chlorophylls is very variable, the absolute amount of chlorophyll in a given plant tissue remains constant for a given environment. There is little difference in the chlorophyll content of a leaf examined

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early in the morning compared with that of the same leaf examined in the middle or at the end of a sunny day. Similarly, the chlorophyll content of leaves of a plant grown in the shade is similar to the content of leaves of a similar plant grown in direct sunlight.

However, its leaves in the sunlight will contain more of the yellow pigments, which has led to the speculation that the rôle of the yellow pigments is to act as a sunscreen for the light-sensitive chlorophyll. But this simple explanation for their presence is far from the whole truth and it is also speculated that the xanthophylls and carotenes are part of a redox system which is an integral part of the photosynthetic process.

Typical leaf material will contain about 0·25% total chlorophylls, 0·03% xanthophylls and 0·015% carotenes. The plant pigments are referred to in a plural context since it has been known for a long time that each of the pigment groups could be separated into at least two different fractions and the separation of plant chlorophyll into its two major components holds a very special place in the history of analytical chemistry.

HISTORICAL SURVEY

The study of chlorophyll has attracted the attention of many of the greatest scientists of the past. It was first described in 1818 by Pelletier and Caventou who devised the name, and the first systematic chemical investigation was made by Berzelius in 1838. In 1851 Verdeil postulated a relationship between the structure of chlorophyll and the blood pigment, haem, which was known to contain iron, and, as late as 1891, it was still believed by some workers that chlorophyll contained iron.

A digression on this subject is that it is now known that plants can only metabolise magnesium to form chlorophyll in the presence of iron and that plants grown in conditions of an iron-deficient, alkaline lime soil require the presence of chelated iron in order to grow healthy leaves.

Returning to the history of chlorophyll, it was studied spectroscopically by Stokes in 1864, who concluded that it was a mixture of two different pigments and in 1873 Sorby isolated the two chlorophylls in a relatively pure form by solvent partition between methanol and carbon disulphide.

Further evidence of the existence of two chlorophylls was described by Borodin in 1882 on the basis of microscopical evidence. He examined the crystal structures of chlorophylls on the surfaces of leaves which had been treated with alcohol. This treatment leached out the chlorophyll from the chloroplasts and, on evaporation of the alcohol, yielded two identifiable crystal types. In fact, the crystals examined were homologues of the chlorophylls produced by ester interchange and were ethyl chlorophyllides *a* and *b*.

In the early years of the twentieth century the subject was examined very extensively by Willstatter who published a considerable number of papers

culminating in a full treatise in 1913. In this treatise he described in great detail methods by which the chlorophyll pigments could be isolated and partitioned to give pure chlorophylls *a* and *b* although, prior to this, opinion was divided on the subject of their separate existence. This was notwithstanding Tswett's work in 1903–1906 and some workers claimed that one chlorophyll was a degradation product of the other.

This view was supported by the established fact that chlorophyll *a* was less stable than chlorophyll *b* although it had not been possible to convert one into the other. It was not surprising, therefore, that, in 1903, when Tswett first published his work and introduced a new word into the scientific vocabularies of the world, the results should be treated with some scepticism.

Some influential workers refused to admit the existence of the two chlorophylls and were unprepared to accept the results and validity of the new 'chromatographic method'. Even Willstatter writing in 1906 described the method as 'odd' although, he had fully accepted its validity. It is interesting to speculate to what extent the course of chromatographic history and its long dormancy was affected by Tswett's choice of pigments to use for his separation. It was also an unfortunate coincidence that the colours were separated in the same order as the dispersion of light by a prism—i.e. xanthophylls, followed by carotenes, then by chlorophyll *a* and chlorophyll *b*—Tswett incorrectly postulating a connection between the two.

The customary explanation for the slow acceptance of the method centres around the obscure nature of the original publication (*Arbeit Naturf. Gesellschaft*, Warsaw, **14** (1903)) but in 1906 a publication in German was made in *Berichte Deutsche Botanische Gesellschaft*, **24,** 384. This is the reference normally quoted as being the original and is widely known. Therefore, the nature of the original publication can, at most, only play a small part in the slow acceptance of the technique. Even Tswett himself may have had some doubts since he clearly described the two separated fractions as chlorophylls alpha and beta and not the usual *a* and *b*. Furthermore, the technique as described by Tswett is very difficult to duplicate and the amounts of separated material are very small.

Today, of course, many different forms of chromatography are recognised and the two chlorophylls may be easily separated by several different methods.

THE CHEMICAL CONSTITUTION OF CHLOROPHYLLS

Attention must now be given to the question of the chemical constitution of the chlorophylls. By 1910 Willstatter had established the basic ring structure containing four pyrrole groups. This is porphin (Fig. 1) from which is derived the slightly more complex basic ring of chlorophyll itself, phorbin (Fig. 2), which is dihydro porphin with the addition of an isocyclic ring. Individual pyrrole nuclei are numbered I to IV and the outer positions are numbered 1 to 8 whilst the outer methine bridge carbon

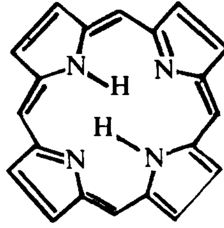

Fig. 1. Porphin.

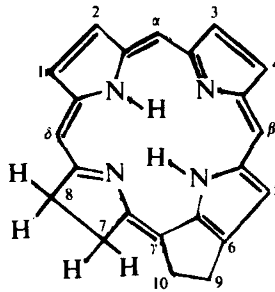

Fig. 2. Phorbin.

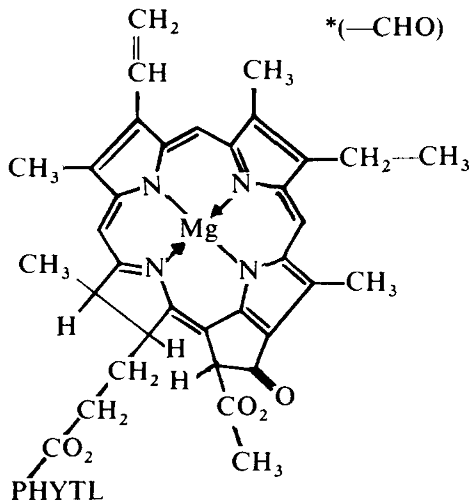

Fig. 3. Chlorophyll *a*. *For chlorophyll *b* replace the methyl at position 3 with formyl.

atoms are designated α–8. By 1940 the structure of chlorophyll itself, both *a* and *b* forms, was finally established by Hans Fischer (Fig. 3).

Some of the more important chemical characteristics of the chlorophylls are: the ease with which magnesium is lost by the action of dilute acids or replaced by other divalent metals and the ease with which the phytyl ester is hydrolysed by dilute alkali or transesterified by the lower alcohols. Stronger alkalis will hydrolyse the methyl ester and cleave the isocyclic ring.

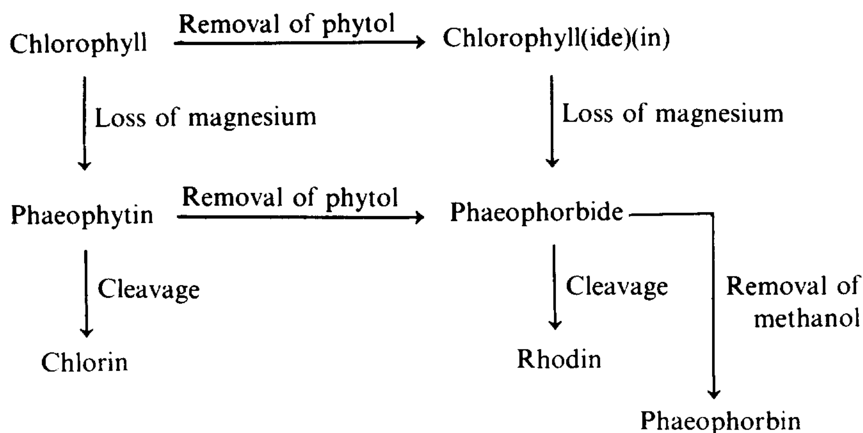

Fig. 4. Chlorophyll. Chemistry nomenclature.

The general scheme of these reactions is shown in Fig. 4. The nomenclature of some of the derivatives causes some confusion inasmuch as any derivative containing magnesium is a phyllin but the commercial, coppered, water-soluble products are described as copper chlorophyllins, whereas they are more correctly described as copper phaeophorbides in which the magnesium has been replaced by copper.

USE OF CHLOROPHYLLS AS FOOD COLOURS

The copper complexes of both phaeophytin and phaeophorbide have the metal very firmly bound and it is not liberated even by the action of concentrated hydrochloric acid. This particular aspect renders the copper complexes safe for food coloration and it has been established that in such use the copper is not absorbed by the body but totally excreted in a complexed form. As a consequence, they are permitted food colours and the current UK law on their use is described in the Colouring Matter in Food Regulations (1973) in which chlorophylls *a* and *b* are permitted, as well as the copper–chlorophyll complex and the copper–chlorophyllin complex. These colours

are given an EEC coding of E140 and E141, respectively. They must conform to the general purity criteria of food additives in respect of their trace metal contents but there is a special limitation on copper such that the colouring shall not contain more than 200 ppm of free ionisable copper. The method of determination is not given. So far as world-wide acceptance is concerned, an E140 product (magnesium chlorophyll) is permitted in all the major countries of the world except Norway. In the USA its use is restricted to certain foodstuffs only. The E141 products, either copper chlorophylls (copper phaeophytin) or copper chlorophyllins (copper phaeophorbide) are permitted in all the European countries except Switzerland. They are also permitted in Chile, Greece, Israel, Japan, Malta, New Zealand, South Africa and Spain. They are not generally permitted in the USA.

COMMERCIAL PRODUCTION OF COPPERED CHLOROPHYLL

The commercial production of coppered chlorophyll products for food colouring has been taking place for a long time and as long ago as 1926 the annual usage of processed chlorophyll in the USA was 20 tons. Present day processes may vary in detail but Fig. 5 gives a general scheme. The starting material is either grass meal or

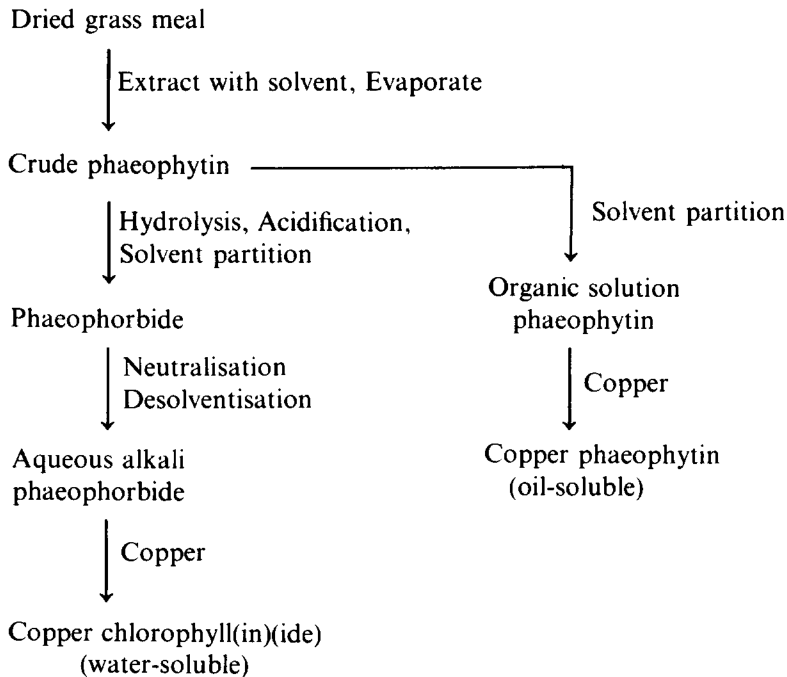

Fig. 5. Commercial chlorophyll products.

lucerne which may be either fresh or, more usually, comminuted and dried. The drying process is rapid to avoid undue loss of magnesium and to minimise the action of a naturally occurring enzyme, chlorophyllase, which hydrolyses the phytol ester linkage, thus rendering the liberated chlorophyllins soluble in dilute aqueous alkali.

The grass or lucerne is extracted with an organic solvent and upon concentration of the solvent extract the magnesium is lost due to the presence of weak organic acids derived from the grass. This loss of magnesium to yield phaeophytin occurs very suddenly in the process and its point of loss is determined by the strengths of acids and the amount of water present. Anhydrous solutions are much more stable but are, of course, impossible to achieve on a commercial scale. The choice of solvent is limited because of undesirable side-effects which can occur. The lower alcohols readily displace the phaeophytin forming the corresponding chlorophyllide. Furthermore, they do not suppress the action of the natural chlorophyllase which catalyses the hydrolysis of the ester group, thus ultimately yielding phaeophorbide.

Aliphatic and aromatic hydrocarbons will not extract the chlorophyll at all, but the most successful solvents are chlorinated hydrocarbons and acetone. But even here the extraction is not simple. Anhydrous acetone with dry grass or lucerne is a poor solvent and requires the addition of water before it becomes effective. If too much water is added it does not suppress the action of chlorophyllase and the control of the strength of the acetone is critical. This control is complicated by the variable water contents of supplies of the grass or lucerne meal.

The action of the water in improving the extraction efficiency is postulated to be because of the effect it has on the colloidal dispersion of the chlorophyll in the chloroplasts. The presence of additional water upsets the electrolyte balance, thus causing the chlorophyll dispersion to agglomerate. The presence of dissolved salts in the extracting solvent has a similar effect. Thus anhydrous ethyl alcohol is a poor solvent but the addition of either calcium chloride or water improves the action considerably. By contrast, methanol is only effective when nearly anhydrous. Since most of the chlorophyll in the chloroplasts is bound with proteins and carbohydrates, it is necessary to choose a solvent which liberates the chlorophyll from these complexes.

The colloidal dispersion of the chlorophyll is also effected by immersion of the plant material in boiling water and this destroys the chlorophyllase but induces acid degradation of the chlorophyll to phaeophytin and, as a consequence, the process is not used commercially even though blanched leaves are much more readily extracted than fresh leaves.

Commercial extraction is carried out rapidly to minimise the formation of degradation products and the extraction arrangements are designed to give the highest concentration of active material in the extract. For those not acquainted with the art—and art it is—of extraction techniques, some further explanation is required. The ratio of solvent to ground grass or lucerne is kept to an optimum. If too much solvent is used the space yield of the product is too low and additional

solvent recovery plant is required with all the energy and cost requirements that are implied. If, on the other hand, too little solvent is used, the total extractive is more difficult to process in the subsequent stages. Dried grass meal yields up to 5 % of its weight to the solvent used and this dissolved resinous material greatly alters the solvent power and extraction properties of the solvent. Nothing dissolves a given resin better than a strong solution of itself in another solvent, and since the amount of chlorophyll or phaeophytin in dried grass varies from 0·15 % to 0·35 %, it can be seen to be important to keep the extraneous resins to a minimum. Considerable differences are found in the products produced from given weights of meal and volumes of solvent according to whether the meal is extracted from a wide, thin bed, a narrow, thick bed or from a suspension, and the effect is due to mutual solubility factors.

The extraneous resins which accompany the chlorophylls and phaeophytins are fats and waxes, phospholipids, organic plant acids, xanthophylls, carotenes and a large number of degradation products and unidentified colourless materials. Both calcium and magnesium are found to be present.

The crude phaeophytin extract is then further processed to give either the water-soluble or oil-soluble coppered chlorophylls of commerce which are sold and used under the colour code E.141.

The oil-soluble products are produced from the phaeophytin without hydrolysis of the phytol and, after purification by a series of solvent partitions, the phaeophytin is converted into its copper complex and sold as a waxy paste or oil. The water-soluble products, on the other hand, are prepared after hydrolysis and removal of the phaeophytin. In this case purification is achieved by both precipitation reactions and solvent partition. Finally, the aqueous alkali phaeophorbide is coppered and the dried salts—sodium, potassium or mixed sodium/potassium—are produced by evaporation.

The colour values of the products are adjusted by the incorporation of suitable diluents. The dose rates used for food colouring vary between 0·005 % and 0·01 % for oil-soluble products and 0·002 % and 0·01 % for water-soluble products.

LABORATORY PREPARATION, ANALYSIS AND CHROMATOGRAPHIC EXAMINATION

Having dealt with the commercial production of chlorophyll, the laboratory preparation of chlorophyll and its analysis and examination by chromatography will be described.

The choice of starting material is important and grass meal, elder leaves and stinging nettle leaves are especially suitable because they have a low concentration of chlorophyllase and the yellow pigments. In contrast, the leaves of heracleums (hog weed) have a high concentration of chlorophyllase and should be avoided for the

preparation of chlorophyll, but are ideal for the preparation of concentrated enzymes.

The grass or stinging nettle meal may be extracted fresh, but more usually after drying. This should be done rapidly under vacuum at a low temperature as soon as possible after harvesting and avoiding any undue exposure to light. The dried meal is then rapidly extracted in thin layers using 80–90 % acetone and this extract is then added to petroleum ether in which it largely dissolves. Small portions of water are added and, after swirling to achieve extraction, are removed. When most, but not all, of the acetone has been extracted in this way the petroleum ether solution is partitioned with 80 % methanol which removes the xanthophylls. The methanol solutions may be worked up for xanthophyll. The petroleum ether solution is then repeatedly washed with water to remove the final traces of acetone and methanol, and, during this process, the petroleum ether solution loses its fluorescence and the chlorophyll separates as a fine suspension. This may then be filtered off using a suitable filter aid and the precipitate dissolved in diethyl ether. Reprecipitation by the addition of petroleum ether may be necessary before a final recrystallisation from ether. In this way it is possible to produce about 6·5 g of chlorophyll in an 80 % yield from 1 kg of good quality dried nettle leaves. (Nettles are preferable to grass because of their higher content of chlorophyll.)

The key steps in the separation rely on the removal of phospholipids during the extraction of the acetone from the petroleum ether solution and the avoidance of emulsion formation. The acetone must not be completely washed out or the chlorophylls and other pigments may be precipitated. Similarly, the washing of the petroleum ether with methanol achieves a further purification and the presence of traces of methanol in the petroleum ether keeps the chlorophylls in solution. It is a characteristic of chlorophylls that they are insoluble in pure petroleum ether, but small traces of the lower alcohols render them freely soluble. The precipitation of the chlorophyll by extensive washing of the petroleum ether solution only occurs provided the earlier purification stages have been efficient and all the methanol and acetone have been removed. The carotenes and any phaeophytins remain in the petroleum ether.

Any severe treatment of solvent extracts, particularly if impure, will result in loss of magnesium and if concentration at any intermediate stage is contemplated the solution must be dehydrated and kept slightly alkaline by the addition of dimethyl aniline, triethanolamine, calcium carbonate or magnesium oxide. The alkali must not be strong enough to hydrolyse the phytyl ester.

Once the magnesium has been lost from the phyllins it is exceedingly difficult indeed to replace it and the only really successful procedure involves the use of a large excess of a Grignard reagent such as methyl magnesium iodide.

The chlorophyll products produced from nettle or grass meal by solvent partition contain a mixture of chlorophylls *a* and *b* in the approximate ratio of three parts of *a* to one part of *b*. This ratio is not greatly affected by external influences on the living

plant such as seasons, time of day of harvesting or degree of illumination. However, chlorophyll *a* is more sensitive to degradation than chlorophyll *b*, by a factor between 3 and 5, and the ratio found in the product will be affected by any degradation which had occurred during the harvesting and drying of the meal or the processing of the solvent extracts.

Chlorophyll *a* is a blue–green colour in solution whilst chlorophyll *b* is a yellow–green. This difference can be quantified spectroscopically. The formyl group in chlorophyll *b* moves the red absorption maximum from 660 nm to 643 nm and lowers the absorption coefficient. One measure of the purity of chlorophylls is the ratio of the absorbancies of the 'blue' and 'red' maxima which, in the case of chlorophyll *a*, is 1·31 to 1·32.

Separation of the two chlorophylls can be achieved by solvent partition between petroleum ether and 90% methanol. The *a* form has a higher partition into the petroleum ether than the *b* form and a countercurrent extraction will ultimately achieve a good separation, but the method is tedious and the likelihood of degradation is high. The method is largely superseded by chromatographic separation.

The chromatographic examination of chlorophylls

The special place that chlorophyll holds in the history of chromatography has already been mentioned and some of the original techniques are still applied. Chlorophylls may be separated on columns of sugar, cellulose, starch and inulin, but the separations are slow and the loadings are low. Tswett's original investigations examined over a hundred different absorbents and the calcium carbonate eventually used took a long time to elute. Tswett's method was to continue elution only to the point where sufficient separation had occurred and then to push out the packing, with the absorbed bands in it. The bands were then mechanically separated and the pigments extracted with polar solvents.

Today, columns of alumina, silica gel and polythene powder are used, as well as paper- and thin-layer chromatography. However, a further example of the unique character of chlorophyll is demonstrated by its behaviour on certain gel permeation packings. Although the molecular weight difference between chlorophylls *a* and *b* is only 14 Daltons, the two may be readily separated if the correct gel and solvent system are chosen. The system will also separate phaeophytins and the copper derivatives.

Thin-layer chromatography on silica gel is also a convenient method of separation and the positions of the plant pigments are self-evident from their colours. However, treatment of the plates with anisaldehyde/sulphuric acid reveals the presence of a considerable amount of colourless material appearing in bands at almost all positions in the chromatogram.

The TLC and column separations may be made quantitative by a variety of additional techniques. In the case of the columns, the bands are eluted and collected

separately and in the case of the TLC the spots are removed mechanically. The chlorophylls may then be estimated spectrophotometrically. If the pigments are phyllins they may be estimated by determination of the magnesium content and, if they are coppered, by the determination of copper. In both cases atomic absorption is a very suitable technique. If the bands contain compounds retaining phytol this may be split off with alcoholic potassium hydroxide and determined by gas–liquid chromatography.

The spectrophotometric examination of chlorophylls

Direct determination of chlorophyll in commercial products and plant extracts has been reviewed extensively in 1955 by Smith & Benitez in *Modern methods of plant analysis* but most routine methods rely on colour measurement of one sort or another. Commercially this may be by using a Tintometer and expressing the results in terms of arbitrary colour values, or by using the AOAC (1960) recommended method using a colorimeter.

Spectrophotometric techniques rely on absorption measurements at different wavelengths, usually in solutions of acetone or diethyl ether, and various methods are discussed by Smith & Benitez.

More recently, a paper by White *et al.* (*J. Agric. Food Chem.*, **25**, 143, 1977) described a method for the determination of copper phaeophytins, chlorophylls and phaeophytins in complex mixtures. The method is simple to apply and involves spectrophotometric measurements before and after treatment with oxalic acid to convert the phyllins into the phaeophytins. However, the mathematical treatment of the measured absorption figures is formidable, but the results are reliable, except in cases where one or more of the components is present in minor amounts when it can happen that a negative figure is obtained.

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ANTHOCYANINS—OCCURRENCE, EXTRACTION AND CHEMISTRY

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ABSTRACT

This paper deals with those aspects of anthocyanins particularly related to beverages. The anthocyanins are briefly discussed and those present in some fruits are listed. pH is shown to affect the colour of anthocyanins, but various means of stabilisation are given. The reactions of anthocyanins with heat and sulphur dioxide are described and means of stabilisation suggested. A list of extractives from various fruits and flowers is given and the difficulties of assessment are discussed. Background knowledge of flower, fruit and vegetable pigments is being collected and assessed at Long Ashton.

INTRODUCTION

The anthocyanins are the water-soluble pigments which are largely responsible for the attractive colours of flowers, fruits and leaves—colours ranging from strawberry through red to violet and blue. Because they are so widespread in nature, a great deal is consumed by the human race in foods and drinks. Probably the greatest source of anthocyanins in fruits is in black grapes. In 1976, annual world production of all grapes was 57 million tons (Anon, 1977); black grapes probably accounted for about 70 % of this figure, or 40 million tons. Assuming an average anthocyanin pigment content of 0·025 %, this amounts to some 10,000 tons per annum. Not all of this quantity is consumed but it does give some idea of the quantities involved and of course this amount does not include consumption of anthocyanins in other fruits.

This short paper cannot fully review the anthocyanins, so aspects have been selected which seem most pertinent to beverages. These fall into five parts: the known types of anthocyanins; the various chemical forms in which they occur; the stabilisation of some of these by physico-chemical effects; their reactions with some

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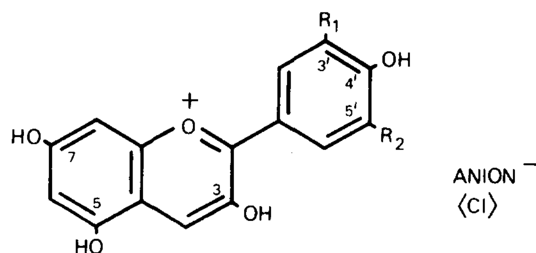
other food components and, finally, comments on the identification, analysis and exploitation of anthocyanin compounds as food colours.

ANTHOCYANIN TYPES

The anthocyanins are part of the C15 group of compounds known collectively as flavonoids and are glycosides of anthocyanidins (or aglycones).



	R_1	R_2	λ_{max} nm
Pelargonidin	H	H	520
Cyanidin	OH	H	535
Peonidin	OCH_3	H	532
Delphinidin	OH	OH	546
Petunidin	OCH_3	OH	543
Malvidin	OCH_3	OCH_3	542

Fig. 1. Major anthocyanidins (aglycones).

The major anthocyanidins and the numbering of their carbon atoms are shown in Fig. 1; others have been documented (Timberlake & Bridle, 1975). The wavelength maxima are those in methanol–hydrochloric acid (0·01 %) (Harborne, 1967). Increasing hydroxylation or methoxylation of the aglycone makes it more violet.

The anthocyanins themselves contain sugars and acylated sugars. There are four main monosides (glucose, galactose, rhamnose and arabinose) and four main biosides (rutinose, sambubiose, lathyrose and sophorose); others are comparatively rare. The triosides which occur can be linear or branched chain. The main acylating groups are the phenolic acids, *p*-coumaric, caffeic and ferulic acids, in the form of acylated sugars. A typical anthocyanin substitution pattern is shown in Fig. 2 which shows the formula of salvianin to be pelargonidin-3-(6-caffeoyl-glucoside)-5-glucoside. Acylated sugars occur, without exception, at the 3 position

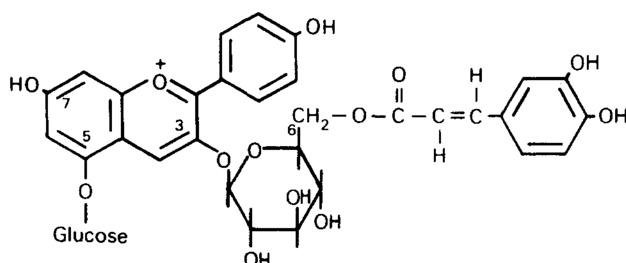


Fig. 2. Anthocyanin substitution patterns. Typical acylated anthocyanin—salvianin—from *Salvia splendens* petals. (Pelargonidin 3-(6-caffeoyl-glucoside)-5-glucoside.)

of the aglycone. This position 3 is always occupied by a sugar (whether acylated or not) and glucose can occur additionally in positions 5, 7, 3′ and possibly 4′. Sugars confer stability and solubility on the aglycone. The nature of the individual sugars has little general effect but their positions in the molecule can have a profound influence on its reactivity.

The anthocyanins in some fruits are listed in Table 1. Most contain 3-glycosides. The most complex are those in grapes of *Vitis vinifera*. Grapes from American hybrids contain additionally the corresponding 3,5-diglycosides and their acylated derivatives.

TABLE 1
ANTHOCYANINS IN SOME FRUITS

Apple	Cy 3-galactoside, 3-glucoside, 3-arabinoside, 3-xyloside, acylated derivatives
Blackberry	Cy 3-glucoside and 3-rutinoside
Blackcurrant	Cy and Dp 3-glucosides and 3-rutinosides
Cherry (sour)	Cy 3-glucoside, 3-rutinoside, 3-sophoroside and 3-glucorutinoside
Cherry (sweet)	Cy and Pn 3-glucosides and 3-rutinosides
Cranberry	Cy, Pn, Dp, Pt and Mv 3-galactosides, 3-arabinosides, 3-glucosides
Elderberry	Cy 3-sambubioside, 3-glucoside, 3-sambubioside-5-glucoside
Grapes *V. vinifera*	Mv, Dp, Pt, Pn, Cy 3-glucosides and acylated with *p*-coumaric and caffeic acids
(hybrid)	(additionally 3,5-diglucosides and acylated)
Plum	Cy and Pn 3-rutinosides, 3-glucosides
Raspberry	Cy and Pg 3-glucosides, 3-rutinosides, 3-sophorosides and 3-glucorutinosides
Redcurrant	Cy 3-glucosides, 3-rutinosides and others
Strawberry	Pg and Cy 3-glucosides

Pg = pelargonidin; Cy = cyanidin; Pn = peonidin; Dp = delphinidin; Pt = petunidin; Mv = malvidin.

ANTHOCYANIN CHEMICAL SPECIES

The anthocyanins are usually represented as flavylium cations (which, of course, must also be associated with anions), because they were first isolated in these forms as chlorides from strongly acidic solutions. But one can argue that the real or

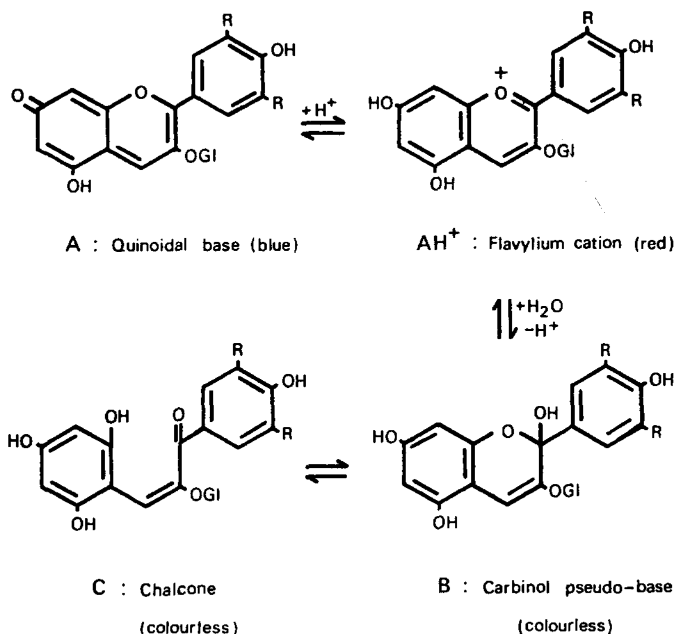


Fig. 3. Anthocyanin structural transformations with pH. Malvidin 3-glucoside (25 °C; 0·2M ionic strength) (Brouillard & Delaporte, 1977).

genuine anthocyanin is anion-free and represented by the quinoidal base (Fig. 3—formula A), since many pigments occur naturally in this form in flower petals. Figure 3 also shows the various other anthocyanin chemical species, the natures of which have been clarified by recent excellent work by Brouillard and his collaborators (Brouillard & Dubois, 1977; Brouillard & Delaporte, 1977; Brouillard & Delaporte, 1978) at the University of Paris. Protonation of the blue quinoidal base (A) of malvidin 3-glucoside gives the red flavylium cation (AH^+), which can hydrate to a colourless carbinol pseudo-base (B) which itself can exist in tautomeric equilibrium with its chalcone (C), also colourless, formed by opening the heterocyclic ring. Some interesting conclusions can be drawn from the distribution of these four structures with pH when equilibrium conditions have been attained (Brouillard & Delaporte, 1978) (Fig. 4). In very acidic solution (pH < 0·5), the red cation AH^+ is the sole species. With increasing pH its concentration and the colour of the anthocyanin is decreased as it hydrates to the colourless carbinol base (B), the equilibrium between these two forms being characterised by a pK value—in this case 2·6—when equal amounts of both forms exist. Already, at this pH, there is some formation of the colourless chalcone (C) and of the blue quinoidal base (A) and the proportions of these and the carbinol base continue to increase with increasing pH at the expense of the red cationic form (AH^+) up to about pH 4·5. Between pH 4 and 5

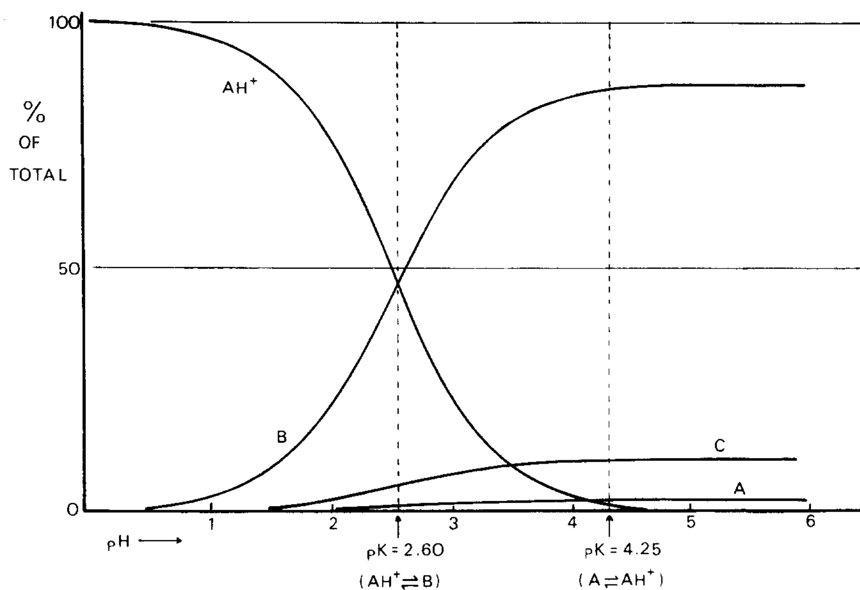


Fig. 4. Distribution of structures with pH. (Malvidin 3-glucoside: 25°C). AH^+ = red cation, B = colourless carbinol base; C = colourless chalcone; A = blue quinoidal base.

there is very little colour left in the anthocyanin since the amounts of both coloured forms—i.e. the cation and the quinoidal base—are very small. The equilibrium between them is characterised also by a pK value, in this case 4·25, when their concentrations are equal. Between pH 5 and 6 a very small amount of the blue quinoidal base is the only coloured form present. Figure 4 thus indicates the most disadvantageous feature of anthocyanins when considered as food colours—that the red colour fades with increasing pH. On this basis, the anthocyanins would appear to have little future for colouring foods or beverages above, say, pH 4. However, there are mechanisms by which the cationic and the quinoidal base forms can be augmented and stabilised so that, given suitable conditions, considerable colour is still possible, even at the higher pH range.

STABILISATION BY PHYSICO-CHEMICAL EFFECTS

The first of these mechanisms is the so-called 'self-association' of the cationic form. Colour increases more than proportionally to its concentration. Thus, in the example shown in Fig. 5, increasing the concentration of pigment ten times (from 1 to 10) increases the absorbance more than twenty times. This effect emphasises two points. First, that strongly coloured anthocyanin solutions should be measured

without dilution using very small absorption cells if necessary, and, secondly, how vital it is to prevent any pigment loss, since a small decrease in pigment can result in a proportionately greater loss of colour.

The second effect is colour augmentation by co-pigmentation—believed to be a hydrogen bonding mechanism. Thus, anthocyanin colour—for example, that of cyanidin 3,5-diglucoside at pH 3·3—is increased in intensity (A_{max}) with shift in the

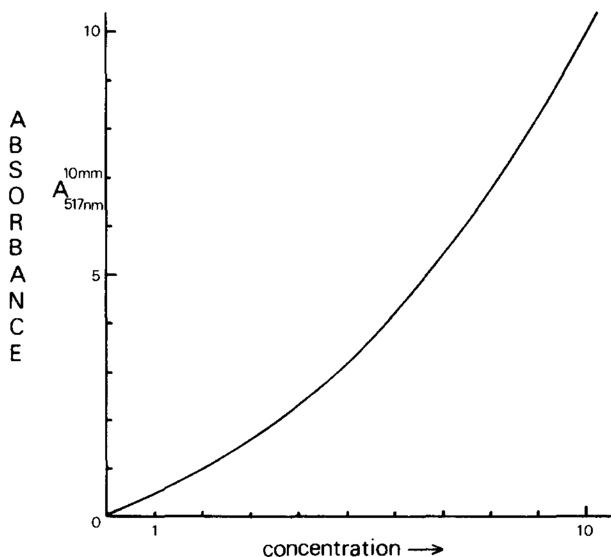


Fig. 5. Colour augmentation by self-association. (Malvidin 3-glucoside in aqueous buffer, pH 3·5, 20 °C.) At concentration 1, $A^{10} = 0·43$; at concentration 10 expect $A^{10} = 4·3$ but find $A^{10} = 9·4$.

peak wavelength (λ_{max}) towards the blue on addition of other compounds, notably flavonols (quercetin and rutin), aurisidin (an aurone) and particularly *C*-glycosyl flavones such as swertisin (Asen *et al.*, 1972). Co-pigmentation caused bathochromic wavelength shifts with both the flavylium cation and the quinoidal base, but it appeared that the colour increase was due only to stabilisation of the quinoidal base. The effect increased with anthocyanin concentration and with the ratio of co-pigment to anthocyanin.

In this connection, recent work by Scheffeldt & Hrazdina (1978) is of interest. These workers measured the colours of solutions containing a constant amount of the flavonol, rutin, as co-pigment over a wide range of anthocyanin concentration. They plotted the percentage increase in absorbance at the peak wavelength against anthocyanin concentration (10^{-5} to 10^{-2}M), for three anthocyanins at pH 3·3. At low anthocyanin levels, colour augmentation by co-pigmentation was very pronounced (about ten times with malvidin 3,5-diglucoside), but it decreased as the

concentration of anthocyanin was increased. The explanation suggested was that, with increasing concentration, the flavylium cation became stabilised by self-association. The anthocyanins so associated were then not available for co-pigmentation with rutin, so that its effect was diminished. It is evident that the two mechanisms can act in opposition and equally evident that both should be considered in any anthocyanin solution containing other components.

Effects such as those just described must be very widespread and effective in nature. How else can the vivid colours of many flowers be explained, when at the pH of cell sap, 3·5–5·5, they may be expected to have very little colour? Thus, Asen *et al.* (1971*a*,*b*) were able to reproduce the spectra of intact cells of iris, azalea and rose petals by combination of co-pigments and anthocyanins at appropriate concentrations and pH values. Much effort has been devoted to the blue flower pigments in which the quinoidal base can be stabilised by several mechanisms.

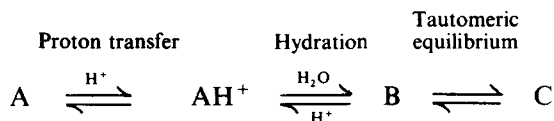
(i) Co-pigmentation—The blue iris pigment contains the quinoidal base of an acylated delphinidin 3,5-diglucoside stabilised by co-pigmentation with *C*-glycosyl flavones such as swertisin (Asen *et al.*, 1970).

(ii) Metal complexing—the blue pigment (commelinin) from flowers of *Commelina communis* is stabilised by metal complexing between magnesium, the anthocyanin—again an acylated delphinidin diglycoside—and 'flavocommelin' which is swertisin 4'-glucoside (Hayashi & Takeda, 1970). 'Commelinin' can be synthesised from its component parts (Takeda & Hayashi, 1977), as can similar blue pigments with the magnesium replaced by other metals (Takeda, 1977).

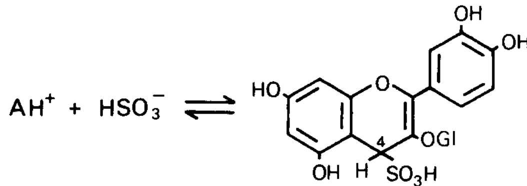
(iii) Finally, if the anthocyanin is of a suitable constitution, its blue quinoidal base can be very stable without benefit from co-pigmentation or metal complexing. Several blue flower pigments recently isolated from Chinese bell flower (Saito *et al.*, 1972), cineraria (Yoshitama & Hayashi, 1972), lobelia (Yoshitama, 1977), morning glory (*Ipomoea tricolor*) (Asen *et al.*, 1977) and *Tradescantia reflexa* (Yoshitama, 1978) owe their remarkable stabilities to some sort of interaction between the acylating groups (caffeic acid) and the phenolic groups in the B ring of the anthocyanin.

REACTIONS

Some reactions of anthocyanins are now considered and return made to the four structures (Fig. 3) and their equilibria as summarised below:



From kinetic studies (Brouillard & Dubois, 1977; Brouillard & Delaporte, 1977; Brouillard & Delaporte, 1978) it is known that the rate of deprotonation of the



$$\text{Formation constant} \quad K_s = \frac{[AH^+.SO_2]}{[AH^+][SO_2]} = 25{,}700$$

for cyanidin 3-glucoside at pH 3.2 and 20°C

$$\text{When half decolourised} \quad \frac{[AH^+SO_2]}{[AH^+]} = 1$$

$$\text{Then } [SO_2] = \frac{1}{25{,}700} \text{ M} = 0.039 \times 10^{-3} \text{ M } SO_2$$

or 2.5 ppm SO_2 (free)

Fig. 6. Bleaching of anthocyanins by sulphur dioxide.

cation (to form the quinoidal base, A) is significantly faster than its rate of hydration (to form the carbinol base, B). Ring opening of B to form chalcone (C) is significantly slower again. However, because the magnitude of the hydration equilibrium constant is much greater than that of the proton transfer equilibrium, the quinoidal base is unstable (unless stabilised by mechanisms already discussed) and rearranges (through AH^+) into the carbinol base and chalcone to extents determined by pH and illustrated in Fig. 4.

From left to right, all the reactions are endothermic, so that heating shifts all equilibria to the right, i.e. towards the chalcone. On cooling and acidification—the usual method of investigating so-called thermal degradation of anthocyanins—the quinoidal base (A) and the carbinol base (B) very quickly transform to the cationic form (AH^+), but the change of the chalcone (C) is very slow. Thus, sufficient time for re-equilibration should be allowed; this has been rarely done in previous investigations of thermal degradation. Changes occurring at 100 °C (pH 2–4) were studied by Adams (1973) who demonstrated the slow hydrolysis of the glycosidic bond in position 3, with formation of the aglycone (cyanidin). Both anthocyanin and aglycone were transformed into chalcones, that from cyanidin also forming an α-diketone. The chalcone glycoside and α-diketone undergo degradation, particularly in the presence of air.

Further reactions of anthocyanins will be confined to those of the red flavylium cation which is probably the most reactive species because of its amphoteric nature.

Sulphur dioxide is widely used in the food industry; its bleaching effect is illustrated in Fig. 6. The formation constant of the bisulphite addition compound of a typical anthocyanin, cyanidin 3-glucoside, is 25,700 at pH 3·2 and 20°C (Timberlake & Bridle, 1967). This means that at equilibrium the pigment is half decolorised when the concentration of free SO_2 is only 2·5 ppm. The total SO_2, of course, is higher, but the example nevertheless illustrates another disadvantageous feature of anthocyanins—their susceptibility to bleaching by sulphur dioxide. However, just as the pH disadvantage can be partly overcome (by co-pigmentation and ion-association), so can the bleaching effect of SO_2.

Thus, if the anthocyanin already contains a substituent in position 4 then it becomes more resistant to SO_2 (Timberlake & Bridle, 1968).

This occurs when other flavonoids such as flavan 3-ols (catechins and proanthocyanidins) can condense with anthocyanin at position 4. The substitution results in stabilisation of the quinoidal base as well as improved resistance to SO_2. Such reactions can occur naturally during red wine ageing.

Further interactions between anthocyanins and flavan 3-ols, such as catechin, can be induced by adding acetaldehyde. Highly coloured new compounds are formed in which anthocyanin and catechin are linked by CH_3CH bridges, followed by stabilisation of the quinoidal base forms and considerable augmentation of colour (Timberlake & Bridle, 1976, 1977).

Figure 7 indicates the extent and speed of this interaction between malvidin 3-glucoside, catechin and acetaldehyde at pH 3·5. With malvidin 3,5-diglucoside, colour augmentation up to seven times was obtained. When these reactions were

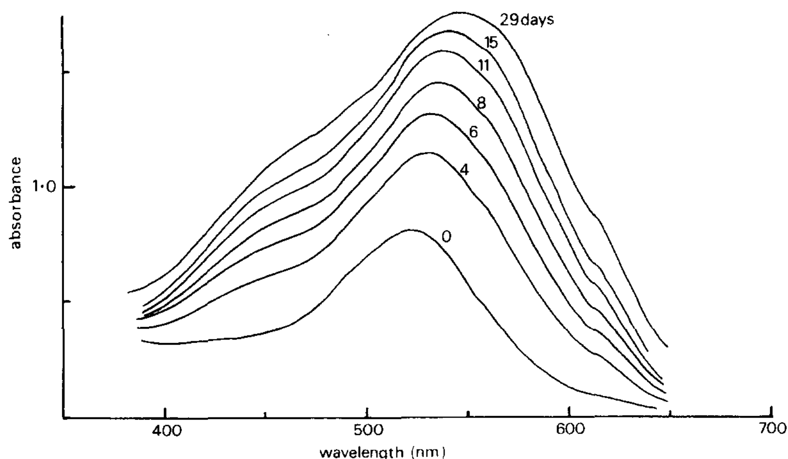


Fig. 7. Reaction of malvidin 3-glucoside, catechin and acetaldehyde.

applied to a crude mixture of elderberry pigments, the newly formed augmented pigments were much more coloured between pH 4 and pH 6 than those unchanged and should thus be more effective food colorants.

ANTHOCYANINS AS FOOD COLORANTS

The use of anthocyanins as natural food colour additives is now consolidated. The first approach is to add extractives of highly pigmented sources amongst which are:

(i) Grape skins or pomace, which has been extracted with alcoholic solvents, usually with addition of mineral acid, tartaric acid (Philip, 1974) or sulphur dioxide (Peterson & Jaffe, 1969).
(ii) Grape juice lees, particularly of the Concord grape (*Vitis labrusca*) (Wallin & Smith, 1977). The pigment is recovered by acid–alcohol extraction of the tank bottoms or filter-press cake (Calvi & Francis, 1978).
(iii) Roselle—a tropical *Hibiscus sabdariffa*—by hot water extraction of the calyces (Esselen & Sammy, 1975).
(iv) Cherry plum—*Prunus cerasifera*—by acid–alcohol extraction of leaves and skin (Baker *et al.*, 1974).
(v) Cranberry pomace—*Vaccinium macrocarpon*—by alcoholic extraction and purification using ion-exchange resins (Chiriboga & Francis, 1970, 1973).
(vi) Red cabbage and blueberries—by SO_2 extraction and a methanol extraction–ion exchange procedure (Shewfelt & Ahmed, 1977, 1978).
(vii) Bilberry—*Vaccinium myrtillus*. A French factory manufactures high grade anthocyanin from bilberries of the Central Massif; used for pharmaceutical purposes (Anon, 1975).
(viii) Miracle fruit—*Synsepalum dulcifum*—the pigment can be obtained as a by-product of production of the taste-modifier (Buckmire & Francis, 1978).
(ix) Flowers of *Clitorea ternatia*—Malaysian rice-cakes are coloured with extracts (Lowry & Chew, 1974).
(x) Other sources—*Vibernum dendatum* (pigment is 1 % berry fresh weight) (Francis, 1975) elderberries, blackberries, other fruit pomace, etc.

These products are likely to be of varying quality, some perhaps containing anthocyanins partly degraded or condensed or polymerised with other substances. They may contain other components simultaneously extracted, such as tannins. Some of these may act as co-pigments and enhance colours; others may be undesirable from the points of view of taste, stability or toxicology.

They present problems of: (a) identification—the types of pigment, simple or complex, and (b) quantitative measurement.

The anthocyanins are not the easiest compounds to measure for several reasons.

(i) Suitable, simple, pure standard compounds are not easily available and,

even if they were, they may not be very similar to the complex type of pigment which might be present in the extract.

(ii) Anthocyanins are difficult to measure independently of other flavonoids since they also react with the usual reagents used for phenolic analysis such as Folin-Ciocalteau, vanillin, etc.

(iii) Methods based on colour are possible, such as:

(a) differential methods—relying on differences in colour produced by pH change (Lees & Francis, 1972), bleaching by sulphur dioxide (Ribereau-Gayon & Stonestreet, 1965) or hydrogen peroxide (Swain & Hillis, 1959). It must be assumed that the reagents SO_2 and peroxide have no effect on other components (e.g. background brown colour),

(b) conversion of all chemical forms to the red flavylium cation by adding normal acid (Niketić-Aleksić & Hrazdina, 1972). This is suitable for the simple monomeric anthocyanins but condensed or polymerised pigments can give low colour response.

(iv) The best method would be to separate and measure the individual components by chromatography—low pressure liquid gel filtration on Sephadex or, better still, high pressure liquid chromatography. Such methods are being developed (Wulf & Nagel, 1978; Williams *et al.*, 1978).

However, even if one could measure accurately the amount of pigment present, this may bear little relation to the amount of colour perceived at the beverage pH because of operation of the effects which I have already discussed. The pigment extracts would boost the existing colour but, being of similar constitution, would tend to suffer the same fate as the original pigments of the foodstuff, although this could be minimised by adding at the last stage of processing.

The second general approach to anthocyanin food colours and the one which we are attempting at Long Ashton is to investigate and understand all factors affecting colour so that we can exploit natural media in which the anthocyanin structures are modified to advantage, e.g. flower, fruit and vegetable pigments and red wine. The background knowledge so obtained could then be used to manipulate extracts to produce natural pigments with required properties.

There is still a lot to learn about the mechanism of anthocyanin colour augmentation and stabilisation, but if sufficient effort is devoted to unravelling these effects, there would seem to be every prospect of developing pigments of improved characteristics, suitable as colour additives.

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OCCURRENCE, CHEMISTRY AND APPLICATION OF BETANIN

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ABSTRACT

Anthocyanins and betalaines are mutually exclusive in plants. The chief red pigment of beetroot is betanin. Its extraction gives low yield and a large proportion of sugar. This may be removed by aerobic fermentation. The chemistry of betanin and the synthesis of betanidin are described. Betanin is stable between pH 3·0 and 7·0; outside this range there are appreciable losses. Heating can also change the red to light brown. The presence of air and light accelerates degradation. Water also aids degradation, suggesting its use in foodstuffs of low moisture content.

INTRODUCTION

The two classes of pigment, anthocyanins and betalaines, in plants are mutually exclusive. Most plant orders have developed anthocyanins as the colouring matters of flowers, etc. Only one plant order, the Centrospermae, containing ten families, has elaborated the betalaines as red and yellow pigments, presumably serving the same purposes as the anthocyanins in the other plant families. The term 'betalaines' includes two classes of pigment—the betacyanins, which are red, and the betaxanthins, which are yellow. Betanin itself is the chief red pigment of beetroot, *Beta vulgaris*. Its structure, together with that of *iso*-betanin, is given in Fig. 1.

EXTRACTION OF THE COLOUR

Generally, the milled beet is acidified and the liquor separated, frequently by pressing or centrifugation. One report (Wiley & Lee, 1978) has appeared using an

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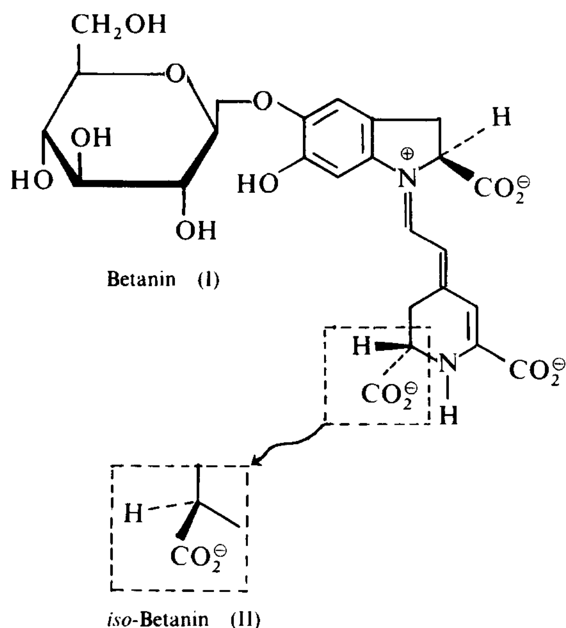

Fig. 1.

extraction similar to that used to obtain sugar from sugar beet. This is a countercurrent liquid/solid extraction which involves screwing beet cossettes up an incline against a counter-flow of acidified water. These processes give a liquor which contains betanin and other pigments, but also large quantities of other material such as sugar. Some processes take this liquor and spray-dry it, which gives a powder of about 1 % betanin concentration. Because of this low concentration and the special flavour of the product, for food colouring purposes it is desirable to remove the sugar and as much as possible of the other contaminants. One process (Adams *et al.*, 1976) utilises the aerobic fermentation of the liquor to remove the sugar as carbon dioxide. The product may be evaporated to a syrup and used in that form or spray-dried to give a powder, when it assays about 8 % betanin.

CHEMISTRY OF BETANIN

Although betanin had been under investigation in several laboratories for some years, it was not until 1957 that two independent groups first reported success in crystallising betanin. They were Wyler & Dreiding (1957) and Schmidt & Schönleben (1957). Both groups used an electrophoretic procedure for purification of the betanin.

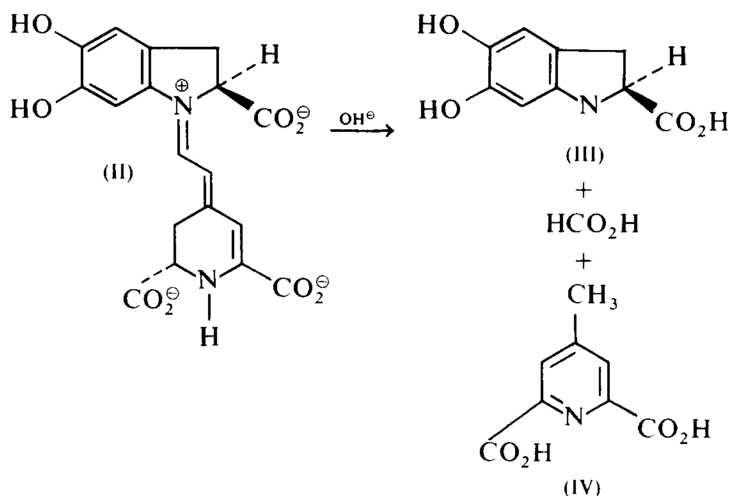

Fig. 2.

During the period 1959 to 1962 Wyler & Dreiding (1959) identified three products from the alkaline degradation of betanidin (II): these were 5,6-dihydroxy-2,3-dihydroindole 2-carboxylic acid (III) (otherwise known as *S*-cyclodopa), formic acid and 4-methylpyridine-2,6-dicarboxylic acid (IV) (Fig. 2). When put into the correct relationship with one another, these give the carbon skeleton of betanidin and also the configuration of the 2 carbon atom.

POSITION OF THE β-D-GLUCOSYL GROUP

The reaction of betanin (I) (Mabry *et al.*, 1962; Mabry *et al.*, 1967) with diazomethane in the presence of air gave the tetramethyl derivative (V) of the neobetanidin series which could be converted by hydrolysis and acetylation to 6-methoxy-neobetanin-trimethylester (VI). Neo-Betanin could be cleaved by alkali to give 5-hydroxy-6-methoxyindole 2-carboxylic acid (VII, R = H) (Fig. 3). The methyl ester of the same acid (VII, R = Me) was obtained from the acetylated material by degradation with alkali followed by esterification and oxidation. These reactions, taken together, demonstrated the position of attachment of the glucosyl residue. In addition, hydrolytic studies with β-glucosidase (Piattelli *et al.*, 1964; Wilcox *et al.*, 1965) and the NMR of betanin in trifluoroacetic acid demonstrated that betanin was an *O*-β-D-glucopyranoside.

The presence of the connecting vinylene group in the neobetanidin derivatives was confirmed by a palladium catalysed disproportionation reaction that converted the yellow 5,6-di-*O*-methylneobetanidin trimethyl ester (VIII) to the colourless 5,6-di-*O*-methyl-2,3-dehydro-11,12-dihydro-betanidin trimethyl ester (IX) (Fig. 4).

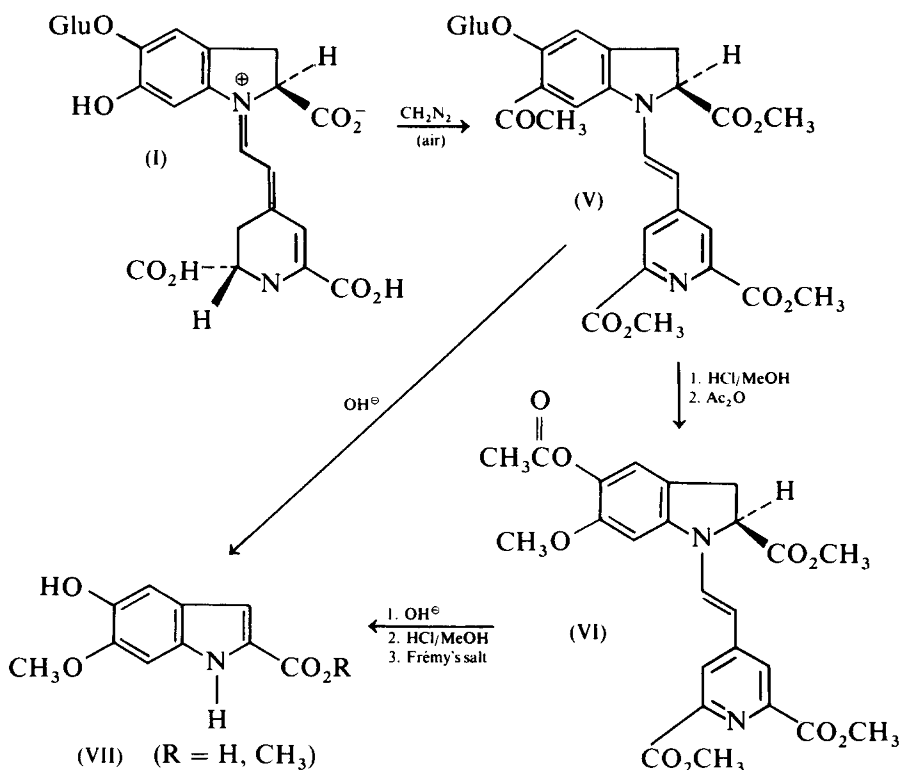

Fig. 3.

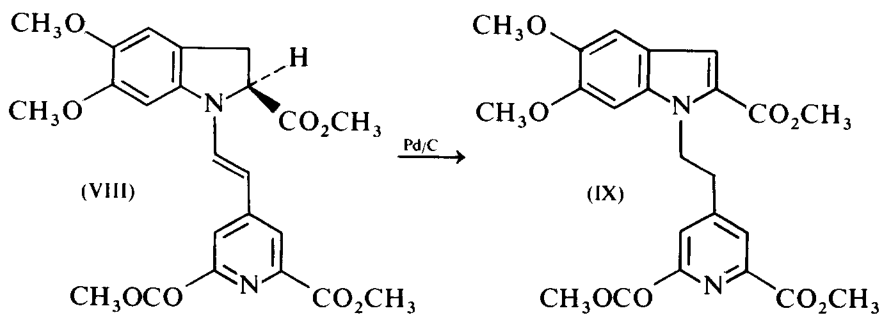

Fig. 4.

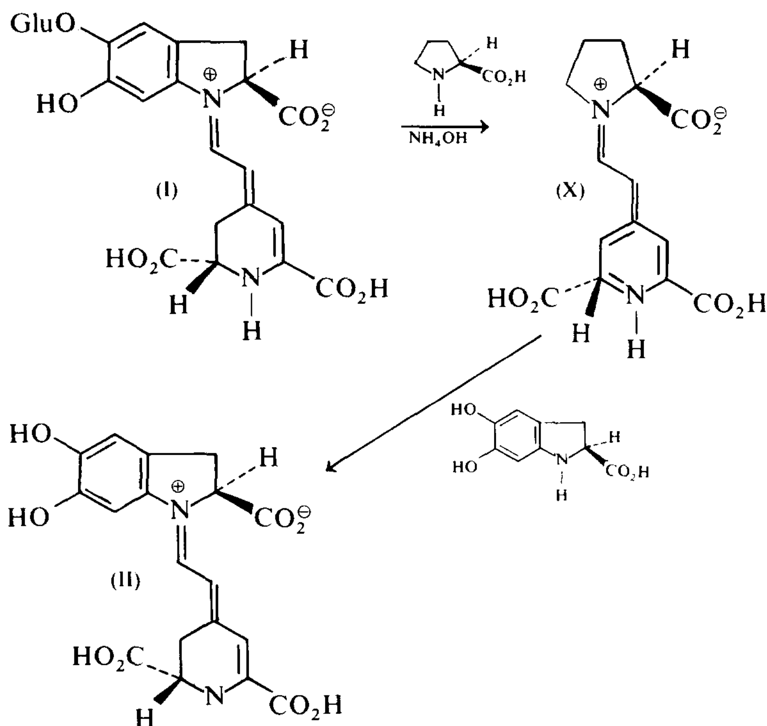

Fig. 5.

The only other reaction of betanin–betanidin to be considered here is its interconversion with indicaxanthin—a yellow betaxanthin. In the presence of dilute ammonia and excess L-proline, betanin (I) was converted to indicaxanthin (X). In the presence of excess *S*-cyclodopa, indicaxanthin was converted to betanidin (II) (Fig. 5).

TOTAL SYNTHESIS OF BETANIDIN (HERRMANN & DREIDING, 1975)

Hydrogenation of 4-hydroxypyridine-2,6-dicarboxylic acid (XI) followed by esterification gave the all *cis* products (XIII) shown (Fig. 6). The secondary hydroxyl group was converted to a ketone (XIII) using the Pfitzner–Moffat oxidation. Following this, the Horner–Wittig reagent gave the semi-carbazide (XIV) which was hydrolysed to an unsaturated ketone (XV). Another variation of the Pfitzner–Moffat oxidation was used to convert this to the betalamic acid (XVI). The reaction of this with L-cyclodopa methyl ester converted the betalamic acid to the betanidin trimethyl ester (XVII) which was hydrolysed with concentrated hydrochloric acid to betanidin (XVIII).

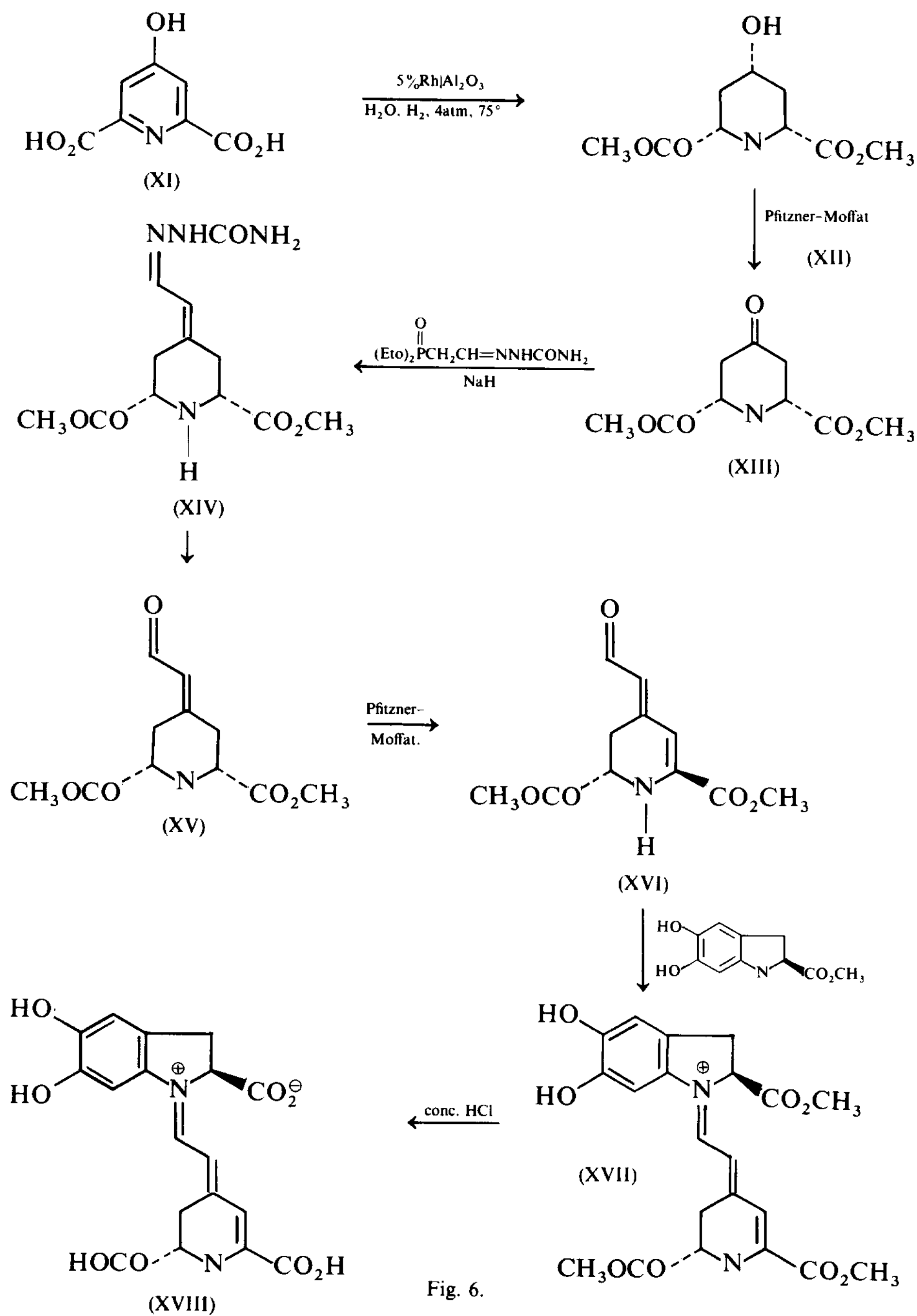

Fig. 6.

STABILITY OF BETANIN IN USE

Effect of pH (*von Elbe* et al., *1974*)

Purified betanin was stored for 7 days at 4 °C in buffers of pH 2–9. The visible spectra were measured at the beginning and end of this period. Between pH 4 and pH 7 no shifts in the betanin absorption maxima were seen. Figure 7 shows spectra in three buffers at pH 2, 5, and 9. Below pH 4 a shift of 2 nm to a shorter wavelength occurred and the intensity of absorbance decreased. The spectrum increased slightly

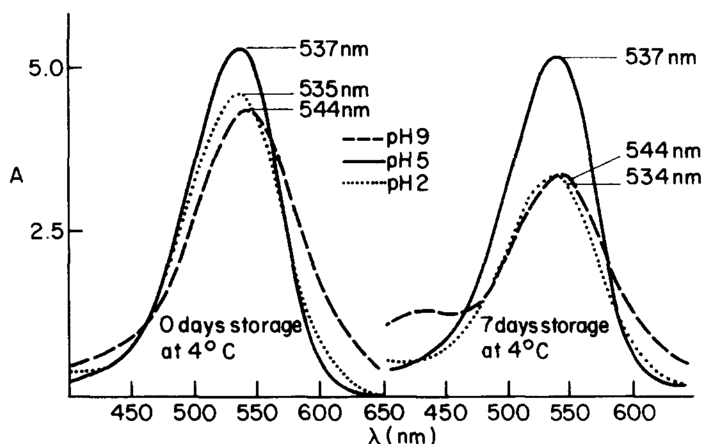

Fig. 7. Visible spectra of betanin at pH 2, 5 and 9. (From: von Elbe *et al.*, 1974).

in absorbance in the 575–650 nm region and the colour of the solution changed from red to red–violet.

Above pH 7 the absorption maximum shifted towards a longer wavelength (544 nm at pH 9) and the intensity decreased. A considerable increase in absorbance occurred in the 575–650 nm and 400–450 nm regions. The colour changed from red to violet. Storage had no effect on solutions between pH 3 and 7. pH values above and below these figures were associated with considerable losses of betanin. Most foodstuffs, however, would lie within the most stable range.

Effect of temperature (*von Elbe* et al., *1974*)

Heating betanin solutions caused the red colour to diminish and eventually turn light brown. The loss of colour was followed by the betanin assay and the rates indicated first order reaction kinetics as shown in Fig. 8. This graph, drawn for a temperature of 100 °C, shows that the rate of degradation at pH 5 is still less than it is at pH 3 and pH 7.

Figure 9 shows the half lives of betanin at pH 3 to 7 and for beet juice at pH 3, 5 and 7 when both were heated at 100 °C. Evidently, betanin is most stable between

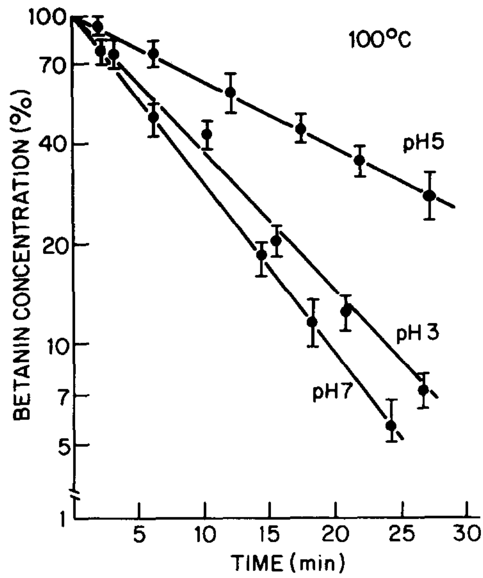

Fig. 8. Degradation rates for betanin in a model system at 100 °C at pH 3, 5 and 7. (From: von Elbe *et al.*, 1974).

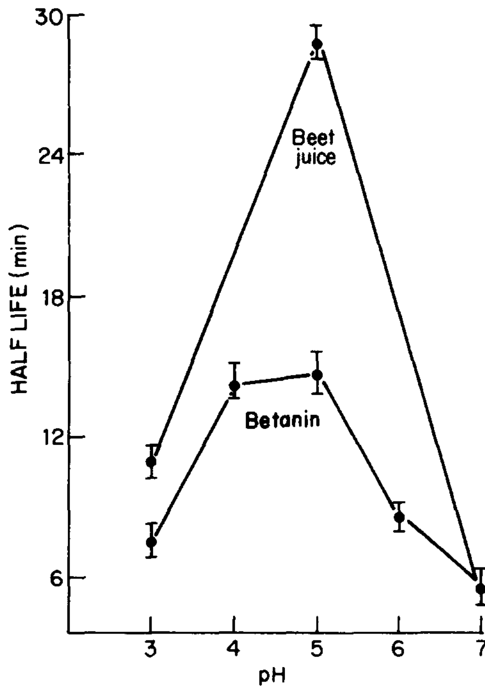

Fig. 9. Half-life for betanin in a model system and betanin in beet juice as a function of pH at 100 °C. (From: von Elbe *et al.*, 1974).

pH 4 and 5. At pH 5 betanin in beet juice is far more stable, which indicates a protective effect by the juice constituents.

Effect of light (*von Elbe* et al., *1974*)

At pH 7 at 15 °C, the presence of air (rather than nitrogen) increased the rate of degradation by 14·6 % and light by 15·6 %. Air and light together increased the rate by 28·6 %.

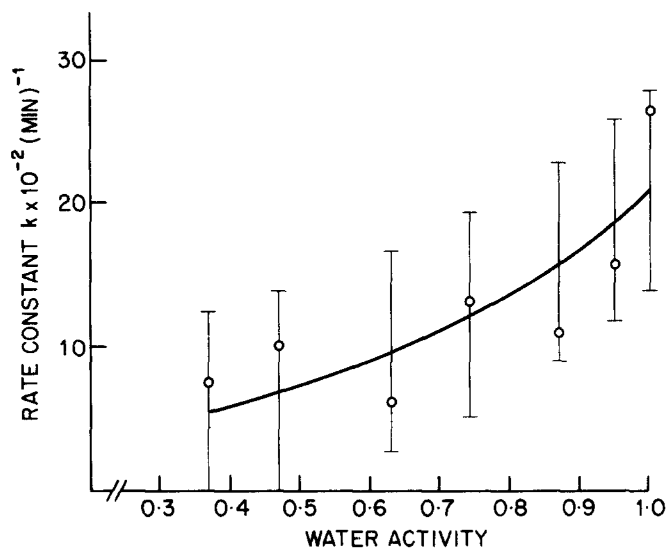

Fig. 10. Water activity versus rate constant for betanin degradation at 75 °C. 90 % confidence intervals are shown. (From: Pasch & von Elbe, 1975).

Water activity (a_w) (*Pasch & von Elbe, 1975*)

This is the ratio of the vapour pressure of water in the specific system to the vapour pressure of pure water at the same temperature. Pasch and von Elbe (1975) have looked at the behaviour of betanin in model systems composed of glycerol and water, the water activity being varied by altering the ratio of glycerol to water. Down to a water activity of 0·37 at 75 °C the rate constant for degradation of betanin decreased as the water activity was reduced. The half life of betanin at 75 °C is nearly four times greater at a_w 0·37 than at a_w 1·0. (Fig. 10). This suggests applications in foodstuffs with low or moderately low moisture contents.

Finally, although we have been considering the instability of betanin, the study of this was influenced by the knowledge that it was less stable than the materials it would replace. However, a sense of proportion must be kept, if the consumer demands natural colours in his food. Beetroots themselves have been sold for a long time in bottled form, both whole and sliced. Precautions are no doubt taken to

exclude air, but the clear glass of the bottle will certainly admit light, and the water activity related to the betanin is certainly high. In addition, such beets are generally cooked, which exposes them to high temperatures. Nevertheless, they are accepted in this form by a public to which the colour of fresh beet is familiar.

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EXTRACTION AND CHEMISTRY OF COCHINEAL

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ABSTRACT

The study of 'cochineal' for use in food involves a wide range of unexpected disciplines. The importance of the historical, geographical and entomological backgrounds is emphasised. The chemistry of carminic acid and related hydroxyanthraquinones is shown to have been confused by the early work of Liebermann & Voswinckel (1897), but evidence for the structure of the anthraquinone nucleus of carminic acid is given. The biosynthetic mechanisms in each species remain a task of considerable proportions. The chemistry of the carmines of cochineal is also described, including the ability of carminic acid to complex with metals, especially aluminium. Amino acid analyses of commercial carmines are given.

INTRODUCTION

Anyone wishing to gain insights into aspects of the commercial exploitation of products derived from 'cochineal' for food and beverage uses should be prepared to enter a field embracing not only the physical, chemical and biological sciences but also a range of other disciplines including archaeology, economics, geography, history, linguistics and even the visual arts. No short paper could hope to do justice to this intricate and fascinating subject.

My own interests originated in the late 1960s whilst I was heading a group at the Queen's University of Belfast which was seeking compounds which might be used to probe the 'active centres' of some enzymes of carbohydrate metabolism and, in particular, certain glycosidases and carbohydrate sulphatases. One of the most promising candidates examined was carminic acid, the major (but perhaps not the only) pigment of 'American cochineal'. It must be admitted that carminic acid was then viewed as consisting of a very large glucose unit to which was attached, purely

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for convenience, an anthraquinone moiety possessing all those characteristics ideally suited to spectrophotometric and spectrofluorimetric microanalysis. The major problem lay, however, in isolating a completely pure preparation of the compound, a difficulty which has persisted even to the present day.

The interest generated at that time has expanded considerably and has been invaluable in guiding those toxicological studies on the commercial products known as the 'carmines of cochineal' and, not the least, in attempting to devise a meaningful specification describing the constitution of the articles of commerce for regulatory purposes in the United Kingdom and the European Economic Community. This paper will attempt a broad review of the current state of knowledge and will describe some recent advances in the extraction and chemistry of cochineal.

HISTORICAL, GEOGRAPHICAL AND ENTOMOLOGICAL BACKGROUND

Present day 'cochineal' products should not be considered in isolation. The source from which they are derived is but one of the many thousand species of insect comprising the superfamily *Coccoidea*. In common with the related superfamily *Aphidoidea*, the coccid insects have a world-wide distribution, each type living in close association with specific forms of plant life. In some respects they might be classified as pests and, by way of illustration, Kosztarab (1977) has estimated that losses in the field and extra production costs in agriculture attributable to damage resulting from coccoid infestation exceeds $500 million annually in the United States alone. Conversely, many species are valued as agents for the biological control of unwanted plant species.

From the standpoint of entomology the 'scale' insects are unusual and unique in many ways (Miller, 1977). The male coccoids are described as being pauro-metabolous with an anomalous complete metamorphosis, while the female insects are held to be 'reproductive factories' that are normally sessile and are neotenic.

Man has, from time immemorial, exploited the female of the species of many of these insects as a source of pigments capable of competing favourably with materials of molluscan or pure vegetable origin. As matters now stand, physical and chemical studies have revealed the structures of 26 of the compounds isolated from the insects to date. The pigments are often accompanied by a number of colourless, frequently fluorescent, materials, related spectroscopically to the coloured components (Banks, 1977). The insects have also been used for an equal length of time as sources of waxes and resins.

A full understanding of the geographical and historical bases underlying the use of coccid pigments generally is essential to a complete appreciation of the present day availability of cochineal and to a knowledge of those factors which have contributed to an understanding of its chemistry. Two excellent reviews by Donkin (1977*a*,*b*) dealing with ethnogeographic factors are available. In brief, however, prior to the Spanish conquest of Central and South America the insect pigments of

the 'Old World'—effectively Europe, North Africa, the Middle East and Asia—were 'Armenian Red', 'Kermes', 'Polish Cochineal', the 'Lac dyes' and materials of Central Asian origin—about which very little is known—called 'Pe-La'. (Striking a cautionary note, the taxonomy of the *Coccoidea*, based almost exclusively on the anatomy of the female insect observed after death, has yet to be settled unequivocally and if, in the following annotations, one of those synonyms not currently in vogue amongst 'coccidologists' is used inadvertently, corrections from readers will be greatly appreciated):

(*a*) *Armenian Red*

Prepared from the species *Porphyrophyra hameli*, a coccoid growing on the roots and lower stems of a number of grasses, including *Aeluropus laevis* (syn. *Dactylis littoralis*, *Poa pungens*). From the standpoint of geography, it appears that the distribution of *P. hameli* is restricted to those wet and alkaline regions favouring the growth of the host plant in Azarbaijan and Armenia, particularly in the region of Mount Ararat.

Donkin (1977*a*) surmises that the term 'kirmiz' from *krmi* (Skt. = worm or insect) was first applied to Armenian Red prepared in remote antiquity from *P. hameli* and only subsequently to those products from *Kermes* sp. (syn. *Kermococcus* sp.).

(*b*) *Kermes*

Usually claimed to be the most important of the products obtained from the 'Old World' insects from the standpoint of trade, and is prepared from *Kermes ilicis* (syn. *Kermococcus vermilis*) which breeds exclusively above ground on various species of *Quercus* (particularly *Quercus coccifera*, the 'kermes oak'). With a pattern of use traceable to at least Neolithic times, the name 'kermes', or some earlier variant, is thought to provide the etymological stem for 'crimson', 'scarlet' and possibly 'carmine', while *vermilis* provides an obvious root for vermilion.

Although traded from earliest times, kermes enjoyed the highest reputation and was most widely employed between the 12th and 17th centuries, only to be superceded as an article of commerce in Europe by the introduction of 'American cochineal' (Donkin, 1977*a*).

(*c*) *Polish cochineal*

Extracts of *Margarodes polonicus* (syn. *Porphyrophora polonica*), an insect found on the roots of *Scleranthus perennis*, a grass flourishing in sandy areas of Central and Eastern Europe, have been used as pigments from earliest times (Donkin, 1977*a*). Obviously, harvesting of Polish cochineal is both arduous and time-consuming as the host plants must be lifted for the collection of the insects (with a yield of some 40 to 50 insects per plant) and each plant then replaced to provide future yields. Nevertheless, correspondents in Poland report a revival of interest (albeit somewhat academic at the present time) in the exploitation of this material.

(*d*) *Lac dyes*

The 'lac' insects have been valued historically both as sources of pigments and of resins, the latter providing the basis for traditional 'shellac'. The insects appear to have a geographical distribution embracing the Indian subcontinent through to Malaysia and the species *Laccifera lacca* is usually recorded as being the most important. The host plants in this case are trees, including *Schleichera oleosa*, *Zizyphus mauretania* and *Butea monosperma*. Although probably still most highly prized as a source of shellac rather than colours, commercial use of the latter on a significantly large scale must remain an area for future exploration, not least at the level of toxicological acceptability.

The insect pigments of the Old World seem to have been used largely as dyestuffs for various textiles, leather goods and art works. However, it is well known that 'kermes' was widely used in medicines both for internal and external applications. *Confectio alchermes* was prescribed as a heart stimulant from about the end of the 8th century and continued to find a place in works on *materia medica* until the late 18th century (Donkin, 1977*a*; Baranyovits, 1978). There is mention of the fact that in Eastern Europe at least the use of 'kermes' for this purpose was replaced by extracts of 'Polish cochineal'.

The Spanish conquest and colonisation of Central and South America heralded a new era in the exploitation of insect pigments, providing Europe with the first imports of 'American cochineal' as used today. According to Donkin (1977*b*), 'Cochineal was the most widely traded and, next to gold and silver, the most valuable product of the Spanish Indies'.

'American cochineal' insects are members of the family *Dactylopiidae*, the major species being *Dactylopius coccus* Costa, living as parasites on the fleshy aerial portions (phylloclads) of various cacti belonging to two closely related genera, *Opuntia* and *Nopalea*, and particularly *N. cochenillifera* (Wright, 1963; Donkin, 1977*a*,*b*; Baranyovits, 1978). The females live in close juxtaposition to one another on the phylloclad, with feeding mouthpieces inserted into the surface of the cactus, and are covered by a protective layer of wax. Harvesting is still undertaken by hand and the insects are usually dried in the open, although accelerated drying may be used in certain areas.

Fully developed females are roughly 6 mm long, 4·5 mm in width and 4 mm in height, weighing in the region of 45 mg, but losing approximately 70 % in weight on drying. There are in the region of 80–100,000 insects in each kilogramme of 'raw' dried cochineal as exported. Commercial cochineal is available in two forms, described as 'silver' and 'black', of which the former is the natural insect obtained by gradual open-air drying, while the latter may be produced by accelerated drying or by solvent extraction.

Present world production depends on those geographical conditions which are required to grow and maintain the host plant, overlaid by certain historical events. Obviously following the route of the Spanish conquest, earliest supplies in Europe

were of Mexican origin. Donkin (1977*b*) has traced the development of what was a casual industry in pre-colonial times, with a major centre near Oaxaca, to an actively sponsored industry, involving 'domestication' of the insect, as opposed to its collection in the wild, in the period from the early 17th century to the mid 19th century. However, attempts to raise cochineal outside the traditional growing areas of pre-colonial times in Central America met with little success, except possibly in Guatemala. The post-colonial period has been marked by a significant decline in cochineal production in Central America and, although attempts have been made to revive the Mexican industry (Wright, 1963), output remains negligible in the terms of present-day world trade.

Although the matter cannot be resolved completely (Donkin, 1977*b*) records the view that, contrary to popular belief, cochineal was used from pre-Hispanic times in South America and was not an artefact resulting from the Spanish conquest of Peru. Western and southern regions of South America have climatic conditions ideally suited to the growth of members of the family *Opuntioideae* and Peru remains the world's largest producer of raw cochineal with annual yields in the range of 150–200 tonnes. Attempts are being made to increase this by intentional cultivation (Oscar Rizo Patron, pers. comm.)

Proposals to raise cochineal in areas other than Central and South America featured amongst the colonial ambitions of the British, French and Spanish during the 16th to 19th centuries, an additional stimulus being provided by the decline of the Spanish–American Empire. In consequence, the host cacti and the insects which they support now have a world-wide distribution. However, striking success in commercial cochineal raising was restricted to the Canary Islands with exports of the dried insects rising from a few kilogrammes in 1831 to a peak of over 3000 tonnes in 1875. Cochineal of high quality is still available from the Canary Islands, but collection is now almost exclusively casual and supplies are irregular, the best available information suggesting an annual output in the region of only 10–15 tonnes.

Viewed overall, current world production is a mere fraction of that which was available in the 16th to 19th centuries. Moreover, with a purchase price for Peruvian cochineal in the region of £10 per kilogramme and a market price of £95 approximately per kilogramme for carmines (higher in the United States) current processing can be considered as providing little immediate prospect of direct competition with the synthetic organic colours either in terms of yield or price. Even so, an excellent technological performance in a variety of foods and beverages merits increasing attention being paid to the insect pigments by industry.

With the above in mind, and from the standpoint of historical precedence, there are few direct references to the use of 'American cochineal' in foods and beverages by the indigenous populations of Central and South America, although Donkin (1977*b*) does record a mention of its use in the preparation of *Tamales*. What is known with certainty is that, in common with 'kermes' and 'Polish cochineal',

extracts of 'American cochineal' as medicines to relieve ailments of the head, heart and stomach were first acclaimed in Europe by a physician to Philip II of Spain and were used for this purpose until the middle of the 19th century. There is little doubt that these practices have their origins in American Indian recipes (Donkin, 1977*b*).

THE CHEMISTRY OF CARMINIC ACID AND RELATED HYDROXYANTHRAQUINONES

The biosynthetic origins of the pigments of American cochineal—and indeed of the other insect pigments—remain to be studied at some future date. In the case of American cochineal, the female insects are collected at sexual maturity, at an age of 90–110 days, just before egg-laying begins, when the concentration of pigment is at its highest. Indeed, on dissection and microscopic examination of freshly collected specimens highest concentrations of the colour appear to be associated with the egg yolk, to be incorporated later into the tissues of the embryo (Baranyovits, 1978). However, in view of the high concentrations which are subsequently detected in the mature female, it must be presumed that colour biosynthesis continues during the remaining phases of the development of the organism.

The carminic acid content of a good quality raw cochineal, determined by a standard spectrophotometric procedure, can reach a remarkable 22% of the dry weight of the insect. Much of the carminic acid can be extracted by treatment of the intact dried insects with hot water, but yields can be increased if the dry preparation is powdered, even though subsequent stages of purification become more difficult. In both cases variable amounts of the pigment remain 'bound' to the insoluble residues.

Traditionally, isolation of a 'pure' carminic acid depends on its ability to form an insoluble complex with lead (see Perkin & Everest, 1918 for historical review) and methods based on lead precipitation still appear to be used in the preparation of carminic acid for histological purposes even today. Recent work has established that even better yields can be obtained if the ground insect is treated with aqueous solutions of proteolytic enzymes in the presence of suitable surfactants and that purification is greatly simplified by using ion-exchange chromatography (A. G. Lloyd, to be published). However, with due regard to studies by TLC and HPLC, the author and his colleagues are left to question whether carminic acid has ever been isolated in a truly pure state.

Much of our knowledge of the structure of carminic acid and related compounds results from the original studies of workers of the classical German schools of natural product chemistry and particularly those of Liebermann and Dimroth and their respective co-workers. Although the detailed conclusions drawn by these earlier researchers have been modified in the light of later work (Ali & Haynes, 1959; Overeem & van der Kerk, 1964; Bhatia & Venkataraman, 1965) incorrect structural representations have continued to appear in the literature with astonishing regularity (see, for example, Kirkbright *et al.*, 1966; Mottier, 1974 and Reisfeld *et*

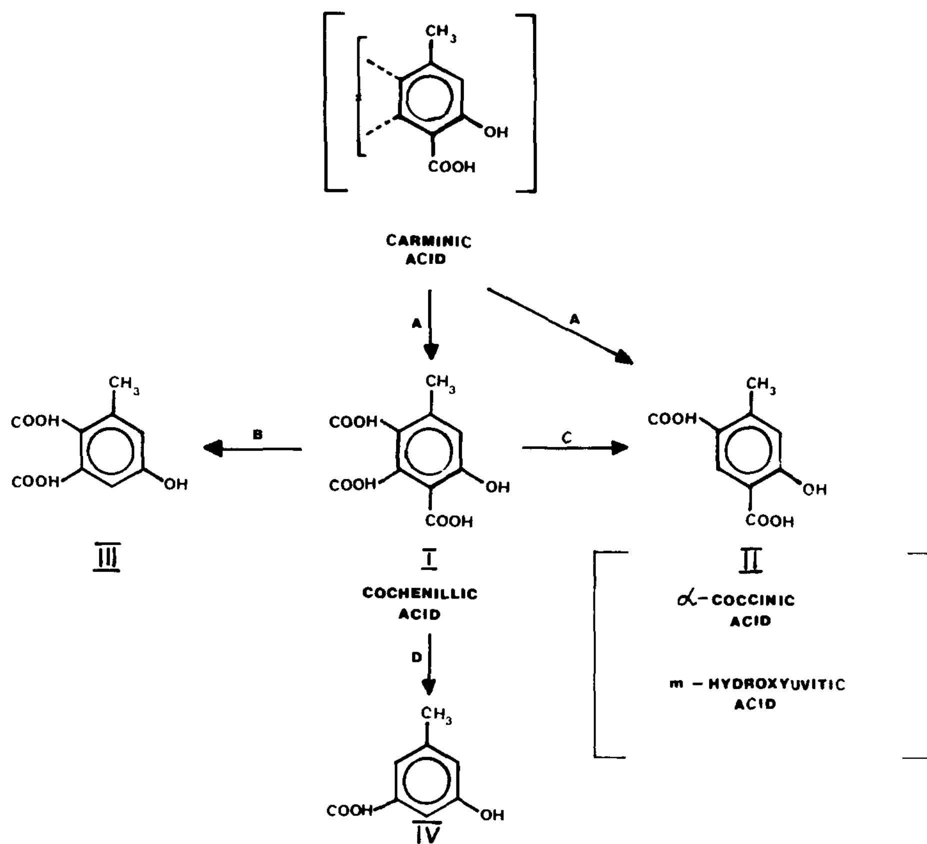


Fig. 1. Structures proposed by Lieberman & Voswinckel (1897) for products obtained after the degradation of carminic acid. Reaction A, oxidation with potassium persulphate. Reaction B, sublimation. Reaction C, 170 °C in water for 1 h. Reaction D, 200–210 °C in water for 2–3 h.

al., 1975), causing considerable confusion. Moreover, while excellent reviews of the subject exist (e.g. Haynes, 1963; Thomson, 1971, 1972; Venkataraman & Rao, 1972; Brown, 1975), many workers, particularly in the food and beverage industries, appear to be unfamiliar with these and this opportunity is taken to give a brief résumé of the present state of knowledge, intended to correct current misconceptions.

Present confusion has its origins in the work of Liebermann & Voswinkel (1897) on products obtained following the oxidation of carminic acid with potassium persulphate (see Fig. 1), yielding a mixture of the tricarboxylic acid, cochenillic acid, and the dicarboxylic acid, α-coccinic acid. On the basis of rather tenuous evidence, these workers considered α-coccinic acid to be identical with the known *m*-hydroxyuvitic acid, and for this reason α-coccinic acid was formulated as shown in

Fig. 1 (structure II). Moreover, since cochenillic acid could be decarboxylated to α-coccinic acid by heating with water at 170 °C, it was represented as the precursor of α-coccinic acid as shown in Fig. 1 (structure I). Two products obtained during either the sublimation of cochenillic acid or heat treatment at 200–210 °C in water were also studied and were assigned structures III and IV, respectively. Two of the carboxyls in cochenillic acid were presumed to result from the oxidation of an adjacent ring structure in carminic acid and, consequently, the 'A' ring of carminic acid was formulated showing a single carboxyl in the 4 position (see Fig. 1).

Unfortunately, this misconception was perpetuated in later studies by Dimroth and his co-workers (Dimroth, 1909; Dimroth, 1910; Dimroth & Scheurer, 1913; Dimroth *et al.*, 1913; Dimroth & Fick, 1916; Dimroth & Kämmerer, 1920) on both carminic acid and kermesic acid, the major pigment of 'kermes'. Nevertheless, Dimroth's work on the coloured products, coccinin and coccinone, obtained after the fusion of carminic acid with potassium hydroxide, did establish that a reversible anthrone–anthraquinone relationship existed between these compounds, indicating that carminic acid itself contained an hydroxyanthraquinone nucleus. Meanwhile, in concurrent investigations on kermesic acid it was proposed that the quinone nucleus was in fact a substituted hydroxyanthrapurpurin (1,3,4,6-tetrahydroxy-anthraquinone).

Consequently, from the similarity of their visible spectra and 'dyeing' properties Dimroth & Fick (1916) concluded that carminic acid and kermesic acid had essentially similar anthraquinone nuclei, even to the same pattern of hydroxylation.

The conclusions drawn by Liebermann and Dimroth remained in vogue until questioned by Overeem & van der Kerk (1964). These authors were of the opinion that it was 'highly improbable that heating of cochenillic acid with water at 200–210 ° causes the elimination of carboxyl groups *ortho* and *para* to the hydroxyl group, while a treatment in water at 170 ° should result mainly in the removal of a carboxyl *meta* to the hydroxyl group'. Furthermore, they pointed out that if cochenillic acid is correctly formulated, as shown in Fig. 1, then it should be identical with mollisic acid which they had obtained from the natural compound, mollisin, by oxidation. They established that this was not the case.

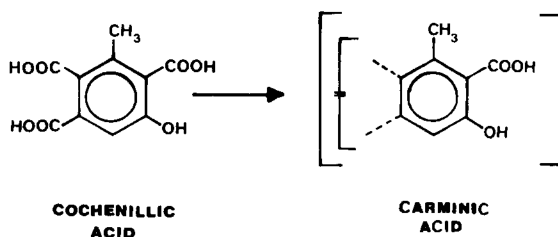


Fig. 2. Corrected structure for cochenillic acid and revised formulation for carminic acid 'A ring' according to Overeem & van der Kerk (1964).

The problem was resolved, however, if cochenillic acid were formulated as shown in Fig. 2, a situation shown to be correct by two syntheses of the cochenillic acid methyl ether (Overeem & van der Kerk, 1964; Bhatia & Venkataraman, 1965). The 'A ring' of carminic acid is thus correctly formulated with the carboxyl in the 2 position—a state of affairs substantiated by NMR studies on carminic acid itself (Bhatia & Venkataraman, 1965).

Accumulated evidence points to the fact that the anthraquinone nucleus of carminic acid has the structure shown in Fig. 3, with the carboxyl in the 2 position.

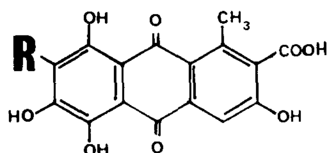
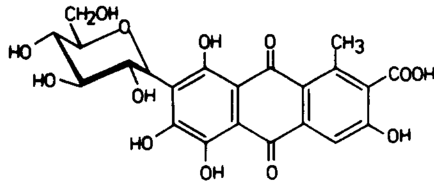


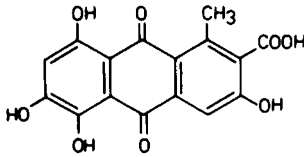
Fig. 3. Presently accepted structure for the anthraquinone moiety of carminic acid.

The exact nature of the substituent grouping at position 7 of the anthraquinone moiety baffled earlier workers although they surmised correctly, on the basis of acetylation studies, that it was likely to be carbohydrate in character. It was left to Ali & Haynes (1959) to establish that the substituent was, in fact, a glucose unit linked to the hydroxyanthraquinone by a (then) unusual *C*-glycoside bond, akin to that found in barbaloin, a natural product obtained from various species of *Aloe* (Haynes, 1963, 1965). In common with other *C*-glycosyl compounds, the glucose link in carminic acid exhibits a remarkable resistance to acid hydrolysis, while the glucose unit itself is not susceptible to modification by many enzymes of carbohydrate metabolism (A. G. Lloyd, unpublished work). The potential of carminic acid as a tool for studies on the 'active centres' of various enzymes normally involved in the metabolism of carbohydrates remains to be explored. In summary, therefore, the structure of carminic acid is correctly described as 7-*C*-D-glucopyranosyl-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracenecarboxylic acid. The stereochemical configuration of the *C*-glycosyl bond, which must be of considerable significance in determining the ability of carminic acid to form complexes with a variety of metals, has yet to be determined, although it is usually presumed to be 'α'.

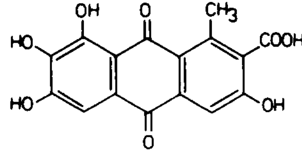
Not surprisingly, there has also been a considerable amount of work on the pigments of other coccid insects (Thomson, 1971, 1972; Venkataraman & Rao, 1972; Brown, 1975; Banks, 1977) and especially 'kermes' and the 'lac dyes'. Much of the most recent information has been obtained by Venkataraman and his colleagues who have established that the pigments adhere to a common structural pattern based on related hydroxyanthraquinone moieties (see Fig. 4). From this it will be obvious that kermesic acid is, in fact, the aglycone of carminic acid. Ceroalbolinic acid, the major pigment produced by *Ceroplastes albolineatus*, growing on the shrub



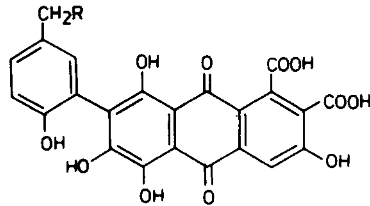
Carminic Acid



Kermesic Acid



Ceroalbolinic Acid



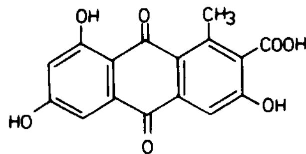
Laccaic Acids

A ; R----CH_2-NH-$COCH_3$

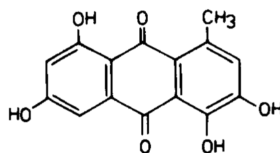
B ; R----CH_2OH

C ; R----CH(NH_2)COOH

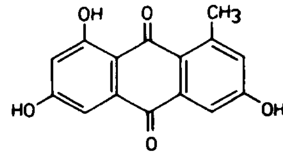
E ; R----CH_2-NH_2



Laccaic Acid D



Erythrolaccin



Deoxyerythrolaccin

Fig. 4. Structures of some of the pigments of the *Coccoidea*.

Senecio praecox, is an isomer of kermesic acid. Evidence has also been obtained that the pigments of 'Polish cochineal' obtained from *Porphyophora polonicus* contain a mixture of kermesic acid and carminic acid, while a material resembling carminic acid has been demonstrated in extracts of *Eumargarodes laingi* (Maskell) although the nature of the sugar has not been established conclusively (Banks, 1977).

With these structural relationships in mind, it is left to question why extracts of cochineal replaced kermes preparations as the major pigments of commerce in the 16th to 18th centuries. Was it a case of superior colour quality and stability (Brunello, 1973; Donkin, 1977*b*) or, more simply, due to the fact that the kermes insect is both difficult to collect and has only one generation each year, while 'American cochineal', apart from living superficially on the plant—a fact which greatly eases collection—also matures in 90–110 days, providing the potential for up to four harvests a year? Work currently in progress, using preparations of kermesic acid obtained by a new method of chemical synthesis (A. G. Lloyd, unpublished work), is designed to examine at least some of those physical and chemical properties of the two anthraquinones which have a bearing on technological performance.

The 'lac dyes', considered by some in the United States as possible alternatives to synthetic organic 'reds' in food and beverage applications, emerge as a complex mixture of pigments (see Fig. 4). The laccaic acids, designated A, B, C and E, differ from kermesic acid and carminic acid in that the 1-methyl is replaced by a carboxyl while the substituent in position 7 is aromatic in character—in the three instances, A, C and E, having a nitrogen-containing side chain. The latter observation inevitably raises the possibility that in nature at least some of the pigments may be linked covalently to protein (see later). Apart from the laccaic acids, A, B, C and E, the pigments of *Laccifer lacca* also contain laccaic acid D, which is substantially a 5-deoxy kermesic acid, as well as erythrolaccin and deoxyerythrolaccin which possess the 1-methyl function but are devoid of the 2-carboxyl.

All the hydroxyanthraquinones shown have been isolated during studies on the raw articles of commerce and it may well be that the true state of the pigments in nature has not been reflected. It will be obvious that the primitive methods of collection, drying, processing and shipping could well favour a variety of *post mortem* changes yielding artefacts. Consequently, in view of the precedents established by D. W. Cameron and H. J. Banks (see Banks, 1977) studies on insects freshly collected in the field and treated specifically to obviate degenerative changes are now highly desirable. On the other hand, Banks (1977) has theorised that each of the coccoid pigments could arise by a series of routes originating from an acetate–malonate or polyketide pathway of biosynthesis. Following cyclisation of the polyketide product, the immediate precursor in the case of those pigments of commercial interest was suggested to be a 1-methyl anthrone derivative from which, by subsequent oxidations, decarboxylations and conjugations with amino acids or carbohydrates, all the known structures could be produced. However, as it is still not known whether the pigments are produced in the tissues of the insects alone or in

association with the various endosymbionts present in each species (see Tremblay, 1977 for most recent review), elucidation of the biosynthetic mechanisms in each case will be a task of considerable magnitude.

THE CHEMISTRY OF THE CARMINES OF COCHINEAL

At the moment purified preparations of carminic acid are not major articles of commerce. While commercial preparations are available on a small scale these are expensive and are most usually used as biological stains or analytical reagents. Improved methods of extraction and purification might yield material at a cost which the food and beverage industries could find attractive but, in the light of present experience, some time is likely to elapse before this objective will be achieved. Similar considerations apply to the commercial preparation of carminic acid by methods of chemical synthesis which have yet to be devised, complicated by the fact that, while the concept of 'nature identity' might be invoked, this is unlikely to achieve favour with any future body of toxicological opinion, with the consequent requirement for the complete spectrum of costly and time-consuming routine testing in laboratory animals. As such, industry will need to rely for the foreseeable future on those materials known generically as the 'carmines of cochineal' that have largely replaced the simple 'extracts of cochineal' which have their origins in prehistory (Donkin, 1977*a*,*b*).

Solutions of carminic acid have little intrinsic colour below pH 7 (where the quinone ring has greatest photostability) and in the region of pH 4 have a 'pale straw' tint depending on concentration. However, carminic acid shares with other quinones, such as alizarin (see Keil & Heertjes, 1963), the property of forming complexes with several metals, a state of combination which is frequently accompanied by a shift in the position of maximum absorption in the visible range to higher wavelengths (Jain, 1960) and apparent increase in colour intensity when viewed by eye. Technologically speaking, the most 'brilliant' colours are produced with tin and aluminium.

The ability of carminic acid to complex with metals is exploited in the manufacture of carmines, although the origins of the technique are lost in antiquity. Many 'recipes' for the preparation of carmines were published at the turn of the present century (Bersch, 1901; Harrison, 1930) but details of current commercial procedures used by individual manufacturers are jealously guarded trade secrets. Processes are designed to obtain the maximum colour yield and the best pigmentation properties at the minimum cost, obviously affecting technological performance and competitive pricing in the marketplace. Carmines may be isolated in forms directly soluble in water-based solvents over a wide pH range or (depending on concentration) in forms insoluble below pH 7. In commercial practice, however,

'insoluble carmines' may be solubilised by treatment in aqueous media above pH 7·5 before addition to a food or beverage.

Viewed broadly and depending on the skill of the manufacturer, some 4–5 kg of raw cochineal are required to produce 1 kg of commercial carmine. Processes may involve either batch or continuous extraction of raw cochineal at temperatures between 90° and 100° in aqueous media before clarification of the extract. Subsequent stages of the process always involve treatment of the extract with an aluminium salt. According to the intended end-use, the resultant complexes may be treated with ethanol at high concentration to precipitate a 'soluble' carmine or, alternatively, they can be isolated in that form which is 'insoluble' below pH 7 by the addition of a calcium salt to the final solution. Although more expensive to produce on a commercial scale, the 'soluble' carmines have certain distinct technological advantages, not least in their solubility over a wide pH range even in aqueous ethanol mixtures. Moreover, my colleagues and I have shown that by simply adjusting the carminic acid/aluminium ratio it is possible, at least on the laboratory scale, to produce soluble colours ranging from pale 'strawberry' to near 'blackcurrant', certainly covering the spectrum of 'reds' normally preferred in foods and beverages. Our recent work has also established that a considerable reduction in the cost of producing carmines soluble over a wide range of pH, coupled with a more precise adjustment of colour for a variety of applications, can be obtained using spray-dried products both with and without the incorporation of other approved food additives as diluents and carriers. Some of the latter greatly increase the photostability of the pigment at pH values above 7 in the presence of oxygen (A. G. Lloyd, unpublished work).

At the same time, it should be borne in mind that in future commercial practice it is proposed that the 'carmines' of cochineal will be expected to conform to the minimum specification summarised in Table 1 if they are intended for food use in the United Kingdom, with the prospect of the endorsement of these proposals by other members of the European Economic Community. It is not clear how the proposed specification for food use will affect alternative applications such as in

TABLE 1
TENTATIVE SPECIFICATION PROPOSED FOR THE 'CARMINES OF COCHINEAL' INTENDED FOR FOOD AND BEVERAGE USES IN THE UNITED KINGDOM

Description	Red to bluish-red pieces or powder
Content	Not less than 50 % of carminic acid by absorption at 494 nm in dilute hydrochloric acid
Proteinaceous material (non-ammoniacal N × 6·25) derived from *D. coccus*	Not less than 15 % and not more than 25 %
Volatile matter	Not less than 10 % and not more than 18 % after drying at 105 °C to constant weight
Ash	Not more than 15 % determined at 800 °C
Matter insoluble in dilute ammonia	Not more than 1 %

cosmetics (including those which may be ingested) and pharmaceuticals (OTC or otherwise).

There can be no doubt that the key to the technological performance and pigmentation properties of all carmines is the presence of carminic acid/metal complexes, most usually now involving aluminium. The physical and chemical factors underlying the formation and eventual structure of these complexes have intrigued many workers. As might be expected, the majority of views have their

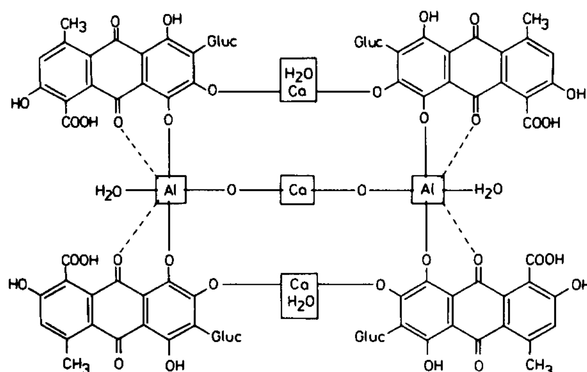


Fig. 5. Suggested structure for a fully complexed calcium/aluminium carminic (after Harms, 1957*b*).

origins in studies on the corresponding complexes of alizarin (see Keil & Heertjes, 1963*a* for historical background). The structure of an 'insoluble' fully complexed calcium/aluminium derivative of carminic acid shown in Fig. 5 is due to Harms (1957*a*) and is based on the Rutishauser (1940) representation of the calcium/aluminium complex of alizarin. Harms (1957*b*) proposed a marginally modified version but this retained many of the features introduced earlier. Later studies on the metal complexes of alizarin and some of its derivatives (Keil & Heertjes, 1963*a*,*b*,*c*,*d*) and studies by Meloan *et al.* (1971) on the carminic acid/aluminium complex called this view into question, not least because it uses the outmoded structure for carminic acid. Exercises with molecular models (Meloan *et al.*, 1971) established that the presence of a carboxyl in the 4 position would, in fact, prevent complex formation through steric hindrance. This was not the case if the carboxyl were inserted in the correct 2 position in a model of the carminic acid complex based on the alizarin/aluminium derivative as represented by Keil & Heertjes (1963*a*). On the other hand, Meloan *et al.* (1971) were careful to point out that the structure favoured by them, involving chelation of two molecules of carminic acid by one of aluminium (see Fig. 6), is speculative and that further confirmation must await experimental work on the metal complexes of a pure carminic acid. In the interim, preliminary evidence has been obtained that while 'insoluble' carmines are likely to be fully

complexed, 'soluble' carmines may contain variable amounts of free carminic acid (A. G. Lloyd and associates, to be published).

Finally, it is well known that all commercial carmines contain varying amounts of protein derived from the source-insect during the extraction of the pigment (see, for example, Marshall & Horobin, 1974). Equally, in my experience, many commercial samples of 'pure' carminic acid also contain residual amounts of organic nitrogenous components. Some early recipes for the preparation of carmines

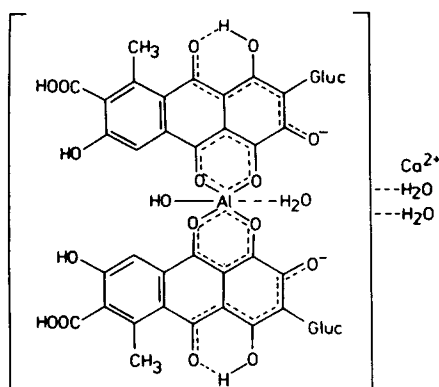


Fig. 6. Revised structure for the aluminium complex of carminic acid suggested by Meloan *et al.* (1971).

frequently included steps involving the addition of proteins from other sources, e.g. gelatin, egg albumin, milk proteins, etc. (Harrison, 1930; Meloan *et al.*, 1971), but this seems not to be a feature of present commercial practice. Until now, no attempt has been made to characterise the protein components derived from the insect nor to assess the way(s) in which carminic acid may be associated with them. In this latter respect, it has been shown that while purified carminic acid is freely dialysable, a substantial proportion of the carminic acid content of 'soluble' carmines does not diffuse across conventional membranes even on exhaustive dialysis, suggesting some form of combination between the hydroxyanthraquinone and high molecular weight components (J. Thomas and A. G. Lloyd, unpublished work).

With this in mind and as a preliminary to more extensive investigations, amino acid analyses on five random batches of a commercial 'soluble' carmine have been undertaken in two different laboratories. Table 2 records the average results obtained in the studies, expressed as amino acid residues per 1000 residues. It will be immediately obvious that the most striking features include:

(i) The low content of sulphur-containing and aromatic amino acids.
(ii) The fact that the contents of aspartic acid, glutamic acid, glycine and lysine taken together constitute more than half of all the amino acids detected.

TABLE 2
TYPICAL AMINO ACID ANALYSIS FOR COMMERCIAL 'SOLUBLE' CARMINES (EXPRESSED AS AMINO ACID RESIDUES PER 1000 RESIDUES)

Asp	116	Met	12
Thr	46	Ileu	20
Ser	56	Leu	29
Pro	53	Tyr	24
Glu	151	Phala	16
Gly	171	His	50
Ala	56	Lys	123
Val	29	Arg	40
Cys	8		

Successive studies show that these observations constitute a virtual 'fingerprint' for 'soluble' carmines (J. Thomas and A. G. Lloyd, unpublished work). Although less obvious, the same trends are apparent in amino acid analyses of 'insoluble' carmines. At the risk of being accused of speculation, it must be said that this pattern is more reminiscent of 'structural' proteins than of their globular 'enzymic' counterparts.

CONCLUDING REMARKS

It is recognised amongst food technologists that 'the best of all natural red pigments is probably cochineal' (Riboh, 1977). The paradox is complete in the sense that the commercial use of cochineal products and related insect pigments, which declined directly as a result of the invention of the synthetic organic red colours, now faces a revival due to those pressures resulting from toxicological attitudes towards materials such as Amaranth (FD & C Red 2), Ponceau 4R, Carmoisine and Allura Red (FD & C Red 40).

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NON-PROTEIN NITROGEN COMPOUNDS IN THE HIGHER FUNGI—A REVIEW

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ABSTRACT

The total non-protein nitrogen content and the amounts of individual non-protein nitrogen compounds, as well as factors affecting the amounts of these compounds in the higher fungi, are reviewed. The non-protein nitrogen compounds most significant in this respect are free amino acids, chitin, nucleic acids and urea; however, urea has only a limited occurrence in the higher fungi. In addition to these compounds, nucleotides and related compounds, ammonia, several kinds of amines and quaternary ammonium compounds, volatile nitrogen compounds (other than amines) and nitrogen-containing vitamins are found in the higher fungi.

It is widely agreed that misleading information is obtained when the protein content of mushrooms is estimated from the nitrogen content as determined by the Kjeldahl procedure using the conventional 6·25 conversion factor. Possibilities of using a factor other than the 6·25 factor are discussed.

INTRODUCTION

The higher fungi have generally been considered to be a good source of protein but for a long time scientists have been aware of the misleading information obtained when nitrogen (N) × 6·25 is used in calculating their protein content. The increasing demand for more exact information on the true protein content of especially the edible fungi initiated this search for published information on the non-protein nitrogen fraction (i.e. compounds not possessing the chemical properties of proteins) in the higher fungi.

Several papers relating to the topic have been published in Eastern Europe in rare native languages. Due to our difficulties with these languages no complete coverage

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of this literature has been accomplished. The review here presented is not aimed to be a critical presentation of the topic discussed but rather a collection of information available on the topic.

TOTAL NON-PROTEIN NITROGEN CONTENT OF THE HIGHER FUNGI

Interpretations of the concept of non-protein nitrogen vary and this fact, together with differences in the analytical methods used, explain in part the wide variations in published data for the non-protein nitrogen content of mushrooms. The content seems to vary also according to the species and age of mushrooms analysed (Table 1). As is shown later, some other factors as well (for example, the strain of mushroom studied, growth conditions, part of the mushroom sampled, storage time and conditions, and manner of processing) can significantly affect the amount of

TABLE 1
TOTAL, PROTEIN AND NON-PROTEIN NITROGEN CONTENT OF THE HIGHER FUNGI REPORTED IN THE LITERATURE

Species	*Total N (% of dry wt)*	*% of total N*		*Reference*[a]
		Protein N	*Non-protein N*	
Agaricus bisporus	0·5[b]	63	37	1
	6·2	90[c]	10[c]	2
Boletus badius	4·2[d]	29	71	3
	3·0[e]	84	16	3
	6·4	69	31	4
Boletus edulis	2·2[d]	93	7	3
	6·4	71	29	5
Cantharellus cibarius	3·6	67	33	4
Gyromitra esculenta	5·0	70	30	4
Lactarius vellereus	4·0	57	43	6
Leccinum scabrum	5·6	72	28	4
Lepiota procera	7·0[f]	47	53	7
Paxillus involutus	4·2[d]	66	34	3
	3·9	65	35	8
Pleurotus ostreatus	3·6	70	30	9
Suillus bovinus	4·3	61	39	4
Suillus luteus	3·2[d]	80	20	3
	3·7[e]	88	12	3
	5·1	77	23	4
Tricholoma equestre	2·6[d]	62	38	3
	2·1[e]	55	45	3
Tricholoma nudum	8·8	43	57	10

[a] 1. Fitzpatrick *et al.* (1946); 2. Hayes & Haddad (1976); 3. Schupman (1961); 4. Młodecki *et al.* (1968*a*); 5. Młodecki *et al.* (1968*b*); 6. Karkocha (1968); 7. Karkocha (1969); 8. Lasota (1969); 9. Ginterová & Maxianová (1975); 10. Stefanov & Zapryanov (1969).
[b] On a fresh weight basis.
[c] Calculated from the data reported in the paper.
[d] Small fruiting bodies.
[e] Large fruiting bodies.
[f] Only cap analysed.

different non-protein nitrogen compounds and presumably also the total amount of non-protein nitrogen in mushrooms.

FREE AMINO ACIDS AND PEPTIDES

Free amino acids

The same amino acids as have been detected in the mushroom protein hydrolysates have been found also to occur as free amino acids in the alcohol extracts of mushrooms studied (Hughes *et al.*, 1958; Orillo & Carangal, 1961; Bano *et al.*, 1963; Aalto & Kreula, 1972). In addition to the amino acids that can be found in the protein molecules a great number of other amino acids have been reported to occur in the higher fungi (Table 2).

Yokohata & Yamauchi (1959) reported that 38–50 % of the total nitrogen present in *Armillaria Matsutake Ito et Imai* was non-protein nitrogen, mainly amino acids. Flegg & Maw (1976) concluded on the basis of their study of the literature that free amino acids in the cultivated mushroom, *Agaricus bisporus*, represent about 20 % of the total amino acid content. Dudareva & Solowjewa (1973) studied the free amino acids of *Boletus edulis* and *Boletus versipellis*. On a fresh weight basis they contained 1·03 and 0·63 % free amino acids, respectively. In a recent study by Kreula *et al.* (1976) the total amount of free amino acids varied in some cultivated and wild mushrooms from 1·5 to 3·5 % of the dry matter. According to the data collected in Table 3 the total amount of free amino acids in mushrooms varies approximately from 2 to 11 % of the dry matter.

The most common and most abundant free amino acids seem to be glutamic acid and α-alanine. In some mushroom species great amounts of free proline, glutamine, arginine or aspartic acid have also been detected (Table 3).

Factors affecting the amount of free amino acids: There appears to be an increase or a decrease in the amounts of certain free amino acids in the fruiting bodies with the growth breaks (Hughes *et al.*, 1958; Kissmeyer-Nielsen *et al.*, 1966). Krupa & Bränström (1974) reported differences in the proportions of individual amino acids in the bound, as well as in the free, amino acid fraction at different stages of growth of *Boletus variegatus* mycelium.

The effect of storage temperature on the amounts of free amino acids in *Agaricus bisporus* has been studied by Le Roux & Danglot (1972). The changes observed in the quantities of individual amino acids in mushrooms stored at 3° and 20 °C for three days did not exhibit a uniform pattern (Le Roux & Danglot, 1972). Murr & Morris (1975*a*,*b*) have studied changes in free α-amino nitrogen content as affected by storage temperature and storage atmosphere.

The effects of different drying methods on the free amino acid content of higher fungi have been studied by Dudareva & Solowjewa (1973) and Dudareva (1974). Freeze-drying of *Boletus edulis* and *Boletus versipellis* caused an insignificant

TABLE 2
SOME AMINO ACIDS OF NON-PROTEIN ORIGIN DETECTED IN THE HIGHER FUNGI

Amino acid[a]	*Species*[b]	*References*[c]
Agaritine (β-*N*-(γ-L(+)-glutamyl)-4-hydroxymethylphenylhydrazine)	*Agaricus bisporus*	Levenberg (1961)
β-Alanine	Several species	Hughes *et al.* (1958) Aalto & Kreula (1972)
α-Aminoadipic acid	Several species	Craske & Reuter (1965) Aalto & Kreula (1972)
α-Aminobutyric acid	Several species	Craske & Reuter (1965) Hatanaka & Terakawa (1968)
γ-Aminobutyric acid	Several species	Hughes *et al.* (1958) Hatanaka & Terakawa (1968)
L-2-Amino-4-chloro-4-pentenoic acid	*Amanita pseudoprophyria*	Hatanaka *et al.* (1974)
L-2-Amino-3-formyl-3-pentenoic acid	*Bankera fuligineoalba*	Doyle & Levenberg (1968)
L-2-Amino-3-hydroxy-hex-4-ynoic acid	*Tricholomopsis rutilans*	Niimura & Hatanaka (1974)
L-2-Amino-3-hydroxy-methyl-3-pentenoic acid	*Bankera fuligineoalba*	Doyle & Levenberg (1968)
β-Aminoisobutyric acid	*Agaricus bisporus*	Altamura *et al.* (1967)
L-2-Amino-4-methyl-5-hexenoic acid	*Boletus* sp.	Rudzats *et al.* (1972)
L-2-Amino-4-methyl-pimelic acid	*Lactarius quietus*	Hatanaka *et al.* (1975)
Cis-3-Amino-L-proline	Several *Morchella* species	Hatanaka (1969)
L-3-(3-Carboxyfuran-4-yl)alanine	*Phyllotopsis nidulans*, *Tricholomopsis rutilans*	Doyle & Levenberg (1974) Hatanaka & Niimura (1975)
Citrulline	Several species	Hughes *et al.* (1958) Aalto & Kreula (1972)
Cysteic acid	*Boletus edulis*, *Agaricus bisporus*	Craske & Reuter (1965) Kissmeyer-Nielsen *et al.* (1966)
α,β-Diaminopimelic acid	*Boletus edulis*	Craske & Reuter (1965)
2,4-Diaminobutyric acid	*Agaricus bisporus*	Altamura *et al.* (1967)
N-Ethyl-γ-glutamine	*Xerocomus badius*	Casimir *et al.* (1960)
Homoserine	*Agaricus bisporus*	Altamura *et al.* (1967)
N-γ-L(+)-Glutamyl-*p*-hydroxyaniline	*Agaricus hortensis*	Jadot *et al.* (1960)
5-Hydroxytryptophan	An Inocybe species (1838)	Robbers *et al.* (1964)
Kynurenine	*Agaricus bisporus*	Altamura *et al.* (1967)
2-Methylenecycloheptene-1,3-diglycine	*Lactarius helvus*	Honkanen *et al.* (1964)
β-Methylene-L-(+)-norvaline	*Lactarius helvus*	Levenberg (1968)
β-Nitraminoalanine	*Agaricus silvaticus*	Chilton & Hsu (1975)
Ornithine	Several species	Altamura *et al.* (1967) Aalto & Kreula (1972)
Pipecolic acid	Several species	Hatanaka & Terakawa (1968)
Sarcosine	*Agaricus bisporus*	Altamura *et al.* (1967)

[a] All amino acids listed here occur presumably mainly as free amino acids. None have been reported to occur as constituents of mushroom proteins.
[b] Single mushroom species have been indicated only when a certain amino acid has been reported to occur in no more than five mushroom species.
[c] References are in many cases only examples.

TABLE 3

QUANTITIES OF SELECTED FREE AMINO ACIDS IN SOME HIGHER FUNGI

Amino acid	Agaricus bisporus				Pleurotus ostreatus		Boletus edulis	Lactarius trivialis	Cantharellus cibarius
	Somycel 11		*Somycel* 500						
	mg/100 g fresh weight	*% of total amino acids*	*mg/100 g fresh weight*	*% of total amino acids*	*mg/100 g dry weight*	*% of total amino acids*	*% of total amino acids*[a]	*% of total amino acids*[a]	*% of total amino acids*[a]
Valine	12·0	2·0	17·4	1·6	82	4·3	2·6	3·19	1·37
Lysine	12·2	2·1	22·0	2·0	132	6·9	1·50	3·05	3·14
Threonine	3·0	0·5	4·0	0·4	57	3·0	4·60	6·55	2·55
Leucine	14·2	2·4	16·4	1·5	178	9·3	3·23	4·19	1·96
Isoleucine	8·4	1·4	12·0	1·1	62	3·2	2·78	1·76	0·91
Phenylalanine	8·6	1·5	17·2	1·6	116	6·0	3·83	4·29	1·58
Methionine	1·4	0·2	2·0	0·2	31	1·6	4·07	0·72	0
Histidine	4·0	0·7	7·4	0·7	41	2·1	2·29	5·98	7·14
Glutamic acid	133·2	22·6	195·6	18·0	290	15·1	9·93	10·45	26·82
Alanine	70·4[b]	11·9[b]	109·0[b]	10·0[b]	173	9·0	21·43[b]	17·55[b]	11·69[b]
Proline	30·8	5·2	157·6	14·5	27	1·4	2·30	2·86	1·59
Serine	26·0	4·4	35·4	3·3	74	3·8	7·80	9·08	3·31
Arginine	30·2	5·1	97·2	8·9	42	2·2	1·41	2·94	11·97
Aspartic acid	73·4	12·4	89·0	8·2	130	6·8	1·81	4·57	3·25
Asparagine	34·0	5·8	50·2	4·6	19	1·0	1·05	0·62	1·56
Glutamine	57·8	9·8	111·6	10·3	300	15·6	—[c]	—	—
Ornithine	46·2	7·8	82·4	7·6	—	—	1·69	4·29	3·69
Total free amino acids	590·4[d]	—	1086·6[d]	—	1924	—	—	—	—
Reference[e]	1	1	1	1	2	2	3	3	3

[a] Includes also the amount of ethanolamine. [b] α-Alanine. [c] The value is not indicated in the paper.
[d] Calculated from the data presented in the paper. [e] 1. Le Roux & Danglot (1972). 2. Ginterová & Maxianová (1975). 3. Aalto & Kreula (1972).

decrease (Dudareva & Solowjewa, 1973), whereas drying at 45–60 °C resulted in a distinct decrease (about 30 %) in the total amount of free amino acids (Dudareva, 1974). The amount of free glutamic acid has similarly been reported to decrease when mushrooms (*Agaricus bisporus*) are dried at 50 °C (Dijkstra, 1976). A moderate increase, however, was observed when mushrooms were sterilised at 110 °C for 20 min (Dijkstra, 1976).

Kissmeyer-Nielsen *et al.* (1966) have studied the effect of the composition of the compost on the amount of different free amino acids in *Agaricus bisporus*. Where gelatin was added to the compost a decrease in the amounts of free alanine and arginine and an increase in the amount of free aspartic acid were noted. When supplementing the compost with hydrolysed casein a decrease in the amount of free arginine was detected.

Peptides

Many reports on peptides and peptide-like substances in the higher fungi deal with the toxic and non-toxic cyclopeptides of *Amanita phalloides* and *Amanita virosa* reviewed, for instance, by Moser (1971). Small amounts of amatoxins have also been reported to occur in several edible mushrooms like *Agaricus silvaticus* (8·5 ng/g), *Cantharellus cibarius* (5·5 ng/g) and *Boletus edulis* (< 0·2 ng/g) (Faulstich & Cochet-Meilhac, 1976).

Remarkably little information is available on peptides in mushrooms other than those mentioned above. Altamura *et al.* (1967) studied the ninhydrin-positive nitrogen compounds in an isolate obtained from *Agaricus bisporus* and out of 53 free ninhydrin-positive compounds registered two free dipeptides, carnosine and γ-L-glutamyl-*S*-methyl-L-cysteine, were identified. In a more recent study by Altamura *et al.* (1970) several pyroglutamyl dipeptides were identified in *Agaricus bisporus*.

CHITIN

Chitin is a polysaccharide which is composed of β-1, 4-linked *N*-acetyl-D-glucosamine residues and which serves as structural material in nature, for instance in the higher fungi, as confirmed by several studies. Recent experiments with fungal mycelium have shown that chitin is present in the cell walls of the higher fungi in close association with β-glucan, a glucose polymer other than cellulose (O'Brien & Ralph, 1966; Wessels & Vries, 1973; Angyal *et al.*, 1974; Michalenko *et al.*, 1976; Siehr, 1975). Iten (1970), however, reported that the cell walls of vegetative mycelium and fruiting bodies of *Coprinus lagopus* contained practically only chitin.

In many papers (Kreger, 1954; Wessels, 1965; O'Brien & Ralph, 1966; Michalenko *et al.*, 1976) the amount of chitin is expressed as a percentage of the isolated cell walls or as a percentage of the alkali-insoluble cell wall fraction, without indicating the weight of the cell walls isolated from the initial material. In such cases

it is impossible to evaluate the chitin content of the original material. O'Brien & Ralph (1966) examined several representatives of the Basidiomycetes and found that the percentage quantities of chitin (26–65) and glucan (22–67) in the alkali-insoluble cell wall fraction showed a wide variation not related in any way to the taxonomic position. The chitin content of enzymically cleaned cell walls of *Schizophyllum commune* was only 3·1 % and 5·0 % of the dry weight of the primordial and mycelial cell walls, respectively, when the organism was grown for 8 days on synthetic medium (Wessels, 1965).

Values reported in the literature for the chitin content of the cell walls of the common mushroom, *Agaricus bisporus*, differ greatly from those stated by Wessels (1965) for *Schizophyllum commune*. Kreger (1954) found 30 to 35 % chitin in the cell wall material from the stem of a young fruiting body, while the chitin percentage obtained by Temeriusz (1975) in the cell wall material from whole fruiting bodies without peels and gills was slightly lower (25 %). The chitin content of purified cell walls from the mycelium of *Agaricus bisporus* was found to be 43 % (Michalenko *et al.*, 1976).

In the study by Temeriusz (1975) the cell walls were obtained as 1·4 % yield of the starting material (fresh fruiting bodies) which, on 10 % dry matter basis of the mushroom (not specified in the paper), would mean that the initial material contained approximately 3·5 % chitin of the dry matter. Higher figures for the chitin content of champignon have been reported by Proskuriakow (1926) (5·5 % of air-dried material) and Szymczak (1972) (5·8–7·5 % of dry matter, depending on the stage of fruit-body development). The chitin content was shown to increase along with the development of fructification (Szymczak, 1972).

The values for the chitin content of *Armillaria mellea* and *Lactarius volemus* were found to be 2·8 and 3·0 % of air-dried material, respectively (Proskuriakow, 1926), while those for the chitin content of *Lactarius vellereus* (Karkocha, 1968) and *Lepiota procera* (Karkocha, 1969) were reported to be as high as 19 and 18 % of the dry weight, respectively. The mycelium of *Boletus edulis*, on the other hand, was found to contain only 0·3–0·5 % chitin of the dry weight, depending on the age of the culture (Řeháček *et al.*, 1962).

Methods for the quantitative determination of chitin (methods involving purification, those requiring the complete hydrolysis of chitin and determination of the D-glucosamine produced, and those based on the enzymic degradation of chitin) are of limited accuracy (Tracey, 1955; Foster & Webber, 1960). This presumably partly accounts for the great variations in the reported values for the chitin content of the higher fungi.

NUCLEIC ACIDS AND RELATED COMPOUNDS

In addition to nucleic acids, nucleotides and nucleosides, as well as purine and pyrimidine bases, are reviewed under this heading.

Nucleic acids

Relatively little information is available on the nucleic acid content of the higher fungi. It has been reported (Hofsten, 1974) that the nucleic acid content of mushrooms is generally only a few per cent, which is low compared with that of bacteria and yeasts (10–20 %).

Pihakaski (1972) has studied the qualitative and quantitative changes in nucleic acids of *Flammulina velutipes* at different developmental stages. The nucleic acid content varied from 5·5 % (mycelia) to 3 % (fruiting bodies) of the dry weight. The proportion of DNA was small, not over 0·05 % of the dry weight. The total RNA consisted of different types of RNA (over 80 % of the total RNA was rRNA) which showed temporal changes during the morphogenesis of tissues. The total RNA synthesis in mycelia increased to the point when fruiting-body initials became visible but thereafter the RNA content decreased rapidly and remained at the same level during the later development stages of mycelia.

Pure tRNA was isolated from RNA of six-week-old mycelia of *Pleurotus ostreatus* by Nowak *et al.* (1975) who used preparative gel electrophoresis. From 100 g of fresh mycelium 150–200 mg of RNA and 11 mg of pure tRNA was obtained. Different methods of isolation of DNA and RNA from the mycelia of Basidiomycetes have been studied by Venner (1963).

Free nucleotides and nucleosides

In addition to nucleic acids many free nucleotides occur in the higher fungi. According to Kritsky & Kulatv (1963) the total amount of free acid-soluble nucleotides in *Agaricus bisporus* is approximately 8 μmol per gramme of the dry weight. Uridine derivatives predominated in the nucleotide pool of the mushroom. Adenosine and guanosine derivatives were present in smaller quantities, while cytidine compounds were not found at all. Nucleoside diphosphates constituted up to 60 % of the total nucleotide pool (Kritsky & Kulatv, 1963).

Uridine and adenosine derivatives were also the predominating nucleotides in all of the eight mushroom species studied by Bergkvist (1958*a* and *b*). The amounts of 9 to 18 acid-soluble nucleotides identified were determined and only small amounts of cytidine derivatives were detected in some mushroom species (Tables 4 and 5).

Hashida *et al.* (1964) studied the nucleoside 5′-monophosphate concentrations of ten kinds of mushrooms. The total amount of 5′-nucleotides in these mushrooms was no more than about one-tenth of the amount of RNA in them. The amounts of nucleoside 5′-monophosphates of dried *Lentinus edodes* extracted with boiling water were higher than the amounts extracted with cold perchloric acid or sulphuric acid (Table 4). Shimazono (1964) also reported that the amounts of nucleoside 5′-monophosphates in the hot water extract of *Cortinellus shiitake* were significantly higher than those in the perchloric acid extract of the mushroom. This is presumably due to the breakdown of intracellular RNA during heating in water by relatively thermostable RNA-decomposing enzymes (ribonucleases) contained in the mushroom (Shimazono, 1964; Mouri *et al.*, 1966).

TABLE 4

QUANTITIES OF NUCLEOSIDE 5′-MONOPHOSPHATES IN SOME HIGHER FUNGI AS DETERMINED BY ION EXCHANGE CHROMATOGRAPHY (I) OR BY ENZYMIC METHODS (E). EXTRACTIONS HAVE BEEN PERFORMED WITH BOILING WATER (W) OR WITH 5 TO 10% PERCHLORIC ACID (P) OR WITH 0·1N SULPHURIC ACID (S)

Nucleotide[a]	Agaricus bisporus	Pleurotus ostreatus	Flammulina velutipes	Lentinus edodes (*died*)			Polyporus squamosus	Coprinus comatus
	I,W *μmoles/g dry weight*	*I,W* *μmoles/g dry weight*	*I,W* *μmoles/g dry weight*	*I,W* *μmoles/g dry weight*	*I,P* *μmoles/g dry weight*	*I,S* *μmoles/g dry weight*	*I,P* *μmoles/1000 g fresh weight*	*I(E),W*[b] *mg/litre*[c] *of extract*
5′-AMP	3·35	0·61	1·95	2·28	0·66	1·00	17·3	99
5′-CMP	trace	0·07	0·28	1·58	trace	trace	—[d]	—
5′-GMP	trace	0·36	1·60	3·96	0·67	1·79	—	122
5′-IMP	0	0	0	0	0	0	—	—
5′-UMP	2·02	0·42	0·65	3·62	0·64	0·99	68·3	88
Reference[e]	1	1	1	1	1	1	2	3

[a] Abbreviations used: A = adenosine, C = cytidine, G = guanosine, I = inosine, U = uridine, MP = monophosphate.
[b] 5′-AMP was determined enzymically. [c] Fresh mushrooms were homogenised with one part of water in a Waring blender. The resulting slurry was heated to boiling, kept at 50 °C for one hour and filtered.
[d] Not reported in the paper. [e] 1. Hashida *et al.* (1964). 2. Bergkvist (1958*a*). Dijkstra & Wikén (1976*b*).

TABLE 5

QUANTITIES OF NUCLEOSIDE DI- AND TRIPHOSPHATES IN SOME HIGHER FUNGI ACCORDING TO BERGKVIST (1958*b*). CONCENTRATION IN μmoles PER 1000 g FRESH WEIGHT[a]

Nucleotide[b]	Polyporus squamosus	Amanita muscaria	Lycoperdon pratense	Armillaria mellea	Lactarius vellereus
ADP	37·9	30·8	50·2	27·3	54·8
GDP	9·5	4·8	15·5	4·0	10·9
UDP	31·5	36·6	46·0	48·5	31·6
ATP	122·0	113·0	113·5	138·5	158·0
CTP	—	—	—	—	3·7
GTP	11·5	6·6	14·8	6·9	16·4
UTP	—	—	11·0	14·5	15·4
UDPG	13·5	18·5	18·0	55·5	55·3
UDPAG	185·5	248·5	160·0	174·0	170·7

[a] Nucleotides were extracted with 5 to 10% perchloric acid and determined by combination of ionophoresis and paper chromatography.
[b] Abbreviations used: A = adenosine; C = cytidine; G = guanosine; U = uridine; DP = diphosphate; TP = triphosphate; UDPG = uridine diphosphate glucose; UDPAG = uridine diphosphate acetylglucosamine.

Mushrooms do not contain, in measurable amounts, inosine 5′-monophosphate, which is the main nucleotide of meat (Shimazono, 1964; Hashida *et al.*, 1964; Mouri *et al.*, 1967*a*).

There are relatively few reports in the literature on the occurrence of free nucleosides in mushrooms. Most deal with uridine (Bergkvist, 1958*a*; Kritsky & Kulatv, 1963; Hashida *et al.*, 1964) but cytidine and guanosine (Hashida *et al.*, 1964), as well as adenosine (Mouri *et al.*, 1967*b*) have also been reported. Kritsky & Kulatv (1963) found in *Agaricus bisporus* 0·33–1·37 μmol of uridine per gramme of dry weight, depending on the part of the mushroom studied.

Factors affecting the amount of free nucleotides: The composition of the nucleotide fraction varies both qualitatively and quantitatively in different parts of the fruiting body. Kritsky & Kulatv (1963) found close similarities in the amounts of different free nucleotides in the stipe and the cap and, on the other hand, in the part of the cap attached to the gills and in the gills. The base of the stipe of *Agaricus bisporus* had a specific nucleotide composition.

Mouri *et al.* (1967*a*) reported that the amount of ATP decreased gradually and the amounts of both 5′-AMP and adenosine increased correspondingly during storage of *Agaricus bisporus* at −20 °C for six months. Freeze-drying did not affect the 5′-nucleotide content of the mushroom, whereas during the storage of freeze-dried samples in cans *in vacuo* at 5° or 30 °C for six months a slow increase was observed in the 5′-AMP content of the mushroom (Mouri *et al.*, 1967*b*).

Drying at 50 °C and, to a smaller extent, sterilisation at 110 °C for 20 min were found to cause a decrease in the amount of 5′-GMP in *Agaricus bisporus* (Dijkstra, 1967).

Free purine and pyrimidine bases and their derivatives

Only qualitative data are found in the literature on the purine and pyrimidine bases in the higher fungi. Thus there are reports on the occurrence of adenine and guanine (List, 1958; List & Hetzel, 1959; List & Reinhard, 1962; Lasota, 1970) as well as of xanthine and hypoxanthine (List & Hetzel, 1959; Lasota, 1970) in some higher fungi. The occurrence of uracil, a pyrimidine base, in higher fungi has been reported by Hashida *et al.*, 1964.

From dried Shii-take mushroom (*Lentinus edodes*) two closely related purine derivatives, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid and 2(*R*)-hydroxy-4-(9-adenyl)-butyric acid, have been identified. The former of these compounds has been found to reduce plasma–cholesterol level in rats, while the hypocholesterolemic effect of the latter compound has been found to be almost negligible (Tokita *et al.*, 1972). Saito *et al.* (1975) studied the occurrence of the former compound (trivial name, eritadenine) in several mushroom species, but detected the compound only in *Lentinus edodes* and in trace amounts in *Agaricus bisporus*.

UREA

Urea, carbamide, is synthesised in the fruiting bodies of the higher Basidiomycetes mainly along the reactions of the Krebs–Henseleit ornithine cycle (Reinbothe & Tschiersch, 1962). Experiments with *Agaricus bisporus* and *Lycoperdon perlatum* have revealed that urea in the higher fungi is synthesised also via aerobic purine degradation (Reinbothe & Tschiersch, 1962) and obviously indirectly from pyrimidines (Wasternack & Reinbothe, 1967). It has been suggested that the urea in puff-balls of *Lycoperdon* might be the special form of a nitrogen reserve of the mushroom and in fruiting bodies of *Agaricus* a typical end product of catabolic pathways (Iwanoff, 1927; Reinbothe *et al.*, 1967).

The pioneering studies of Iwanoff (1923, 1927, 1928) indicated that some mushroom species (members of *Agaricus*, *Lycoperdon* and *Bovista*) may contain considerable amounts of urea, even up to 50% of the total nitrogen content of the mushroom. In a comprehensive research by Tyler *et al.* (1965) in which 344 mushroom species were examined for the presence of urea, characteristic distribution and accumulation patterns in various taxa were revealed. The authors classified the examined species according to their capacity to produce and store urea as urea-accumulating ($\geq 0{\cdot}33\%$ urea of dry weight), urea-non-accumulating ($< 0{\cdot}33\%$ urea of dry weight) or urea-negative.

Urea-accumulating species were found only in the orders Agaricales and Lycoperdales. With one exception (*Montagnea* sp. of the order Podaxales) urea was entirely absent from all species of other examined orders of Basidiomycetes (Polyporales, Hymenogastrales, Gautieriales, Podaxales, Phallales). It was also absent from a number of species of Ascomycetes representing the families

Morchellaceae, Helvellaceae and Pezizaceae. A single exception, *Helvella infula*, apparently contained a very small amount (<0·33%) of urea (Tyler *et al.*, 1965).

Members of the families Agaricaceae (representing the genera *Chlorophyllum*, *Macrolepiota*, *Agaricus*, *Lepiota* and *Cystoderma*) and Lycoperdaceae (representing the genera *Bovista* and *Lycoperdon*) tested were consistently large accumulators of urea. Urea-accumulating species were also found in the genera *Pleurotus*, *Lyophyllum*, *Clitocybe*, *Lepista*, *Omphalina*, *Melanoleuca*, *Armillaria*, *Collybia*, *Marasmius*, *Mycena*, *Amanita*, *Limacella*, *Coprinus*, *Psathyrella*, *Panaeolina*, *Panaeolus*, *Anellaria*, *Conocybe*, *Stopharia*, *Rhodocybe* and *Suillus* (only one species, *Suillus piperatus*, of nine species examined was a urea-accumulator). All species of other genera of Agaricales examined (for example all members of the families Hygrophoraceae, Cortinariaceae, Paxillaceae, Boletaceae, with the exception of *Suillus piperatus*, and Russulaceae) were either urea-negative or non-accumulators of urea (Tyler *et al.*, 1965).

Reinbothe & Tschiersch (1962) and Reinbothe *et al.* (1967) have reported urea contents of some *Agaricus* and *Lycoperdon* species that are in good agreement with the results of Tyler *et al.* (1965). Aalto & Kreula (1972), on the other hand, detected urea in *Cantharellus cibarius*, *Lactarius trivialis*, *Lactarius torminosus* and *Boletus edulis* in quantities (0·6–0·7% of dry weight) large enough to make these species, according to the classification of Tyler *et al.* (1965), urea-accumulating, which is in disagreement with the results of the latter study.

Factors affecting the amount of urea

In addition to the variations within different species, the urea content of mushrooms varies within several different parameters, as with age (Iwanoff, 1923, 1927; Reinbothe & Tschiersch, 1962; Reinbothe *et al.*, 1967) as well as according to the part (Iwanoff, 1927; Reinbothe & Tschiersch, 1962; Reinbothe *et al.*, 1967) and the break (Kissmeyer-Nielsen *et al.*, 1966) of the mushrooms studied. These studies have been performed mainly with *Agaricus* and *Lycoperdon* species.

Iwanoff (1923, 1927) reported that the urea content of *Agaricus bisporus* which in very young fruiting bodies was only 2·6% of the dry weight, increased with the age of the fruiting body and reached its maximum in the mature fruiting body (up to 13·2% of dry weight), although the urea content of the spores was only 1·1%. The amount of the spores in the mature fruiting bodies of *Agaricus* is small, however (Iwanoff, 1927). Reinbothe & Tschiersch (1962) investigated the urea content in five stages of development of *Agaricus xanthodermus* and found the maximum in the third stage.

Lycoperdon pyriforme has been reported to reach its maximum in urea content at the moment the spores begin to form. In ripe sporulating fungi the urea content practically decreases to zero because mature puff-balls contain many spores with, at the most, very small amounts of urea (Iwanoff, 1927; Reinbothe *et al.*, 1967).

The highest urea content of a mushroom is found in the hymenophore (Iwanoff, 1927; Reinbothe & Tschiersch, 1962; Reinbothe *et al.*, 1967) which evidently also contains the highest urease activity (Reinbothe *et al.*, 1967).

According to Iwanoff (1923, 1927) an excess of nitrogen supplementation in the growth medium leads to high urea contents, whereas excess of carbohydrates in the medium will have the reverse effect. Kissmeyer-Nielsen *et al.* (1966) have investigated the changes in the urea content of *Agaricus bisporus* as influenced by nutrient supplementation of the compost during the growth cycle.

AMMONIA

Ammonia has been reported to be a common compound in mushrooms (Stein von Kamienski, 1958; Schormüller, 1974). Stein von Kamienski (1958) studied the occurrence of ammonia and volatile amines in 105 species representing 18 families of the higher fungi and found that ammonia was present in all species studied. Dijkstra & Wiken (1976*a*) found ammonia (12 mmol/litre) in water extract (fresh mushrooms were homogenised with one part of water) of *Agaricus bisporus*. Aalto & Kreula (1972) did not, however, detect ammonia in ethanol extracts of four kinds of mushroom but found it in acid hydrolysed samples.

The ammonia content of mushrooms has been reported to increase generally with increased maturity (Stein von Kamienski, 1958). Changes in the amount of ammonia in *Agaricus bisporus*, as influenced by nutrient supplementation of the compost during the growth cycle, have been studied by Kissmeyer-Nielsen *et al.* (1966).

AMINES AND QUATERNARY AMMONIUM COMPOUNDS

Several amines and quaternary ammonium compounds, besides the ones already discussed, have been detected in the higher fungi (Table 6). List & Hetzel (1959) summarised the literature published until that time on the biogenic amines in mushrooms, including the earlier studies of List and his co-workers. In addition to the imidazole derivatives listed in Table 6 (histamine, dimethylhistamine, herzynine, ergothioneine) the occurrence of some de-aminated imidazole derivatives (imidazolylacetic acid, urocanic acid, imidazolylpropionic acid, imidazolylethanol) in mushrooms has also been reported (List & Menssen, 1959*b*; List & Reith, 1960; List, 1960; List & Reinhard, 1962).

List (1957, 1958) isolated from *Coprinus comatus* 55·7 mg of ergothioneine and 300 mg of tyramine per approximately 10 kg of fresh mushrooms. Kotomska & Młodecki (1962) studied the choline contents in sixteen mushroom species and found that *Boletus edulis* and *Lactarius deliciosus* were good sources of choline (0·44 % and 0·46 % choline of the dry weight, respectively). Some species (*Agaricus bisporus*, *Boletus rufus* and *Boletus scaber*), on the other hand, did not contain any choline (Kotomska & Młodecki, 1962).

TABLE 6

SOME AMINES AND QUATERNARY AMMONIUM COMPOUNDS IDENTIFIED IN THE HIGHER FUNGI

Compound	*Species*[a]	*References*[b]
Betaine	*Coprinus comatus*	List (1958)
N-Butylamine	*Coprinus micaceus*	List & Hetzel (1960)
Choline	Several species	List (1958); Kotomska & Młodecki (1962)
Dimethylamine	Several species	Stein von Kamienski (1958); List & Menssen (1959*a*)
Dimethylhistamine	*Coprinus comatus*	List (1958)
Ergothioneine	*Coprinus comatus*, *Coprinus micaceus* *Phallus impudicus*	List (1957); Hetzel (1960); List & Reinhard (1962)
Ethanolamine (Colamine)	Several species	List & Menssen (1959*a*); Altamura *et al.* (1967)
Ethylamine	*Polyporus sulphureus*, *Coprinus micaceus*, *Phallus impudicus*	List & Menssen (1959*a*); List & Hetzel (1960); List & Reinhard (1962)
Herzynine (Betaine of histidine)	Several species	List (1958); List & Hetzel (1959); List & Reinhard (1962)
Histamine	*Coprinus comatus*, *Phallus impudicus*	List (1958); List & Reinhard (1962)
Isoamylamine	Several species	Stein von Kamienski (1958); List & Reinhard (1962)
Methylamine	Several species	Stein von Kamienski (1958); List & Menssen (1959*a*)
N-Methyltyramine	Several species	Lee *et al.* (1975)
N-Nitroethylenediamine	*Agaricus bisporus*	Chilton & Hsu (1975)
β-Phenylethylamine	Several species	Stein von Kamienski (1958); List & Reinhard (1962)
N-Propylamine	*Polyporus sulphureus* *Coprinus micaceus*	List & Menssen (1959*a*); List & Hetzel (1960)
Putrescine	*Phallus impudicus*	List & Reinhard (1962)
Spermidine	*Coprinus comatus*	List (1958)
Taurine	*Agaricus bisporus*	Kissmeyer-Nielsen *et al.* (1966); Altamura *et al.* (1967)
Trimethylamine	Several species	Stein von Kamienski (1958)
Tryptamine	*Coprinus micaceus*	List & Hetzel (1960)
Tyramine	Several species	List (1958); Lee *et al.* (1975)

[a] Single mushroom species have been indicated only when a certain compound has been reported to occur in no more than five species.

[b] References are in many cases only examples.

OTHER VOLATILE NITROGEN COMPOUNDS

In addition to the volatile amines, some other volatile nitrogen compounds have been identified in mushrooms. List & Freund (1967) found two sulphur-containing dinitrophenyl derivatives in *Phallus impudicus*. The characteristic aroma of this mushroom was assumed by the authors to be partly due to these compounds.

Thomas (1973) identified several volatile compounds in dry *Boletus edulis*, including nine pyrazines and seven 2-formylpyrroles. The drying process, however,

could encourage the formation of substances in a higher state of oxidation, e.g. pyrazines and pyrroles (Thomas, 1973).

Among the volatiles of *Gyromitra esculenta* four toxic nitrogen compounds, gyromitrin (acetaldehyde *N*-methyl-*N*-formyl hydrazone) (List & Luft, 1967; Pyysalo, 1975), and pentanal, 3-methylbutanal and hexanal *N*-methyl-*N*-formyl hydrazones (Pyysalo, 1975) have been identified.

NITROGEN-CONTAINING VITAMINS

The higher fungi are the source of several nitrogen-containing vitamins (Table 7). Zerova *et al.* (1972) reported that four mushroom species (*Boletus edulis*, *Leccinum scabrum*, *Leccinum aurantiacum*, *Suillus luteus*) grown in the Ukraine contained (depending on the kind, age and part of the mushroom studied) thiamine traces up to 7·93 mg, pyridoxine 0·14–0·78 mg, niacin 189–991 mg and biotin 0·03–0·48 mg per kilogramme dry weight. Shivrina *et al.* (1965) detected vitamin B_{12} in only nine out of 38 species of Polyporaceae and Agaricus investigated, the highest amount (0·14 mg/100 g dry weight) being found in *Pleurotus ostreatus*.

Factors affecting the amounts of vitamins B

According to Fedorova & Milova (1974) the thiamine and riboflavin contents of Agaricales and Aphyllophorales species are generally higher in the mycelia than in the fruiting bodies, with the exception of the wood-destroying fungi. There are also differences in the vitamin B contents between different parts of the fruiting body. Higher contents have been detected in the pileus than in the stipe of the fruiting body (Zerova *et al.*, 1972; Karosiene, 1975, 1976; Mäkinen *et al.*, 1978). Young fruiting bodies, on the other hand, seem to have higher vitamin B contents than old ones (Zerova *et al.*, 1972; Karosiene, 1975, 1976).

The effect of storage time and conditions on the thiamine content of several mushroom species has been studied by Mäkinen *et al.* (1978). For example, the cultivated mushroom *Agaricus bisporus* contained 0·39 mg thiamine per 100 g dry matter when analysed within one day after picking. Storage for one week in polyethylene bags at 4 °C doubled the content, while two weeks' storage increased it threefold. The thiamine content of six kinds of fresh wild mushroom varied from 1·16 mg (*Lactarius necator*) to 0·43 mg (*Trichloloma portentosum*) per 100 g dry matter when the mushrooms were stored for various times in polyethylene bags at 4 °C. Storing for two months in the freezer (−20 °C) decreased, or increased significantly, the thiamine content or maintained the content nearly unchanged.

Młodecki *et al.* (1973*a*) reported that the riboflavin content (in dry matter) was higher in dried than in fresh mushrooms (*Leccinum scabrum* and *Xerocomus badius*). The highest values (3·5 and 2·3 mg % of dry weight, respectively) were obtained when mushrooms were dried at 50° and 70 °C. Drying at room

TABLE 7

CONTENTS OF NITROGEN-CONTAINING VITAMINS IN SOME HIGHER FUNGI (mg/100 g DRY WEIGHT)

Species	*Thiamine*	*Riboflavin*	*Folic acid*	*Niacin*	*Pantothenic acid*	*Biotin*	*Vitamin B_6*	*Vitamin B_{12}*	*Reference*[a]
Agaricus bisporus	1·1	4·0	—[c]	55·7	22·6	0·0019	—	—	1
Agaricus campestris[b]	1·1	4·8	0·33	67·4	22·8	0·17	0·54	—	2
Cantharellus cibarius[b]	0·2	2·7	—	76·5	—	—	—	—	2
Morchella esculenta, mycelium[d]	0·392	2·46	0·348	8·20	0·87	0·075	0·58	0·00036	3
Morchella hortensis, mycelium[e]	0·518	1·31	1·09	12·4	12·6	0·015	2·62	—	4

[a] 1. von Hofsten (1974); 2. Souci *et al.* (1969); 3. Szuecs (1956); 4. Litchfield (1964).
[b] In edible portion.
[c] Not reported in the paper.
[d] Purely synthetic growth medium, where the only organic component was glucose and the only nitrogen source was ammonium phosphate.
[e] A glucose–ammonium phosphate–cornsteep liquor–calcium carbonate medium.

temperature for some days and at 105 °C to constant weight were other drying methods used.

Zhuk & Rod'kina concluded from their data that blanching should be performed within two minutes because, when five minutes' blanching at 65 °C was used, decreases of 50 % of the thiamine, 60–85 % of the riboflavin and 14–27 % of the niacin were observed. High temperature and short time were suggested when sterilising the canned product. According to Młodecki *et al.* (1973*b*) peeling or slicing of the mushrooms before blanching causes higher losses of riboflavin during blanching than in those blanched whole. When mushrooms (*Cantharellus cibarius*) were blanched whole in distilled water at 90° to 95 °C for five minutes the losses of riboflavin were on the average 33 %, but when mushrooms (*Suillus luteus* and *Tricholoma flavovirens*) were peeled or sliced before blanching the losses of riboflavin were much higher (61 and 58 %, respectively).

CONCLUSIONS

The compounds that most significantly contribute to the non-protein nitrogen content of the higher fungi are free amino acids, chitin, nucleic acids and, in some species, urea. Several factors, such as the species, strain and age of mushroom, growth conditions, part of mushroom sampled, storage time and conditions, as well as the manner of processing, can, however, greatly affect the amounts of these compounds and thus presumably the proportion of non-protein nitrogen in mushrooms.

The protein content of a mushroom is commonly estimated from the nitrogen content as determined by the Kjeldahl procedure, using the 6·25 factor which is the generally-used conversion factor for foodstuffs, although it is widely agreed that it gives too high values for the true protein in mushrooms. Some scientists (Karkocha & Młodecki, 1962; Souci *et al.*, 1969), on the other hand, have preferred the use of the 2/3 × 6·25 factor to the conventional 6·25 factor. On the basis of this literature survey a more accurate estimation can be assumed to result from the use of the former rather than of the latter factor, although it seems impossible to find any universal factor applicable to mushrooms because of the great variations reported in their non-protein nitrogen content. To obtain more accurate estimations the non-protein nitrogen content should be determined separately and deducted from the total nitrogen content before using the 6·25 factor (*cf.* Młodecki *et al.*, 1968*a*,*b*; Lasota, 1970). If only the nutritive value of a mushroom is assessed, the protein content can be calculated from the weights of the amino acids (*cf.* Hayes & Haddad, 1976; Weaver *et al.*, 1977), thus including the free amino acids in the protein figure. Since, however, free amino acids are of special nutritional and technological interest, because of their water solubility and participation in the Maillard reaction, it is often necessary to obtain information also about their amounts in mushrooms.

Digestibility values ranging from 44 to 92% (determined *in vivo* or *in vitro* enzymic techniques) have been reported for mushroom crude protein (6·25 × N) (Lintzel, 1941; Karkocha & Młodecki, 1962; Karkocha, 1968, 1969; Hayes & Haddad, 1976). Since the protein figure thus obtained also includes variable amounts of unavailable non-protein nitrogen compounds, the digestibility of the true mushroom protein is obviously better. This has to be borne in mind when evaluating the data of the nutritional value of mushroom protein.

The great discrepancies observed between different papers in the data for non-protein nitrogen compounds suggest that more uniformly accepted, well defined methods are needed for reliable and comparable data. Systematic investigations of the factors that affect the contents of non-protein nitrogen compounds in mushrooms should also be continued.

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PROPAGATION OF *LENTINUS EDODES* ON MODIFIED MALT SPROUTS MEDIUM FOR AMYLASE PRODUCTION

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ABSTRACT

The moisture, ash, total nitrogen and total organic carbon contents of malt sprouts extract and a synthetic medium used for fungal amylase production were determined and compared. The results revealed that these constituents varied greatly in the two media. Accordingly, various sources of carbon (glucose, maltose, dextrin and soluble starch), of nitrogen (ammonium nitrate, ammonium sulphate, peptone and yeast extract) and of mineral salts (calcium carbonate, potassium dihydrogen phosphate, magnesium sulphate, sodium chloride and zinc sulphate) were introduced into the media in order to modify the medium composition for Lentinus edodes *propagation to attain maximum production of amylase. High amylolytic activity and mycelium yield were attained in the presence of dextrin* (2·0%) *and yeast extract* (0·5%), *potassium dihydrogen phosphate and calcium carbonate* (0·05%).

INTRODUCTION

The amounts of various enzymes produced by micro-organisms in liquid media are affected by different variables. Of these variables, the type and components of the culture media are important. Underkofler (1954) and Barton-Larry *et al.* (1969) investigated the influence of nitrogen and carbon sources on the production of glycoamylase by *Aspergillus niger*. Corn steeping liquor and nutrient broth proved to be the most suitable nitrogen sources. Of various carbon sources tested, glucose yielded the highest level of enzyme. Kundu *et al.* (1973) studied the cultural and nutritional requirements of *Aspergillus oryzae* for the production of amylase under submerged conditions. Starch and sodium nitrate or ammonium nitrate were the best carbon and nitrogen sources, respectively. K^+, Na^+, Cl^- and SO_4^{--} ions had no effect on amylase yield but Ca^{++} was inhibitory. Hayashida (1975) investigated

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the optimum cultural conditions for the submerged production of three types of glucoamylase by *Aspergillus awamori*. Malt sprouts extract medium was demonstrated by El-Zalaki & Hamza (1979) to be promising for amylase production by the edible mushroom *Lentinus edodes*. Accordingly, the present work aimed to support and modify the malt sprouts extract to attain a medium suitable for propagating *Lentinus edodes* for maximum amylase production. The mycelium yield was also determined during propagation.

MATERIALS AND METHODS

Sources of materials

A culture of *Lentinus edodes* IFRI 2571 c, furnished by the Indian Forest Research Institute, was used. Malt sprouts were obtained from El-Ahram Beer Factory, Giza, Egypt.

Preparation of inoculum

A spore suspension of *Lentinus edodes* containing $2{\cdot}6 \times 10^8$ spores per millilitre was used as the inoculum. The number of spores in the suspension was determined by the indirect cell count technique according to De Moss & Anbard (1957).

Malt sprouts medium

Malt sprouts (120 g) were boiled with distilled water (500 ml) for one hour. The extract was filtered through cheese-cloth and diluted to one litre.

Different concentrations of various carbon, nitrogen and mineral salts were added separately to the basic extract to investigate their effect on the mycelium yield, protein content and amylase activity.

Lentinus edodes *propagation*

Portions of the malt sprouts extract (100 ml) were transferred to Erlenmeyer flasks (*ca.* 500 ml), sterilised at 121 °C for 20 min and cooled. Each flask was inoculated with one millilitre of the spore suspension then incubated at 30 °C (El-Zalaki & Hamza, 1979).

Synthetic medium

The medium recommended by Mahmoud *et al.* (1973) for fungal amylase production was used. Chemical reagents used were of the highest available laboratory grade.

Chemical methods

The amylolytic activity of the culture filtrate was determined colorimetrically according to the method described by Pmston (1964). Total organic carbon was

determined according to the method of Black *et al.* (1965). Total nitrogen, total solids and ash content were determined according to the methods of the AOAC (1970).

RESULTS AND DISCUSSION

Main chemical constituents of basic media

As the organic carbon and the total nitrogen content, as well as the ash content of the medium, are important factors affecting the microbial growth, these components were determined in the two media. The results, given in Table 1, reveal that the total organic carbon of the synthetic medium was four times that of malt sprouts extract

TABLE 1
MAIN CHEMICAL CONSTITUENTS OF SYNTHETIC AND MALT SPROUTS MEDIA (USED FOR *Lentinus edodes* PROPAGATION)

Chemical constituents	*Synthetic medium* (%)	*Malt sprouts extract* (%)
Moisture	94·7	87·5
Total organic carbon	32·4	8·18
Total nitrogen	0·12	1·16
(C/N ratio)	(22:1)	(7:10)
Ash	0·35	0·80

while the total nitrogen content of the latter medium was more than tenfold that of the first; this resulted in obvious variation of the C/N ratio of the media. The malt sprouts extract medium also contained a higher ash content compared with the synthetic medium.

Effect of carbon sources

Figure 1 illustrates the effect of incorporating various carbohydrates (at a concentration of 1 %) in the malt sprouts extract medium on the amylolytic activity of *Lentinus edodes*. The amylolytic activity reached its maximum on the fourth day of incubation upon the incorporation of dextrin. Lower amylolytic activity was obtained when either glucose or soluble starch were added. It was observed that the medium colour was changed to light brown in the presence of maltose.

The effect of adding different concentrations of dextrin and maltose to the malt sprouts extract medium was investigated as these two carbohydrates showed a pronounced effect on the induction of amylases by *Lentinus edodes*. The results,

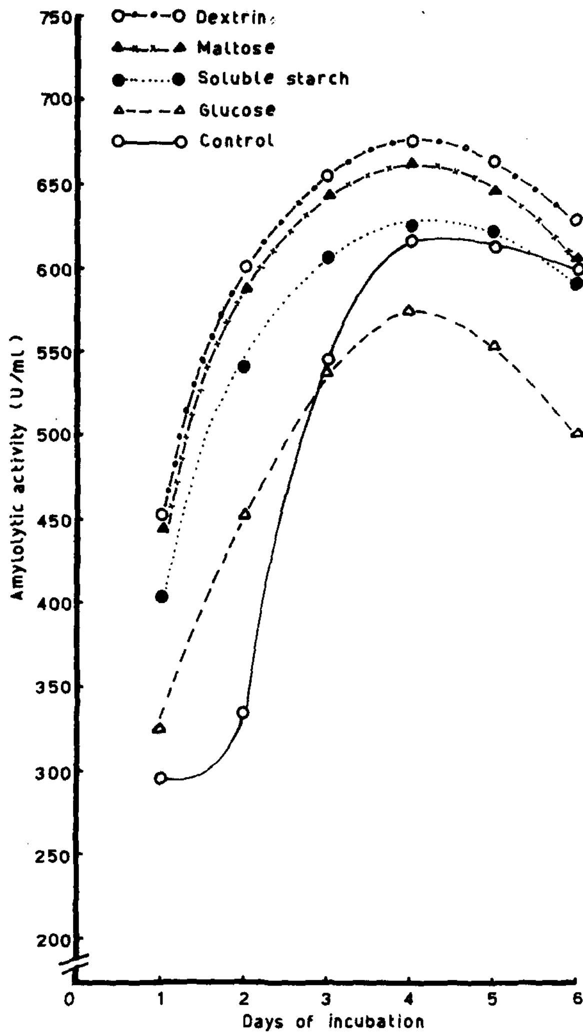

Fig. 1. Effect of some carbon sources on the amylolytic activity of *Lentinus edodes*.

given in Table 2, indicate that the maximum amylolytic activity and mycelium yield were noted after four days of incubation when either dextrin or maltose were added to the malt sprouts extract medium at concentrations of 1–2 %.

Effect of nitrogen sources

Ammonium nitrate, ammonium sulphate, peptone and yeast extract were added to the malt sprouts extract at concentrations of 1 %. The results, illustrated in Fig. 2,

TABLE 2

EFFECT OF DIFFERENT DEXTRIN AND MALTOSE CONCENTRATIONS ON AMYLOLYTIC ACTIVITY AND MYCELIUM YIELD OF *Lentinus edodes*

Concentration (%)	*Amylolytic activity (U/ml)*						*Mycelium yield (g/litre)*					
	Days of incubation											
	1	2	3	4	5	6	1	2	3	4	5	6
0·0	297·7	336·0	557·7	633·4	610·2	602·9	4·60	5·85	6·18	6·51	7·31	7·18
Dextrin												
0·5	450·3	532·0	597·0	617·0	590·5	583·1	6·00	7·31	7·72	8·34	7·12	6·00
1·0	471·6	598·1	646·2	678·2	668·7	631·2	5·91	7·87	8·53	9·87	7·70	6·91
2·0	476·9	599·2	649·4	684·2	630·1	599·4	5·93	8·16	8·30	10·51	9·27	8·00
3·0	499·4	518·0	620·0	685·4	600·0	538·2	6·00	7·90	8·27	10·51	8·64	7·00
0·0	292·6	228·1	553·3	618·2	614·0	600·0	4·65	5·81	6·00	6·77	7·73	6·81
Maltose												
1	444·6	578·9	637·0	682·1	648·8	606·8	5·09	7·43	7·91	8·62	7·00	5·54
2	445·0	593·2	643·2	680·0	629·8	603·2	5·21	7·21	8·87	8·82	7·00	6·84
3	442·0	590·0	638·4	663·7	621·7	602·2	5·29	7·00	8·66	9·60	7·00	6·40

TABLE 3

EFFECT OF YEAST EXTRACT CONCENTRATION ON AMYLOLYTIC ACTIVITY AND MYCELIUM YIELD OF *Lentinus edodes*

Concentration (%)	*Amylolytic activity (U/ml)*						*Mycelium yield (g/litre)*					
	Days of incubation											
	1	2	3	4	5	6	1	2	3	4	5	6
0·0	298·5	336·0	550·4	623·8	618·1	609·2	4·70	5·92	6·10	6·50	7·50	7·0
0·5	355·6	520·0	616·0	658·0	609·0	539·0	8·22	9·30	9·83	8·24	7·00	5·90
1·0	353·1	532·0	628·0	550·0	638·0	629·0	8·36	9·61	9·98	8·40	6·88	6·00
2·0	351·0	548·0	645·0	663·0	653·0	578·0	8·58	9·83	10·40	9·69	7·20	6·60

TABLE 4

EFFECT OF SOME MINERAL SALTS ON AMYLOLYTIC ACTIVITY AND MYCELIUM YIELD OF *Lentinus edodes**

Mineral salts	*Amylolytic activity (U/ml)*						*Mycelium yield (g/litre)*					
	Days of incubation											
	1	2	3	4	5	6	1	2	3	4	5	6
None added	299·7	338·1	547·4	619·2	623·1	612·5	4·60	5·88	6·20	6·39	7·40	7·19
Calcium carbonate	351·6	430·0	586·2	660·0	625·6	608·3	8·00	8·32	8·80	8·10	7·92	6·12
Potassium dihydrogen phosphate	359·0	420·7	613·1	677·7	621·7	579·3	8·32	8·42	8·70	8·58	7·73	6·71
Magnesium sulphate	319·7	350·9	581·4	630·6	601·0	55·8	8·01	7·38	7·98	6·00	6·92	6·11
Sodium chloride	345·1	310·8	562·4	582·2	612·8	604·0	8·40	7·70	8·00	7·20	6·80	5·42
Zinc sulphate	—	—	218·8	307·1	—	—	—	—	Weak growth	—	2·44	—

* Grown on malt sprouts extract medium.

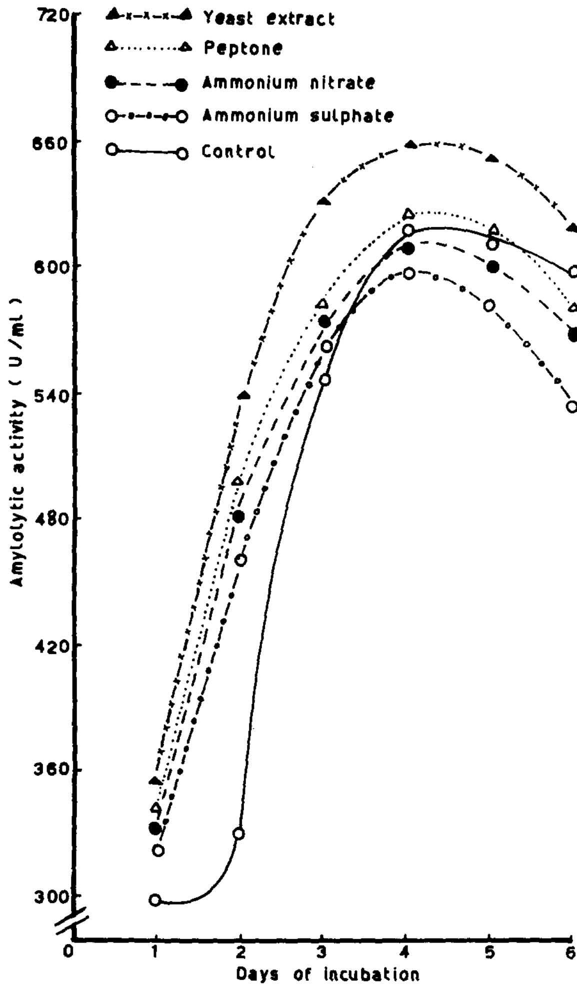

Fig. 2. Effect of some nitrogen sources on the amylolytic activity of *Lentinus edodes*.

indicate that yeast extract proved to be the best nitrogen source as it yielded the highest production of amylolytic activity after four days of incubation. Further studies revealed that addition of yeast extract to the malt sprouts extract at a concentration of 0·5 % resulted in an obvious increment of the amylolytic activity and increased the mycelium yield as shown in Table 3. A slight increase in the amylolytic activity was detected upon the addition of 2 % yeast extract.

Effect of mineral salts

The effect of adding some mineral salts at a concentration of 0·1 % on amylase

TABLE 5
EFFECT OF CALCIUM CARBONATE AND POTASSIUM DIHYDROGEN PHOSPHATE CONCENTRATION ON AMYLOLYTIC ACTIVITY AND MYCELIUM YIELD OF *Lentinus edodes*

Concentration (%)	*Amylolytic activity (U/ml)*						*Mycelium yield (g/litre)*					
	Days of incubation											
	1	2	3	4	5	6	1	2	3	4	5	6
None added												
0·0	295·7	330·1	552·5	610·9	619·0	598·4	4·51	5·78	6·22	6·20	7·40	7·10
Calcium carbonate												
0·02	340·2	420·2	600·5	658·4	640·3	540·3	7·97	8·23	9·87	8·65	7·01	5·95
0·05	342·0	430·7	612·9	660·0	609·0	581·0	8·02	8·30	9·87	8·68	7·30	6·00
0·10	347·0	440·0	596·0	660·0	611·4	594·4	9·30	9·30	10·00	9·10	7·92	6·10
Potassium dihydrogen phosphate												
0·02	319·5	400·1	588·2	612·0	592·7	540·6	9·52	9·52	9·98	8·70	6·80	5·70
0·05	319·5	402·2	600·6	638·0	596·7	566·7	8·18	9·70	10·50	9·32	7·22	6·43
0·10	349·0	421·0	617·0	670·0	630·0	583·0	8·32	9·92	10·20	9·50	7·72	6·72

production by *Lentinus edodes* grown on malt sprouts extract medium was investigated. The results, given in Table 4, show that potassium dihydrogen phosphate markedly increased the amylolytic activity. Maximum activity was attained on the fourth day of incubation. The amylolytic activity slightly increased in the presence of magnesium sulphate and calcium carbonate but slightly decreased in the presence of sodium chloride. No amylolytic activity was detected during the first two days after the addition of zinc sulphate but slight amylolytic activity was attained on the third and the fourth days of incubation, after which the amylolytic activity diminished. Further studies on the effect of various concentrations of potassium dihydrogen phosphate and calcium carbonate, which proved to be the most effective mineral salts for amylolytic activity and mycelium yield, revealed that a concentration of 0·05 % was preferable (Table 5).

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STABILITY OF SUGAR ACETATES IN CITRIC ACID SOLUTION

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ABSTRACT

The stability of six sugar acetates in 10% citric acid solution was investigated. Quantitative thin-layer chromatography was used to monitor the hydrolysis of acetate groups. The times taken for 50% hydrolysis ($t_{0.5}$) *varied from 35 h for sucrose octaacetate to 110 h for raffinose hendecaacetate. The stability of sucrose octaacetate was determined at concentrations of 0·5, 1·5, 4 and 10% citric acid. The rate of hydrolysis showed a marked dependence on the pH of the citric acid solution. Primary acetate groups are hydrolysed more readily than secondary acetate groups.*

INTRODUCTION

Increase in the lipophilicity of a sugar molecule by chemical modification (e.g. esterification, etherification, glycosidation or the formation of certain deoxy-halo derivatives) results in the molecule becoming insoluble (Birch & Lee, 1976). Sugar acetates have already been referred to as possible food additives with peculiar solubility properties, due to the combined lipophilic and polar characteristics of the acetate group (Birch *et al.*, 1970; Lee, 1977). In general, the greater the number of acetate groups in a particular sugar molecule, the more soluble is it likely to be in fats and oils. However, an increase in the number of acetate groups generally leads to a decrease in the solubility of the compound in water.

Sugar esters are already recognised as useful surface active additives for food purposes, but their use may be limited by their interaction with other food

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components such as minerals and acids which might catalyse some degree of hydrolysis. Generally sugar esters are unstable in alkaline environments but exhibit good stability in acids. However, such chemical generalisations may not be applicable to the long periods of shelf-life expected of pasteurised beverages or other foodstuffs. The stability of sugar acetates in citric acid solution was therefore investigated.

MATERIALS AND METHODS

Sugar acetates

Commercially available sugars or their alditols were acetylated with acetic anhydride in pyridine at 50–60 °C for 1 to 3 h and recovered in the usual way. Where necessary, the acetates were purified by column chromatography on silica gel. 6,1′,6′-Tri-*O*-methylsucrose pentaacetate was synthesised as described previously (Lindley *et al.*, 1975).

Preparation of solutions

Hydrolysis studies were performed at both high and low concentrations of acid and sugar acetate, keeping the ratio between the acid and sugar acetate constant (50:1).

(A) *High concentration:* A sample (100 mg) of the sugar acetate was dissolved in 10 % citric acid (50 ml). Ethanol (2 ml) was used to facilitate the dissolution of the sugar acetate. Aliquots (5 ml) were withdrawn at 24-h intervals and analysed by quantitative thin-layer chromatography as described below.

(B) *Low concentration:* The sugar acetate (20 mg) was dissolved in ethanol (0·5 ml) and 0·5 % citric acid (200 ml) was added. Aliquots (20 ml) were withdrawn at weekly intervals and analysed by quantitative thin-layer chromatography as described below.

For sucrose octaacetate, two additional solutions containing 80 and 30 mg sucrose octaacetate in 100 ml citric acid solutions (4 % and 1·5 %, respectively) were prepared. All hydrolyses were monitored at room temperature.

Quantitative thin-layer chromatography (tlc)

Each aliquot was extracted with chloroform (2 × 10 ml), washed with water (2 × 10 ml) and concentrated (temperature below 40 °C) to a small volume. Each of these extracts was analysed by thin-layer chromatography on pre-coated glass plates of silica gel 60 (0·25 mm; Merck) using *n*-butyl acetate-pyridine-water (5:3:1) as eluant. Quantities of an extract in the ratio of 1:2:3 were applied to the plate, developed in the above solvent system and the components detected with dilute sulphuric acid (140 °C). The proportion of sugar peracetate remaining after a certain period was determined quantitatively by comparison of the intensity of the spot

corresponding to the initial sugar peracetate with those of the slower moving de-*O*-acetylated compound in the same solution. From this ratio of sugar peracetate to hydrolysis product(s), the percentage of the initial sugar peracetate remaining was calculated.

De-O-acetylation of sucrose octaacetate in citric acid solution

Sucrose octaacetate (2 g) was dissolved in ethanol (50 ml) and citric acid (1 %, 150 ml) was added. After three months at room temperature, no sucrose octaacetate was detected (tlc) and approximately equal quantities of three slower moving compounds (on tlc) had formed. The solution was diluted with water (200 ml) and extracted with chloroform (2 × 200 ml). The combined chloroform extracts were washed with water, dried (sodium sulphate) and concentrated to a syrup. The residue was absorbed onto silica gel, applied to a column of dry silica gel and eluted with benzene-ethyl acetate (15:1). The three products of hydrolysis (in order of elution) were: (a) A syrup, which was shown by nmr spectroscopy and elemental analysis (Found: C, 49·5; H, 5·5 %. Calculated for $C_{26}H_{36}O_{18}$: C, 49·1; H, 5·7 %) to be a sucrose heptaacetate. It had $[\alpha]_D$ +49·7° (*c*. 1·0, chloroform), in good agreement with that reported (Buchanan *et al*., 1972; Ballard *et al*., 1972) for 2,3,4,6,1′, 3′, 4′-hepta-*O*-acetylsucrose. The identity was confirmed by treatment with triphenylmethyl chloride which produced a crystalline mono-*O*-trityl derivative having a melting point of 116–118° and $[\alpha]_D$ +58° (*c*. 1·5, chloroform), identical to those reported in the literature (Buchanan *et al*., 1972). (b) A syrup, which had the nmr spectrum and elemental analysis (Found: C, 47·9; H, 5·8 %. Calculated for $C_{24}H_{34}O_{17}$: C, 48·5; H, 5·7 %) of a sucrose hexaacetate. Tritylation produced a crystalline di-*O*-trityl derivative having a melting point of 103–105° and $[\alpha]_D$ +65·2° (*c*. 1·2, chloroform), in good agreement with those reported (Hough *et al*., 1972) for 2,3,4,1′, 3′, 4′-hexa-*O*-acetyl-6,6′-di-*O*-tritylsucrose. (c) The third component was also isolated as a syrup; Nmr spectroscopy and elemental analysis indicated that it was a penta-*O*-acetylsucrose derivative. The compound was not further characterised.

^{1}H-Nmr spectra were recorded for solutions in $CDCl_3$ with a Varian HR-220 spectrometer, Me_4Si being the internal standard. The degrees of acetylation or tritylation were determined by comparing the proton integration in the acetyl–proton region (~τ8) or aromatic–proton region (~τ2–3) with the integration for the remaining protons (~τ4–7) of compounds (a), (b) and (c) or their *O*-trityl derivatives (where applicable).

RESULTS AND DISCUSSION

It was found that breakdown of sucrose octaacetate was soon detectable in aqueous citric acid solutions and that some of the acetate groups were slowly being

hydrolysed in the acidic medium. The three products of hydrolysis of sucrose octaacetate were isolated by column chromatography on silica gel. They were shown by nmr spectroscopy and elemental analysis to be (in order of elution from the column) the mono-, di- and tri-hydroxy derivatives of sucrose octaacetate. The two faster moving compounds were identified as 2,3,4,6,1′,3′,4′-hepta-*O*-acetylsucrose and 2,3,4,1′,3′,4′-hexa-*O*-acetylsucrose by characterisation as their mono- and di-*O*-trityl derivatives, respectively. It therefore appears that the primary acetate groups on O-6 and O-6′ of sucrose octaacetate are hydrolysed more rapidly in citric acid solution than the secondary acetate groups. The relative rate of hydrolysis of the primary acetate groups of sucrose octaacetate decreases in the order AcO-6′ > AcO-6 > AcO-1′. These partially acetylated sucrose derivatives are all more soluble in water than sucrose octaacetate (Lee, 1977) and account for the overall loss of sucrose octaacetate on storage in citric acid solution.

Relative rates of hydrolysis of sugar acetates in citric acid solution

Since it was found that the primary acetate groups of sucrose octaacetate were hydrolysed more rapidly than the secondary acetate groups, 6,1′,6′-tri-*O*-methylsucrose pentaacetate was synthesised (Lindley *et al.*, 1975) to compare its stability in acid solution with that of sucrose octaacetate. Methyl ether groups are known to be much more stable than corresponding acetate groups under these acidic conditions.

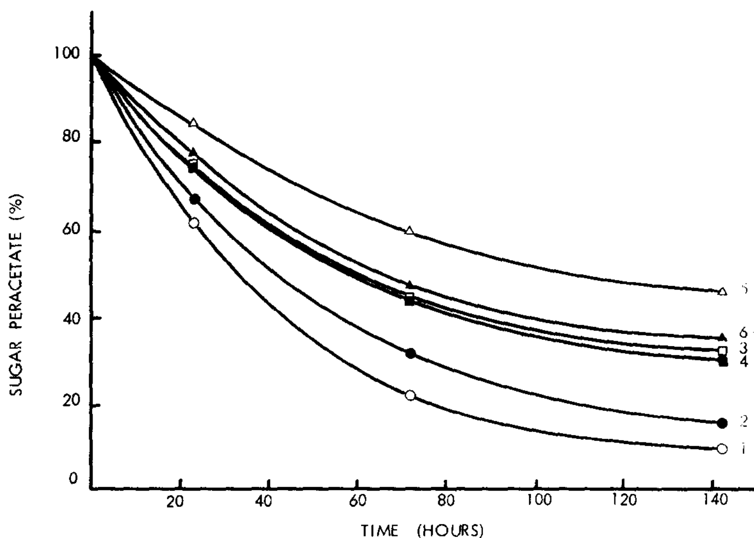

Fig. 1. Hydrolysis of 0·2% solutions of sucrose octaacetate (1), maltitol nonaacetate (2), methyl α-D-glucopyranoside tetraacetate (3), xylitol pentaacetate (4), raffinose hendecaacetate (5) and trimethylsucrose pentaacetate (6) in 10% citric acid solution.

The rates of de-*O*-acetylation of the following six sugar acetates in citric acid solution were determined: sucrose octaacetate (1), maltitol nonaacetate (2), methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (3), xylitol pentaacetate (4), raffinose hendecaacetate (5) and 6,1′,6′-tri-*O*-methylsucrose 2,3,4,3′,4′-pentaacetate (6). The results of this study over a period of six days are shown in Fig. 1 and the times taken for 50 % hydrolysis ($t_{0.5}$) of the sugar acetates are given in Table 1. The trisaccharide

TABLE 1

TIMES FOR 50 % HYDROLYSIS ($t_{0.5}$) OF COMPOUNDS 1–6 UNDER CONDITIONS A

Compound	1	2	3	4	5	6
$t_{0.5}$(h)	35	45	60	60	110	65

peracetate (5) is much more stable than any of the other sugar acetates. As expected, the tri-*O*-methylsucrose acetate (6) is more stable than sucrose octaacetate (1), but the secondary acetate groups are also hydrolysed at a significantly fast rate. Since the glucose acetate (3) is more stable than sucrose octaacetate, it is presumably the lability of the AcO-6′ group (as shown in the previous section) that accounts for the instability of sucrose octaacetate in acid solution.

To determine the stability of sugar acetates in more dilute acid solution, the rate of hydrolysis of sucrose octaacetate (1) was compared with that of maltitol nonaacetate

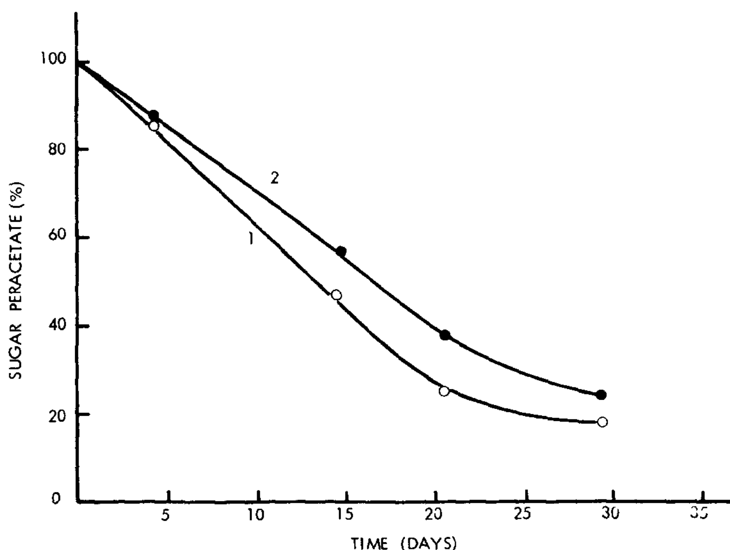

Fig. 2. Hydrolysis of 0·01 % solutions of sucrose octaacetate (1) and maltitol nonaacetate (2) in 0·5 % citric acid solution.

TABLE 2
COMPOSITIONS OF, AND $t_{0\cdot5}$ VALUES OBTAINED FOR, SOLUTIONS a–d

Solution	*Sucrose octaacetate (%)*	*Citric acid (%)*	*pH*	$t_{0\cdot5}$ (*h*)
a	0·20	10	1·70	34
b	0·08	4	1·91	76
c	0·03	1·5	2·13	126
d	0·01	0·5	2·37	220

(2) under condition B. The results of this investigation over a period of four weeks are shown in Fig. 2. As was found under the more concentrated conditions above, maltitol nonaacetate is hydrolysed slower than sucrose octaacetate, but not to a very significant extent ($t_{0\cdot5}$ values of 17 and 13 days, respectively).

Rates of hydrolysis of sucrose octaacetate in various concentrations of citric acid

The rates of hydrolysis of sucrose octaacetate were determined at four concentrations of citric acid, ranging from condition A to B (Table 2). The ratio of acid to sugar acetate was kept constant (50:1) for all four solutions. The pH of each solution was measured using a combination electrode connected to a Pye model 290 pH meter. The results of these hydrolysis studies are shown in Fig. 3 and the

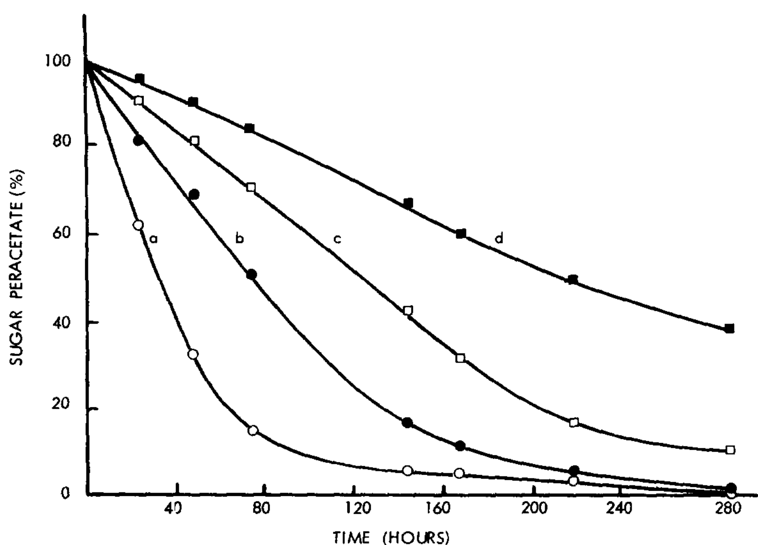

Fig. 3. Hydrolysis of (a) 0·2 %, (b) 0·08 %, (c) 0·03 % and (d) 0·01 % solutions of sucrose octaacetate in (a) 10 %, (b) 4 %, (c) 1·5 % and (d) 0·5 % citric acid solutions.

TABLE 3
VALUES OF LOG V OBTAINED FOR SOLUTIONS a–d

Solution	V	*Log* V
a	$3{\cdot}13 \times 10^{-3}$	$-2{\cdot}504$
b	$5{\cdot}88 \times 10^{-4}$	$-3{\cdot}231$
c	$1{\cdot}08 \times 10^{-4}$	$-3{\cdot}967$
d	$1{\cdot}54 \times 10^{-5}$	$-4{\cdot}812$

corresponding $t_{0{\cdot}5}$ values are given in Table 2. The initial rates of hydrolysis (V) may be determined from the initial slope of each of the curves in Fig. 3. Hence:

$$\text{initial rate } (V) = \frac{\Delta \text{ (sugar acetate) } (\%)}{\text{Time (h)}} \tag{1}$$

From the values obtained for $\log V$ (Table 3) and pH (Table 2), a graph may be drawn relating these two functions (Fig. 4). The slope of the line is $-3{\cdot}42$ and the intercept on the $\log V$ axis (at pH $= 0$) is $3{\cdot}35$. Hence the equation of the graph is:

$$\log V = 3{\cdot}35\text{–}3{\cdot}42 \text{ pH} \tag{2}$$

To illustrate the marked effect of change of pH on the initial rate of hydrolysis of sucrose octaacetate, the following example may be considered. For a solution of sucrose octaacetate having an initial concentration of 0·01 %, the times required for this concentration to decrease to 0·0099 % (i.e. 1 % hydrolysis or Δ(sucrose

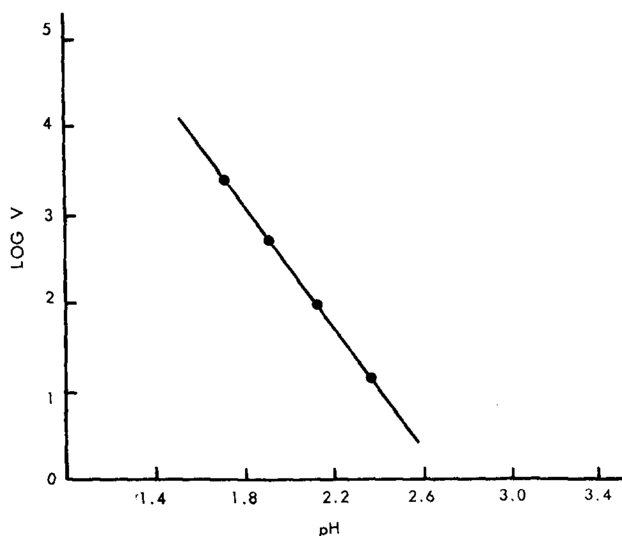

Fig. 4. Graph of log V versus the pH of solutions a–d.

TABLE 4
CALCULATION OF THE TIME REQUIRED FOR 1 % HYDROLYSIS OF SUCROSE OCTAACETATE AT VARIOUS pH VALUES

pH	*Log* V	V	*Time*
2·0	−3·490	$3{\cdot}24 \times 10^{-4}$	0·3 h
2·2	−4·174	$6{\cdot}70 \times 10^{-5}$	1·5 h
2·4	−4·858	$1{\cdot}39 \times 10^{-5}$	7·2 h
2·6	−5·542	$2{\cdot}87 \times 10^{-6}$	34·8 h
2·8	−6·226	$5{\cdot}94 \times 10^{-7}$	7 days
3·0	−6·910	$1{\cdot}23 \times 10^{-7}$	34 days
3·2	−7·594	$2{\cdot}55 \times 10^{-8}$	163 days
3·4	−8·278	$5{\cdot}27 \times 10^{-9}$	2 years

acetate) = 0·0001 %) at various pH values can be calculated from eqns. (1) and (2), assuming eqn. (2) holds throughout the range. From eqn. (1):

$$\text{Time (h)} = \frac{\text{(Sucrose acetate) (\%)}}{V} = \frac{10^{-4}}{V}$$

The results of these calculations are given in Table 4. The extent to which the initial rate of hydrolysis depends on the pH of the citric acid solution can clearly be seen.

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OLIGOSACCHARIDE FORMATION DURING HYDROLYSIS OF LACTOSE WITH *SACCHAROMYCES LACTIS* LACTASE (MAXILACT®): PART 2—OLIGOSACCHARIDE STRUCTURES*

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ABSTRACT

The oligosaccharides produced during the hydrolysis of lactose with Saccharomyces lactis *lactase were isolated and structurally analysed.*

Six oligosaccharides with two to four monosaccharide units were studied. All were linear and had one or two galactosyl residues linked β-(1 → 6)- to glucose, galactose or lactose. Thus, in addition to lactose hydrolysing activity, the enzyme has transglycosylation activity with high specificity for formation of β-(1 → 6)-galactosidic linkages.

INTRODUCTION

Lactose hydrolysis in milk makes it possible for individuals with milk intolerance due to low intestinal lactase activity to consume milk without side effects (Paige *et al.*, 1975; Gudmand-Hoyer & Simony, 1977). It also has certain technological advantages (Bouvy, 1975). Lactose splitting enzymes are present in a wide variety of organisms, but for technical production of the enzyme only selected strains of various microorganisms such as *Saccharomyces torulopsis*, *Saccharomyces fragilis*, *Saccharomyces lactis*, *Escherichia coli* and *Aspergillus niger* have been used (Olling, 1972). It has been known for a long time that during the enzymic hydrolysis of lactose, different oligosaccharides are biosynthesised, some of which have been structurally characterised (e.g. Pazur *et al.*, 1958; Ballio & Russi, 1960). In the preceding paper in this series (Burvall *et al.*, 1979*a*) it was demonstrated that the

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oligosaccharide formation is considerable in concentrated solutions of lactose. It has also been shown that these oligosaccharides are very slowly hydrolysed by human intestinal β-galactosidase (Burvall *et al.*, 1979*b*), eventually leading to intestinal discomfort, mainly flatulence, because of bacterial degradation in the large bowel (Asp *et al.*, 1977). In order to determine which compound or compounds are involved in these side effects it was necessary to isolate the oligosaccharides formed and to establish their structures.

MATERIALS AND METHODS

Preparation of oligosaccharides

Lactose, analytical grade, was obtained from Mallinckrodt Chemical Works. It contained 0·1 % saccharides other than lactose. One thousand millilitres of a 20 % solution of lactose in 0·05M potassium phosphate buffer, pH 6·8, was incubated for 16 h at 37 °C with 80 mg *Saccharomyces lactis* lactase (Maxilact 40 000, Gist Brocades NV, Delft, The Netherlands). The reaction was stopped by heating the reaction mixture at 70 °C for 5 min.

6'galactosyl lactose was isolated from human milk as described by Yamashita & Kobata (1974).

Gel chromatography was performed on a Sephadex G-15 Column (5 × 200 cm; void volume = 1300 ml; flow rate = 45 ml/h; eluted with distilled water). Eluted fractions (15 ml) were analysed for total hexose using the anthrone method (Scott & Melvin, 1953). Gel chromatographic fractions were purified by preparative paper chromatography on Whatman No. 3 papers. The following solvent systems were used:

(A) Ethyl acetate:pyridine:water (10:4:3; v/v)
(B) Butan-1-ol:pyridine:water (6:4:3; v/v)
(C) Ethyl acetate:acetic acid:water (3:1:1; v/v)
(D) Ethyl acetate:pyridine:acetic acid:water (5:5:1:3; v/v)
(E) Propan-1-ol:ethyl acetate:water (6:1:3; v/v)
(F) Propan-1-ol:ethyl acetate:water (42:35:23; v/v)

Papers were stained with a silver dip reagent (Trevelyan *et al.*, 1950). Sugar analysis was performed by GLC (the abbreviations used are: GLC—gas–liquid chromatography; MS—mass spectrometry; Gal—D-galactose: Glc—D-glucose; Me—methyl; R_L—$R_{Lactose}$; *T*—relative retention time) (Sawardeker *et al.*, 1965) and MS (Golovkina *et al.*, 1966) after hydrolysis (4M trifluoracetic acid, 4 h, 100°). Optical rotation was recorded using a Perkin Elmer 241 polarimeter. Methods for methylation analysis and methylation of reduced oligosaccharides have been described elsewhere (Björndal *et al.*, 1970; Lundblad *et al.*, 1975). A Perkin Elmer model 3920 Gas Chromatograph was used under the following conditions: (a) glass

column, 2 m, packed with 3% ECNSSM on Gas Chrom Q (100–200 mesh) at a column temperature of 185–210° for sugar alditol acetates and at 160° for partially methylated alditol acetates; (b) glass capillary column (25 m × 0·25 mm), wall coated with SE-30 (LKB, Stockholm, Sweden) at a column temperature of 160° for partially methylated alditol acetates and at 210–310° for permethylated alditol derivatives of di- to tetrasaccharides. For GLC–MS the same column was used on a Varian MAT 311 A combined GLC–MS instrument. Glass capillary columns were connected directly to the ion source of the instrument. The mass spectra were recorded at an ionisation potential of 70 eV, an ionisation current of 1 mA and an ion source temperature of 120°. All data were processed by an on-line computer system (Spectro-system 100 Varian MAT).

RESULTS AND DISCUSSION

Isolation of oligosaccharides

Forty millilitres of the enzymic hydrolysate, containing about 2 g of sugar, were fractionated on a Sephadex G-15 column. Eluted fractions were analysed for total hexose (Fig. 1) and the material was pooled into fraction A–E. Fraction E was found to contain only monosaccharides (70% of total hexoses in the hydrolysate) and was discarded. The material in each fraction A–D was subjected to preparative paper

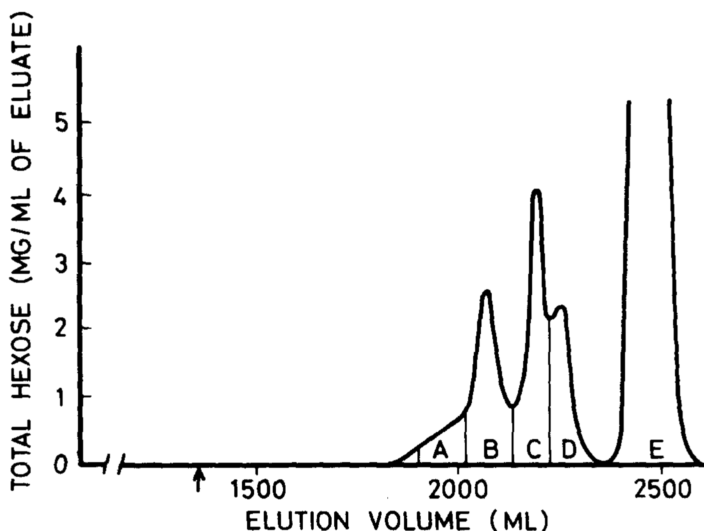


Fig. 1. Gel chromatographic (Sephadex G-15) profile of a hydrolysate of lactose obtained after digestion with *Saccharomyces lactis* lactase. Eluted fractions were anlysed for total hexose. (Void volume = 1300 ml is indicated with an arrow.)

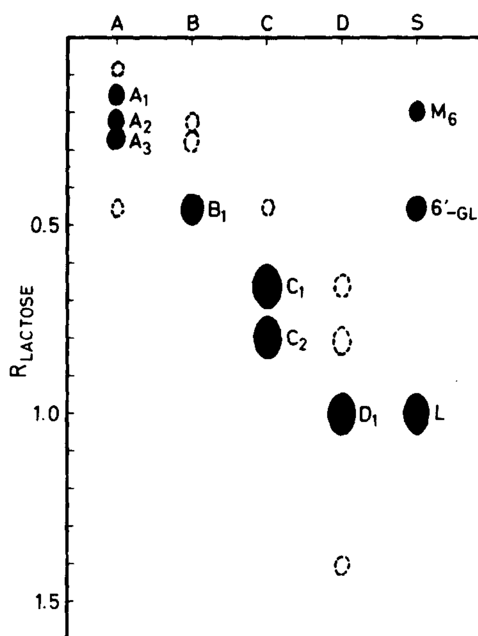


Fig. 2. Paper chromatographic distribution of material in gel chromatographic fractions A to D. The chromatogram was developed in system (A) and visualised with a silver-dip reagent (Trevelyan *et al.*, 1950). Standard compounds (S) used are: Maltohexaose (M_6), G-galactosyl lactose (G′-GL) and lactose (L).

chromatography with solvent (A) for 6, 3, 1·5 and 1·5 days, respectively. Seven major sub-fractions, denoted A_1–A_3, B_1, C_1–C_2 and D_1, were obtained, as shown in Fig. 2. Further purification of the different sub-fractions was achieved by rechromatography as follows: A_1–A_3 in solvent (A) for six days, B_1 in solvent (A) for three days, C_1–C_2 in solvent (B) for three days and D_1 in solvent (B) for three days. Column chromatography on Bio-Gel P-2 was performed as a final step to remove impurities extracted from the chromatography papers and solvent.

Characterisation of oligosaccharides

All seven sub-fractions were homogenous in six different solvent systems (Table 1). The results obtained from sugar analysis, methylation analysis and optical rotation measurements are presented in Tables 2 and 3. No determination of the absolute configuration of the monosaccharides was performed but it is assumed that they all have the D-configuration. All monosaccharides were found by methylation analysis to be in the pyranoside form.

A_1: Fraction A_1 was shown by sugar analysis to contain D-galactose and D-glucose in the relative molar proportions 3:1. Acid hydrolysis of the permethylated and

TABLE 1
MOBILITY IN DIFFERENT SOLVENT SYSTEMS OF ISOLATED FRACTIONS

Solvent system	*R_L values*						
	A_1	A_2	A_3	B_1	C_1	C_2	D_1
A	0·15	0·22	0·26	0·45	0·66	0·80	1·00
B	0·18	0·28	0·33	0·50	0·71	0·84	1·00
C	0·12	0·24	0·26	0·40	0·72	0·86	1·00
D	0·33	0·41	0·46	0·65	0·76	0·88	1·00
E	0·33	0·48	0·53	0·62	0·85	0·96	1·00
F	0·16	0·29	0·32	0·45	0·75	0·89	1·00

TABLE 2
ANALYTICAL DATA ON THE FRACTIONS ISOLATED FROM REGIONS A TO D

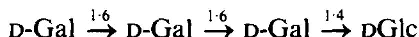
Fraction	*Total yield (mg)*	$[\alpha]_D^{20}$	*Analytical solution (mg/ml)*	*Relative molar proportions*		*% Carbohydrate in dry weight*
				Galactose	*Glucose*	
A_1	4·6	+26°	1·143	2·8	1·0	95
A_2	5·3	+21°	1·068	+	–	100
A_3	9·2	+16°	1·840	1·9	1·0	98
B_1	13·6	+35°	1·359	2·0	1·0	100
C_1	11·3	+31°	1·133	+	–	95
C_2	13·7	+30°	1·367	1·0	1·0	100
D_1	12·5	+52°	1·270	1·0	1·0	100

TABLE 3
METHYL ETHERS OBTAINED IN THE METHYLATION ANALYSIS OF THE REDUCED ($NaBD_4$) FRACTIONS

Methyl ethers	*T-values[a] ECNSS-M*	*Relative molar proportions[b]*						
		A_1	A_2	A_3	B_1	C_1	C_2	D_1
1,2,3,5,6-penta-*O*-Me-Glc-1-*d*	0·38	0·5	—	—	0·8	—	—	0·8
1,2,3,4,5-penta-*O*-Me-Glc-1-*d*	0·40	—	—	0·7	—	—	0·8	—
1,2,3,4,5-penta-*O*-Me-Gal-1-*d*	0·46	—	0·5	—	—	0·8	—	—
2,3,4,6-tetra-*O*-Me-Gal	1·25	1·0	1·0	1·0	1·0	1·0	1·0	1·0
2,3,4-tri-*O*-Me-Gal	3·41	2·1	1·0	1·2	1·0	—	—	—

[a] Retention times of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] Substantial amounts of the penta-*O*-methyl ethers are lost in the methylation procedure because of their high volatility.

reduced ($NaBD_4$) derivative indicated one terminal non-reducing D-galactose residue, two 1- and 6-disubstituted D-galactose residues and one reducing 4-substituted D-glucose residue. From these data it can be concluded that A_1 is a linear tetrasaccharide with the following sequence:



$$\text{D-Gal} \xrightarrow{1\cdot6} \text{D-Gal} \xrightarrow{1\cdot6} \text{D-Gal} \xrightarrow{1\cdot4} \text{DGlc}$$

The low optical rotation ($[\alpha]_D^{20}$ + 26°) indicates that all glycosidic linkages have the β-configuration. The complete structure of compound A_1 is given in Table 4.

TABLE 4

PROPOSED STRUCTURES OF THE ISOLATED OLIGOSACCHARIDES

Fraction	*Structure*
A_1	β-D-Gal-(1 → 6)-β-D-Gal-(1 → 6)-β-D-Gal-(1 → 4)-D-Glc
A_2	β-D-Gal(1 → 6)-β-D-Gal-(1 → 6)-D-Gal
A_3	β-D-Gal-(1 → 6)-β-D-Gal-(1 → 6)-D-Glc
B_1	β-D-Gal-(1 → 6)-β-D-Gal-(1 → 4)-D-Glc
C_1	β-D-Gal-(1 → 6)-D-Gal
C_2	β-D-Gal-(1 → 6)-D-Glc
D_1	β-D-Gal-(1 → 4)-D-Glc (= lactose)

A_2, A_3 and B_1: These fractions were all found by sugar and methylation analysis to be linear trisaccharides. All compounds had low optical rotations, indicating that all glycosidic linkages have the β-configuration. Complete structures are given in Table 4. The analytical data for compound B_1 were identical with those obtained for an authentic sample of 6'galactosyl lactose.

C_1, C_2 and D_1: These three fractions were all disaccharides with β-glycosidic linkages. Their structures are given in Table 4. In addition to these disaccharides, fraction D contained one compound with higher mobility than lactose. This gave a very weak spot in the paper chromatogram and was not studied further.

The formation of oligosaccharides during lactase treatment of lactose has been studied by several authors. Our compounds B_1, C_1 and C_2 were previously found in hydrolysates of lactose using lactase from *Saccharomyces fragilis* (Pazur *et al.*, 1958) and *Penicillium chrysogenum* (Ballio & Russi, 1960). The trisaccharides B_1 and A_3 were also reported to be present (Pazur *et al.*, 1958) whereas compounds A_1 and A_2 have not, to our knowledge, been identified in these types of lactose hydrolysate before.

It is interesting that in the *Saccharomyces lactis* hydrolysate of lactose only 1 → 6 galactosidic linkages had been formed, indicating a more restricted specificity for the galactosidase present in this organism as compared with others, where also significant amounts of oligosaccharides with 1 → 3 galactosidic linkages are formed (Pazur *et al.*, 1958; Ballio & Russi, 1960).

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ENZYME ACTIVITIES IN 'NON-OILSEED' SUNFLOWERS AND SUNFLOWER PRODUCTS (VARIETY GREYSTRIPE)

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ABSTRACT

Amylase, protease, cellulase and hemicellulase activities of 'non-oilseed' sunflower products (unshelled sunflower seeds, sunflower kernels and hulls as well as roasted unshelled sunflower seeds and sunflower kernels) were determined. Amylase and cellulase activities were highest in unshelled sunflower seeds and lowest in the shelled kernels. Protease and hemicellulase activities were highest in the kernels and lowest in the hulls. Enzyme activities were reduced by roasting.

INTRODUCTION

The sunflower, *Helianthus annuus*, is a native American wild flower. It is now a staple crop in many parts of the US (Robertson, 1975; Betschart *et al.*, 1975).

Two types of sunflowers are grown commercially—the small-seeded high-oil types (40–50 % oil) which are processed for oil, and the large-seeded, low-oil (21–33 %) human food and birdseed types referred to as 'non-oilseed' varieties (Robertson, 1975). Both types of sunflowers are grown mainly in Minnesota and North Dakota. California, however, also produces a rather large quantity of low-oil varieties.

The production of 'non-oilseed' sunflowers has increased considerably over the last five years. About 50 % of these sunflowers are used for human food and the other 50 % are sold as birdseed or as feed for hamsters and other pets. For human consumption the seeds may be dehulled and eaten raw or heated in an oven at 150 °C to create a toasted flavour.

Several food product applications for sunflower seeds and meals have been mentioned in the literature. Talley *et al.* (1972) described the use of dehulled and roasted sunflower kernels as a nut substitute in confectionery and bakery

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formulations. In cakes, cookies and pies sunflower kernels were found to be very acceptable. Removal of oil from high-oil sunflower varieties leaves a meal which can be a valuable source of protein, although the protein is somewhat limiting in lysine. The meals from oilseed and non-oilseed varieties of sunflowers have been used in the preparation of protein-enriched breads (Rooney *et al.*, 1972; Lorenz, 1978).

The activities of certain enzymes in sunflower products might have to be considered when such products are used as ingredients in foods. However, with the exception of the lipases (Banu & Serban, 1970), enzyme activities of sunflowers and sunflower products have not been thoroughly investigated.

It was the purpose of this study to determine the activities of different enzymes—amylases, proteases, cellulases, and hemicellulases—in a 'non-oilseed' variety of sunflowers.

MATERIALS AND METHODS

Sample identification and preparation

Sunflower seeds of the 'non-oilseed' variety Greystripe, grown in California, were used in this study. Unshelled sunflower seeds, sunflower kernels and hulls as well as roasted (1 h at 150 °C) unshelled sunflower seeds and sunflower kernels were ground into a flour using a Laboratory Apparatus Co. 'Micro-Mill.' The small grinding chamber of the mill was completely enclosed during the entire grinding procedure, eliminating any possible sample loss. The grinding chamber temperature was maintained by means of a heat exchanger encircling the grinding chamber, which assured a uniformly low temperature during grinding and prevented any possible change in enzyme activity of the samples under study.

Analytical methods

Moisture, protein, ash, crude fat and crude fibre were determined as described in AACC approved methods 44-15A, 46-11, 08-01, 30-10, and 32-15, respectively (AACC, 1962). Protein is expressed as Kjeldahl N × 6·25 %.

Enzyme activity measurements

Amylase activity was determined as described by Bernfeld (1955). One gramme of each ground sunflower product was extracted with 10 ml of 0·1 M sodium acetate buffer (pH 4·75) for 30 min at 37 °C. The mixture was centrifuged at 27000 g for 10 min. Aliquots of the supernatant were incubated with 1 ml of the starch substrate at 37 °C, for periods of 10, 20, 30 and 60 min. Using a maltose standard curve, amylase activity was calculated and expressed as mg of maltose per ml of extract.

Protease activity was determined using a modification of a method described by Bushuk & Hwang (1971). One gramme of each ground sunflower product was extracted with 10 ml of 0·2 M sodium acetate buffer (pH 3·8) for 30 min at 37 °C,

and the mixture was centrifuged at 27000 g for 10 min. One ml aliquots of the supernatant were incubated with 2 ml of the haemoglobin substrate for 10, 20, 30 and 60 min, respectively. Protease activity was calculated using a tyrosine standard curve and expressed as μg of tyrosine per ml of extract. Controls were included in both amylase and protease analyses and consisted of assays of aliquots of extracts boiled to inactivate the enzymes.

For cellulase and hemicellulase activities 1 g of each ground sunflower product was extracted with 20 ml of 0·6 % NaCl for 30 min at 5 °C, followed by centrifugation at 27000 g for 10 min. The supernatants were used for the determination of both cellulase and hemicellulase activity.

For cellulase activity a modified procedure of Schmitz *et al.* (1974) was used. The sunflower extracts (2 ml aliquots) were incubated at 37 °C with 2 ml of carboxymethyl cellulose (1·0 mg per ml in 0·02M sodium acetate buffer, pH 5·0, containing 0·6 % NaCl) to measure viscosity reduction. Viscosities were determined in Ostwald flow-type viscometers of 2 ml sample size. Measurements were taken every 30 min for a total of 3 h. Cellulase activity is expressed as percent viscosity reduction.

For hemicellulase activity, 2 ml of the sunflower extracts were incubated at 37 °C with 2 ml of arabinogalactan (10 mg per ml in 0·02M sodium acetate buffer, pH 5·0, containing 0·6 % NaCl) to measure viscosity reduction. Viscosity measurements were conducted as described above for cellulase activity. Hemicellulase activity is expressed as percent viscosity reduction.

After 3 h of incubation at 37 °C, the solutions in the viscometers used to measure cellulase and hemicellulase activities were boiled for 5 min to destroy all enzyme activity. A qualitative determination of the sugars in the solutions was made. Thin-layer chromatography (TLC) was carried out on silica gel plates in an acetone:water (92:8) solvent. After separation, the sugars were detected by spraying the plates with anisaldehyde-sulphuric acid. All enzyme determinations were carried out in triplicate.

RESULTS AND DISCUSSION

Proximate analysis

The proximate analyses of the different sunflower products used in this study are shown in Table 1. The ash content of the sunflower products was considerably higher than that of most cereal flours even after removal of the hull from the sunflower seeds. The fat content of the unshelled seeds was 32·3 %, which is at the upper end of the range of values reported for 'non-oilseed' sunflower seeds (Robertson, 1975). The shelled product was more than 50 % fat. Crude fibre content was highest in sunflower hulls as would be expected. Unshelled whole sunflower seeds contained about 30 % crude fibre. Dehulling the seeds reduced crude fibre

TABLE 1
PROXIMATE ANALYSES OF SUNFLOWER PRODUCTS

Sample	*Moisture* (%)	*Ash* (%)	*Fat* (%)	*Crude fibre* (%)	*N* (%)	*Protein*† (%)
Unshelled sunflower seeds	7·8	2·74	32·3	28·98	2·60	16·25
Shelled sunflower seeds	5·2	3·36	52·3	2·87	4·67	29·19
Sunflower hulls	4·8	2·10	4·1	62·34	0·42	2·63
Roasted unshelled sunflower seeds	1·5	2·89	30·0	30·51	2·94	18·38
Roasted shelled sunflower seeds	2·6	3·53	54·1	1·27	4·67	29·19

† Protein calculated as N × 6·25.

content considerably. The protein in sunflowers is mainly concentrated in the shelled kernel. As shown in Table 1, the shelled sunflower meal contained the highest amount of protein of the sunflower products.

Enzyme activities

Amylase activity was detected in 'non-oilseed' sunflower products. The enzyme activities in these products are shown graphically in Fig. 1. Maltose values of unshelled sunflower seeds increased steadily with time of incubation of the extract with the starch substrate. From extracts of the sunflower kernels and hulls, the maximum amounts of maltose were liberated after 30 min of incubation. The unshelled sunflower seeds showed the highest enzyme activity, the sunflower kernels the lowest and the hulls intermediate amylase activity after 60 min at 37 °C. Roasting of the sunflower seeds and kernels reduced amylase activity considerably.

Maximum protease activity was found in sunflower kernels as shown in Fig. 2. The smallest amount of protein was solubilised from hull extracts while extracts from unshelled sunflowers produced intermediate values after 60 min of incubation of the extracts with the haemoglobin substrate. For each of the three products, there was an increase in the amount of protein solubilised with time. While roasting of the samples for 1 h at 150 °C caused a reduction of protease activity in the kernels, the activity of the enzyme was essentially unaffected in the unshelled sunflower seeds presumably resulting from the protection of the kernels against the temperature provided by the hull.

Cellulase activity of the sunflower products expressed as percent viscosity reduction is shown in Fig. 3. The viscosity of all extracts decreased with time. Extracts of the unshelled sunflower seeds, however, showed a higher activity than those of the kernels or hulls. TLC showed that glucose was liberated in the reaction. Roasting of the samples caused a decrease in cellulase activity in the unshelled sunflower seeds but produced no effect on the activity of the enzyme in the kernels, the product with the lowest cellulase activity before roasting.

Hemicellulase activity of 0·6 % NaCl extracts of the sunflower products, expressed as percent viscosity reduction, is shown in Fig. 4. The viscosity of all extracts

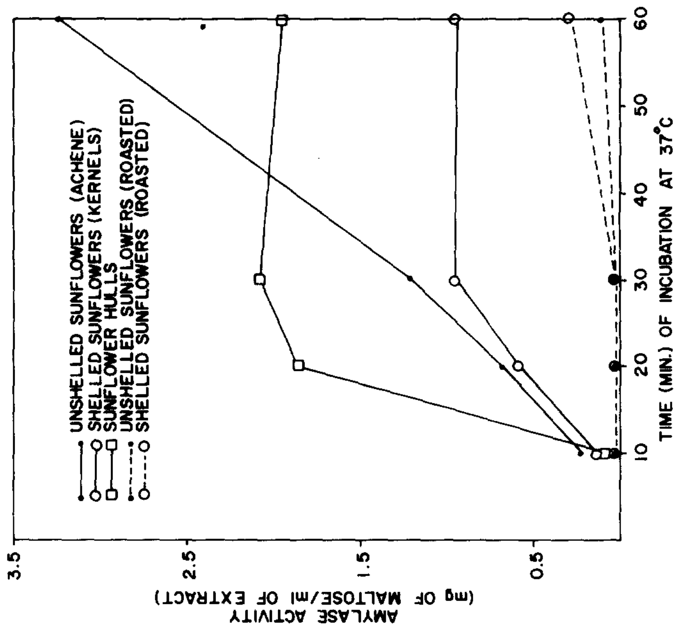

Fig. 1. Amylase activities of sunflower products.

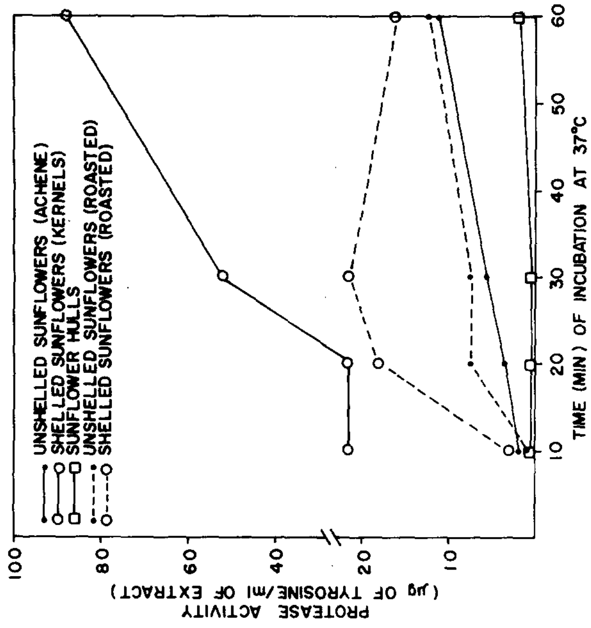

Fig. 2. Protease activities of sunflower products.

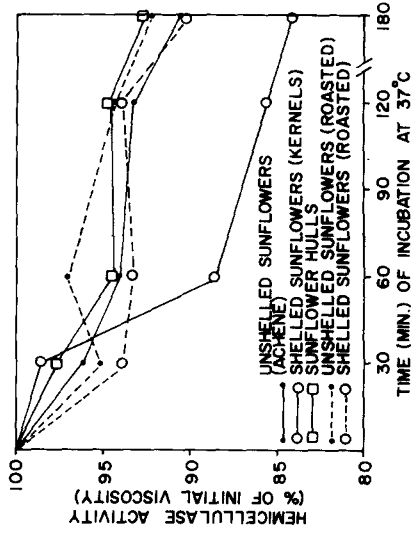

Fig. 4. Hemicellulase activities of sunflower products.

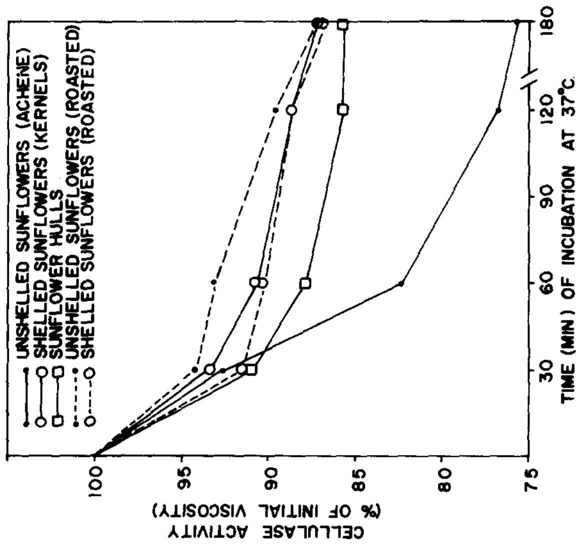

Fig. 3. Cellulase activities of sunflower products.

decreased with time. Extracts of the sunflower kernels caused the greatest viscosity changes; those of the hulls the smallest. TLC showed that arabinose was liberated as the result of hemicellulase activity. Roasting of the samples caused a reduction of hemicellulase activity in extracts of both the unshelled sunflower seeds and the sunflower kernels incubated for 3 h with the arabinogalactan.

CONCLUSIONS

The presence of amylases, proteases, cellulases, and hemicellulases was demonstrated in 'non-oilseed' sunflowers. Maximum activity of the amylases and cellulases was found in unshelled sunflower seeds, while the proteases and the hemicellulases were concentrated in sunflower kernels. Roasting of the sunflower products reduced the activity of each of the enzymes considerably in the product showing the highest activity.

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The technical assistance of J. Lallier is gratefully acknowledged.

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TROPICAL FRUITS AS SOURCES OF VITAMIN C

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ABSTRACT

Samples of nine different fruits grown in Oyo State of Nigeria were obtained from different market locations within Ibadan Municipality and analysed for their vitamin C content. Sweet orange was found to contain the highest amount of vitamin C (56·0 mg/100 g edible portion) whilst sweet banana variety II contained the lowest amount (9·4 mg/100 g edible portion). Lime, grapefruit, pawpaw and 'agbalumo' were found to contain considerable amounts of vitamin C, having 46·5, 47·0, 43·2 and 48·0 mg/100 g respectively. Consumption of these fruits would thus meet the nutritional requirement for vitamin C in this part of Nigeria. It is also recommended that sweet orange, agbalumo, grapefruit, lime and pawpaw, found to contain considerable amounts of vitamin C, should be processed either at home or factory level to make them available to the people all year round.

INTRODUCTION

Various types of fruits are grown in different parts of Nigeria to meet the demands of the population. Though in abundance, the production of these fruits is seasonal, thus putting a limitation on their availability throughout the year since there is no effective processing of them. The importance of vitamin C in the nutrition of people in developing countries need not be over-emphasised, however, the availability of fruits high in vitamin C serves to prevent the manifestation of deficiency diseases.

Little information is available on the vitamin C content of fruits grown and consumed in Oyo State of Nigeria. According to a Food and Agriculture Organisation report (FAO, 1968) the findings for African foods are scanty and often the methods of analysis are not clearly specified. Furthermore, only forty-five of the 208 fruits whose analyses were presented in this report contained any information on their vitamin C content.

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This paper presents the results of a study aimed at determining the vitamin C content of some fruits commonly consumed in Oyo State of Nigeria. Furthermore, those types containing high amounts of vitamin C have been identified and recommended for processing and preservation to make them available throughout the year.

MATERIALS AND METHODS

Nine different fruits were studied. Samples of each fruit were purchased, when in season, from different market locations within Ibadan Municipality in Oyo State, and from different vendors within a market, for randomisation.

In all cases, fruits purchased had been brought to the market a day or two after plucking, except for the sweet banana varieties which were purchased after ripening. Markets in Ibadan Municipality were chosen for sampling because it is a major centre for redistribution of fruits to other parts of the State. The fruits studied were pineapple, lemon, lime, grapefruit, orange, pawpaw, agbalumo (*Chrysophyllum delevoyi*), 'omini' (sweet banana variety I) and 'paranta' (sweet banana variety II).

For each fruit, twenty specimens assembled from the different locations were prepared for analysis as juice in the case of lemon, lime, grapefruit and orange whilst the rest were prepared for analysis by chopping, mixing, and blending. Samples (200 g) of each fruit mixture were then taken for analysis. Vitamin C was determined using an adaptation of the method described by Roe (1954) as adopted by the Association of Vitamin Chemists (1966) based on the oxidation of ascorbic acid to dehydroascorbic acid and its subsequent transformation to diketogulonic acid followed by coupling within 2,4-dinitrophenyl-hydrazine to give a red coloured osazone. A comparison of the absorbance produced in samples (measured at 520 nm) and ascorbic acid standard solutions was then used to determine the ascorbic acid content of the fruits.

This method is accurate for anti-scorbutic assay of many fresh foods when ascorbic acid and dehydroascorbic acid occur in the foods and diketogulonic acid has not been formed in appreciable amounts. It is thus valuable for determining total ascorbic acid content of foods at the time of harvesting. Means and Standard errors were calculated for values obtained after laboratory analysis.

RESULTS AND DISCUSSION

The results of the mean vitamin C content of pineapple, lemon juice, grapefruit juice, orange juice, pawpaw, agbalumo and sweet bananas varieties I and II are presented in Table 1. The results show that the orange juice contained the highest amount of

vitamin C in the group, being higher than values reported by FAO (1968), Oyenuga (1968) or Mudambi & Rajagopal (1977). They do, however, agree with values reported by Akinyele *et al.* (1978) for fresh orange juice obtained from a fruit canning factory before pasteurisation.

Agbalumo, grapefruit juice, lime juice and pawpaw were not significantly different from each other but with the exception of pawpaw, they were significantly higher ($p < 0{\cdot}05$) than FAO (1968) values. The value obtained for pawpaw in this study ($43{\cdot}2 \pm 5{\cdot}4$ mg) was also lower than the 60 mg/100 g reported by Bradley (1972). It does, however, fall within the quoted range of 22–78 mg (FAO, 1968).

TABLE 1
MEANS AND STANDARD ERRORS FOR VITAMIN C CONTENT OF SOME FRUITS

Fruit	*Botanical name*	*Vitamin C (mg/100 g edible portion) with SE*†	*Range (mg/100 g)*	*Vitamin C from FAO Tables*‡ *(mg/100 g edible portion)*
Sweet orange	*Citrus sinensis*	$56{\cdot}0 \pm 8{\cdot}5^a$	41–70	44 (37–54)
'Agbalumo'	*Chrysophyllum delevoyi* (formerly *C. africanum*)	$48{\cdot}0 \pm 6{\cdot}0^b$	38–59	—
Grapefruit	*Citrus paradisi*	$47{\cdot}0 \pm 9{\cdot}4^b$	24–62	43 (29–61)
Lime	*Citrus aurantifolia*	$46{\cdot}5 \pm 4{\cdot}4^b$	39–55	40§
Pawpaw	*Carica papaya*	$43{\cdot}2 \pm 5{\cdot}4^b$	33–58	52 (22–78)
Lemon	*Citrus lemon*	$35{\cdot}2 \pm 2{\cdot}8^c$	30–40	37 (26–50)
Pineapple	*Ananas comosus*	$25{\cdot}2 \pm 0{\cdot}2^d$	25–26	12 (7–18)
Sweet banana variety I	*Musa paradisiaca*	$12{\cdot}5 \pm 1{\cdot}4^e$	10–15	—
Sweet banana variety II	*Musa paradisiaca*	$9{\cdot}4 \pm 1{\cdot}6^e$	8–11	—

† Means in this column with a different superscript are significantly different ($p < 0{\cdot}05$).
‡ Figures in parentheses refer to range in FAO Tables.
§ No range given.

Finally, lemon juice, pineapple, sweet banana variety I, and sweet banana variety II were significantly lower ($p < 0{\cdot}01$) than those for the other fruits in this study. The value obtained for lemon juice was not significantly different from FAO (1968) values but was lower than values reported by Oyenuga (1968). This could be due to the small number of samples analysed by Oyenuga. Similarly, the result for the pineapple was considerably higher than that reported by FAO (1968) and the 16·90 mg for fresh pineapple juice and 14·5 mg for pineapple pieces reported by Akinyele *et al.* (1978) for samples obtained from a fruit canning factory, but was similar to the 24·0 mg reported by Oyenuga (1968). The differences in the result of this study and that reported by the authors elsewhere (Akinyele *et al.*, 1978) with regard to pineapple could have been due mostly to the fact that factory processed fruits were less ripe than those used in this study. The sweet banana varieties I and II contained lower levels of vitamin C than other fruits.

It is evident from the foregoing that the consumption of orange juice, agbalumo, lime juice, grapefruit juice and pawpaw should be encouraged among the people of Oyo State of Nigeria. The main contribution of fruits and their processed products to the nutrition of mankind is their supply of the anti-scorbutic vitamin (vitamin C). It has been pointed out by Mapson (1970) that fruits possess an advantage over many vegetables as sources of vitamin C because of the acidic media of the fruit juices compared to the more neutral vegetables. The fact that many fruits are eaten raw is also an advantage because losses of ascorbic acid are usually incurred during cooking.

Vitamin C deficiency as a nutritional problem seems less evident than deficiencies of other vitamins in developing countries. To prevent shortage of fruits containing considerable amounts of vitamin C, processing conditions that would prevent the loss of the vitamin need to be adopted in Nigeria either at the home or factory level. This would ensure the availability of fruits such as oranges, agbalumo, grapefruit, lime and pawpaw for consumption all year round. Since there is considerable difference of opinion regarding the amount of vitamin C needed by the adult male (the US Food and Nutrition Board quotes 60 mg/day for the average adult male, while the British Department of Health and Social Security suggests 30 mg/day (Davidson & Passmore, 1969); Pauling (1970) has recommended up to 10 g daily), consumption of these fruits should be to taste and unrestricted.

The ability of the tropical fruits studied to supply ascorbic acid to the Nigerian population would depend on the cost of each fruit item in the market place. Production of fruits in Nigeria is restricted to the Southern States and the Northern States with their teeming population depend entirely on the Southern States for their fruit supply. Most of these fruits are not cultivated on a large-scale orchard basis, hence total supply prohibits low selling prices especially in urban centres. Efforts are now being concentrated in Nigeria on the development of early maturing cultivars of tropical fruits and research so far shows good promise. Mapson (1970) has also shown that selective breeding to improve vitamin C content of fruits is possible. It would thus be advantageous to concentrate on high vitamin C cultivars identified in this study for use in breeding programmes in Nigeria. Since these fruits contain not only ascorbic acid but in most cases also provitamin A, the development of better and early maturing varieties should help increase their use in tropical and subtropical countries and lead to obvious advantages for better health and well-being of the people.

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HEAT INACTIVATION AND pH OPTIMA OF PEROXIDASE AND CATALASE IN CARROT, SWEDE AND BRUSSELS SPROUTS

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ABSTRACT

Heat inactivation and pH optima of the enzymes peroxidase and catalase were studied in carrot, swede and Brussels sprouts. There were differences in the heat stabilities of the peroxidases from different vegetables, but all peroxidases were more heat stable than the catalases. From the pH profiles and the heat stability curves it was concluded that both the peroxidases and catalases in the three vegetable species are somewhat different. Lipoxygenase and phenolase activities were not detected by the methods used.

INTRODUCTION

It has been reported recently (Baardseth, 1978) that unblanched leek, onion and swede did not develop detectable off-flavour or off-odour during storage at −20 °C and −30 °C for 15 months. Carrot, cauliflower and French bean, on the other hand, have to be blanched, but a 5 % residual activity of peroxidase did not affect quality during storage.

Some vegetables can be stored as such for months, for example, carrot, swede, onion (8 months at 0 °C) and leek (5 months at 0 °C). Brussels sprouts (6 weeks at 0 °C), beans and cauliflower (2 weeks at 0 °C), however, deteriorate within a relatively short time (Berg, van der & Lentz, 1977). Carrot, which can be stored for months, will start to deteriorate when peeled, cut and frozen, but swede, onion and leek do not.

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The enzymes lipoxygenase and phenolase are involved in undesirable quality changes in food. Peroxidase and catalase, on the other hand, are often reported to cause off-flavour, but the reactions involved have not been conclusively identified (Svensson, 1977). The aim of this investigation was to study the presence, pH optima and heat stabilities of these four enzymes in carrot, swede and Brussels sprouts. These vegetables differ in storage stability, and it was of interest to correlate enzyme activities with shelf life.

MATERIALS AND METHODS

Materials

Carrot (*Daucus carota*, var. Nantes Duke), swede (*Brassica napus* var. *napobrassica*, var. Bangholm) and Brussels sprouts (*Brassica oleracea* var. *gemmifera*, var. Jade Cross E) used in the experiments were obtained locally.

Methods

The vegetable samples were prepared by washing, peeling and cutting (Weisser cutting machine). The cut vegetables (20 g) were homogenised (Ultra-Turrax TP 18/10, Janke & Kunkel KG) with 20 ml 0·1 M phosphate buffer, pH 7·0, at 4 °C. The supernatant obtained after centrifugation (600 000 g_{av}. min at 4 °C with a Sorvall centrifuge and rotor SS 34) was used in the enzyme assays. Enzyme reaction velocities were calculated from the initial slopes of the absorbance/time curves. The enzyme activities were determined at 25 °C in a thermostatically controlled chamber of a recording spectrophotometer (Shimadzu UV 300), and the pH of the reaction mixtures was measured. To cover the actual pH range and elucidate the presence of ionic effects, different buffers were selected as shown in Figs. 1 and 2.

Heat inactivation was performed in triplicate in glass tubes (0·5 mm walls) covered with a marble. The tubes were placed in a circulating water bath at 70 °C for different lengths of time and cooled in ice water before enzyme assays were performed.

The peroxidase activity was determined at 420 nm using guaiacol and hydrogen peroxide as substrates, as described by Lu & Whitaker (1974). Catalase activity was measured at 230 nm using hydrogen peroxide (absorbance at 230 nm = 1·0) as substrate (Bergmeyer *et al.*, 1974). Lipoxygenase was determined essentially as described by Sekiya *et al.* (1977), but Brij 58 (a nonionic detergent, Polyoxyethylene 20 cetyl ether, Sigma, St. Louis, USA) was used to emulsify the linolenic acid. Brij 58 has the advantage that it does not absorb significantly at 230 nm, and the solutions did not show any sign of clouding under the conditions employed. The assay was standardised using lipoxygenase from peas. Phenolase activity was assayed essentially according to Satô (1976) at 500 nm, but 20 mM catechol and sulphanilic acid were used. Potato phenolase was used to standardise the assay. Protein was determined by the method of Lowry *et al.* (1951).

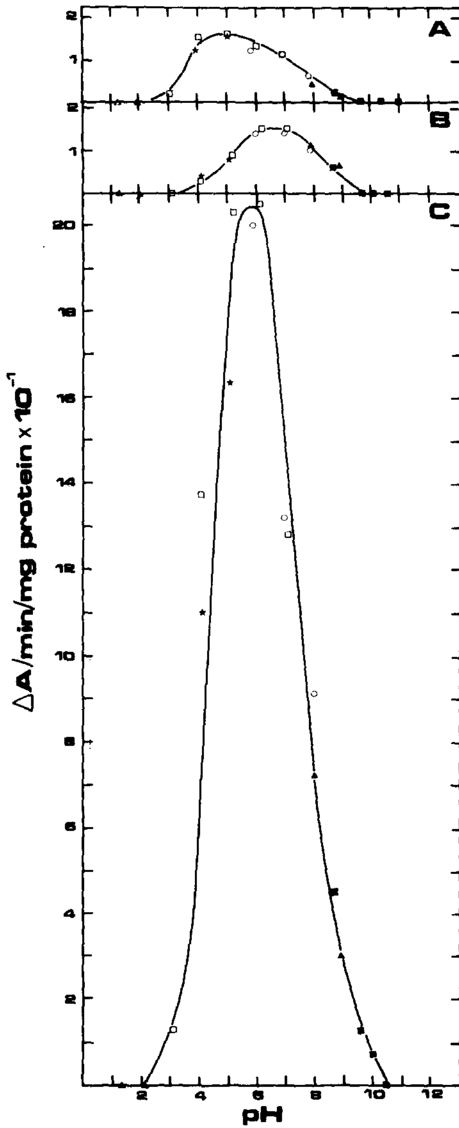

Fig. 1. Peroxidase activity of Brussels sprouts (A), carrot (B) and swede (C) as a function of pH. The buffers (0·1 M) used were: glycine/HCl (△), acetate (★), citrate/phosphate (□), phosphate (○), tris/HCl (▲), glycine/NaOH (■).

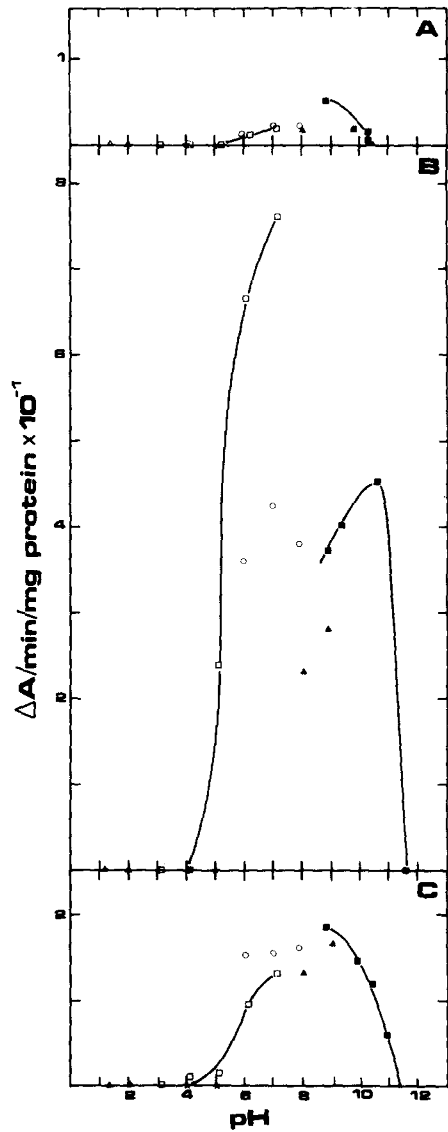

Fig. 2. Catalase activity of Brussels sprouts (A), carrot (B) and swede (C) as a function of pH. The buffers (0·1 M) used were: glycine/HCl (△), acetate (★), citrate/phosphate (□), phosphate (○), tris/HCl (▲), glycine/NaOH (■).

RESULTS AND DISCUSSION

Peroxidase and catalase are widely distributed in higher plants (Whitaker, 1972), and the presence of these enzymes in carrot, swede and Brussels sprouts was confirmed. On the other hand, phenolase and lipoxygenase activities were not detected with the present techniques. Pinsky *et al.* (1971) reported no activity of lipoxygenase in carrot while swede and Brussels sprouts were not investigated.

The amounts of protein in extracts of the three vegetables were determined as carrot, 3·4 mg/ml; swede, 5·5 mg/ml and Brussels sprouts 63·7 mg/ml. The pH values of water extracts were 5·6 for carrot, and 5·8 for both swede and Brussels sprouts.

A number of peroxidase activities exist, and these belong to various classes (for review, see Whitaker, 1972). Since the optimal pH for the peroxidase activities are different in the three vegetables (Fig. 1), indications are that the enzymes responsible for the activities are somewhat different. However, the peroxidase activities of the three vegetables are nearly maximal at the pH values (5·6–5·8) found within the vegetables.

The pH optima of the catalase activities (Fig. 2) were not found to coincide with the pH of the vegetables. Furthermore, a pronounced variation was observed at the same pH with the different buffers especially in the case of carrot (Fig. 2(B)). Thus, the three enzymes seem to be very heterogeneous. A variety of acids react with catalase to give inactive compounds (Schonbaum & Chance, 1976). This may explain the activity differences.

Heat inactivation of the enzymes at 70 °C for 1·5 min gave residual activities of approx. 45 % for peroxidase and 20 % for catalase (Fig. 3). In the response to heat

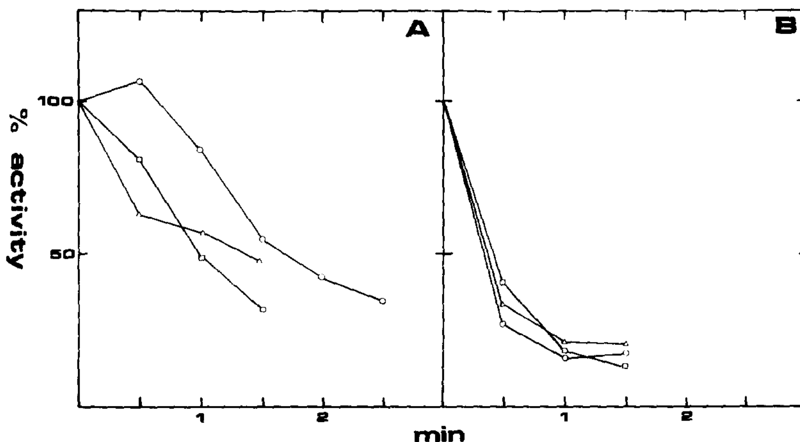

Fig. 3. Heat inactivation (70 °C with 0·1 M phosphate buffer pH 7·0) of peroxidase (A) and catalase (B) from (□) Brussels sprouts, (△) carrot, and (○) swede as a function of time.

inactivation peroxidase shows greater variation among the vegetables than catalase (Fig. 3). Swede peroxidase seems to be more heat stable than peroxidase from Brussels sprouts and carrot.

The living vegetable contains numerous enzymes that are active in different metabolic processes, and in some cases the presence of certain deteriorative enzymes in fresh and harvested vegetables can change the quality within days or weeks (Svensson, 1977). The absence of the deteriorative enzymes lipoxygenase and phenolase in the vegetables investigated favours extended shelf life and may partly explain why they can be stored for the lengths of time stated. On the other hand, the different storage stabilities of the Brussels sprouts, carrot and swede could be due to the differences in their contents of peroxidase and catalase.

For the three vegetables studied, a correlation exists between the storage stability and the activity of catalase and peroxidase, since swede has a high activity of peroxidase and a medium catalase activity, carrot has a medium peroxidase and a high catalase activity, while Brussels sprouts have a relatively low activity of both enzymes. However, it must be kept in mind that the amount of protein is highly different in the three vegetable extracts and that the activity per volume of Brussels sprouts is relatively high for at least peroxidase. In the cut state, swede has a long storage stability in contrast to carrot. When vegetables are injured, the deteriorative enzymes are often released or activated and enzyme reactions utilising oxygen proceed at a much higher rate. The swede contains the highest amount of ascorbic acid per mg protein (Paul & Southgate, 1978). Ascorbic acid is a reducing agent and an endogenous substrate to the respiratory chains which give ATP and remove oxygen. This, in conjunction with the absence of deteriorative enzymes, may partly explain the long storage stability of swede when cut.

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COMPOSITION OF BOLTI (*TILAPIA NILOTICA*) MUSCLE PROTEINS

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ABSTRACT

During the study described in this paper it was found that the edible portion of bolti fish (Tilapia nilotica) *comprises about 36% of its total weight and contains 79·5% moisture, 18·8% crude protein, 0·36% fat and 1·4% ash. The proteins of fresh bolti muscles consist of 66% myosin nitrogen, 25% non-myosin nitrogen and 8·5% insoluble protein nitrogen. The non-protein accounts for 15·3% of the total nitrogen.*

Electrophoreses of fresh bolti muscle proteins showed that three bands migrated towards the cathode and only one towards the anode. Protein-free amino acids consisted of only alanine and glycine. Bolti muscle proteins consisted of lysine, 10·6%, histidine, 1·7%, arginine, 5·8%, threonine, 3·8%, methionine, 1·1%, valine, 5·4%, phenylalanine, 3·2% and leucine/isoleucine, 12·4%.

INTRODUCTION

In Egypt the bolti fish (*Tilapia* spp.) is considered to be one of the best local fish species. It belongs to the family Cichlidae, which constitutes about half of the Egyptian freshwater fish resources (Latif, 1974). Information about the characteristics of its protein is scanty and, to some extent, contradictory. Investigations of the electrophoretic pattern of fresh bolti muscle proteins indicate that it may have five (Nagib, 1963) or six (Hassan, 1964) bands. The degree of freshness may be one source of error. Dyer & Dingle (1961), utilising cod, reported that the yield and nature of the constituents obtained upon extraction depended largely on the fish

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species and on the state of freshness. Bramstedt (1962) claimed that the amino acid chromatograms from different fish species revealed a uniform pattern, although the quantity of individual amino acids may differ with species. The study reported in this paper was therefore carried out to resolve some of these contradictions.

MATERIALS AND METHODS

Materials

Fresh bolti (*Tilapia nilotica*) was obtained during the course of the spring season (April–June, 1975) from Lake Mariut, near Alexandria, Egypt. The fish were caught by means of the ordinary fish traps usually used in the area and were transported live to the laboratory in glass jars filled with water, where they were killed instantly by knocking them on the head. The fish were immediately beheaded and the flesh cut from the backbone. The belly was then removed, thus obtaining the edible portion—i.e. the fish flesh.

Methods of analysis

The proximate composition of the edible portions: Moisture, crude protein, fat, ash, calcium and phosphorus contents were determined according to official methods (AOAC, 1970).

Extraction and fractionation of fish muscle proteins: The soluble protein nitrogen, the myosin nitrogen and the insoluble protein nitrogen (i.e. stroma and denatured proteins) were determined in the edible portions of the fish according to the method of Dyer *et al.* (1950) by extraction with 5 % sodium chloride solution. The insoluble protein nitrogen was determined in the precipitate, while the supernatant contained the myosin and non-myosin nitrogens. The latter two groups were fractionated from each other by diluting the supernatant tenfold with distilled water and allowing it to stand for 24 h in a refrigerator at 0–4 °C before centrifugation. The myosin nitrogen was then determined in the precipitate and the non-myosin nitrogen in the supernatant. The effect on the yield of each fraction of blending the edible portions prior to extraction was also studied according to the method of Dingle *et al.* (1955).

Extraction and fractionation of the muscle proteins were also carried out according to the methods of Aman (1976). This procedure consisted of extraction with a low ionic strength phosphate buffer ($U = 0{\cdot}05$) at pH 7–7·5 to extract the albumins. The globulin nitrogen was then extracted using a phosphate buffer of higher ionic strength ($U = 0{\cdot}5$). The remaining nitrogen in the precipitate represents the non-soluble protein nitrogen.

Protein nitrogen and non-protein nitrogen were determined according to official methods (AOAC, 1970).

Paper electrophoresis: The fish muscle proteins were extracted in a borate buffer (8·8 g borax/litre and 4·65 g/litre boric acid, pH 7·7) using a ratio of 4 ml

buffer/gramme flesh according to the method of Block *et al.* (1958) as modified by Nagib (1963). Phosphate buffer (KH_2PO_4/Na_2HPO_4, pH 6·2, ionic strength, 0·076) was used for the run which was conducted in an E-Cephor Zeurich type apparatus at 110 V for 24 h. The protein bands were located by staining with bromophenol blue, as described by Lederer (1957). The amount of protein located in each band was estimated using a Beckman E electrophoresis scanner.

Paper chromatographic analysis of amino acids: The free amino acids were extracted by 80% ethanol from the fish muscle homogenate according to the technique of Jones (1959). Chloroform was added to the aqueous ethanol extract and the upper aqueous layer—which contained the protein-free amino acids—was used for analysis. The acid hydrolysate (6N HCl) of the protein-bound amino acids was prepared according to the method of Block *et al.* (1958). Quantitative determination of the amino acids was carried out using the descending multiple development technique (six replications). A standard mixture of the amino acids (Merck, West Germany) was applied to the chromatogram along with the unknown sample. *N*-butanol: acetic acid: water (144:13:34) was used for the separation, as suggested by Mikes (1966). Ninhydrin in acetone containing 1% acetic acid was used for colour development (Roland & Gross, 1954). Colour intensity was measured at 515 nm using a Spekol spectrocolorimeter.

RESULTS AND DISCUSSION

Edible portion as percentage of total

The results given in Table 1 show that the edible portion of bolti fish comprises about 36% of the total weight. This is somewhat higher than the value of 30% previously reported by Awadallah (1958). This may be due to differences in the fishing ground.

TABLE 1
PERCENTAGE OF THE EDIBLE PORTION OF BOLTI (*Tilapia nilotica*)

No.	*Total weight (g)*	*Weight of edible portion (flesh) (g)*	*Weight of waste (g)*	*Edible part to total weight (%)*
1	150	55	95	36·6
2	153	56	97	36·6
3	156	56	100	36·0
4	157	58	99	37·0
5	159	56	103	35·3
6	160	55	105	34·3
7	162	57	105	35·2
8	165	58	107	35·1
9	166	62	104	37·4
Average	158 ± 8	57 ± 3·5	101 ± 6	35·9 ± 1·5

Proximate composition of the edible portion

The results given in Table 2 indicate that the protein content did not vary greatly among individual fish caught at different times (April, June) and ranged between 18·1 and 19·7%. This agrees with the results of Abdel-Rehiem (1948) who gave the range 19·4% to 19·6% in February, 18·4% to 20·0% in May and 17·3% to 19·1% in December. The results also agree with those of Awadallah (1958), Nagib (1963) and Hassan (1964) for Egyptian bolti fish. Table 2 shows that bolti fish are relatively lean,

TABLE 2
PROXIMATE COMPOSITION OF THE EDIBLE PORTION OF BOLTI (*Tilapia nilotica*)

Date of capture	Percent				mg/100 g	
	Moisture	*Crude protein*	*Ether extract*	*Ash*	*Calcium*	*Phosphorus*
		On a wet weight basis				
8 April	80·88	18·28	0·24	1·28	62	249
29 April	78·85	18·16	0·29	1·38	80	273
20 May	78·21	19·73	0·42	2·04	—	—
15 June	80·27	18·63	0·48	1·07	68	255
Average	79·55 ± 1·34	18·69 ± 0·78	0·36 ± 0·12	1·43 ± 0·49	71 ± 9	260 ± 12
		On a dry weight basis				
8 April	—	95·66	1·28	6·70	342	1302
29 April	—	84·67	1·39	6·28	372	1272
20 May	—	90·55	1·92	9·51	—	—
15 June	—	94·39	2·43	5·43	343	1293
Average	—	91·32 ± 5·49	1·78 ± 0·57	6·98 ± 2·04	352 ± 15	1289 ± 15

having a fat content ranging between 0·24% and 0·48%. This is in agreement with the results of El-Saby (1934), Abdel-Rehiem (1948), Awadallah (1958), Nagib (1963), Hassan (1964) and Shaheen (1969). The results also clearly show that the proteins are second only to water as the most abundant substance in fish tissue and are, without doubt, the most important constituent of the edible portion.

Extraction and fractionation of the proteins of the edible portion

The results given in Table 3 indicate that the extraction method of Dyer *et al.* (1950) is superior, allowing the extraction of 91·6% soluble nitrogen, while the methods of Dingle *et al.* (1955) and of Aman (1976) permitted the extraction of only 43% and 29·2%, respectively. This property allowed for better determination of myosin nitrogen, since the percentage found when this method was used was 66·7%, while the other two methods gave 19·8% and 4·2%, respectively. The advantage of this method is further exemplified if the figures for the remaining nitrogen are examined. These are 8·5% for this method and 58·2% and 71·0% for the other two, respectively. This remaining nitrogen was considered insoluble according to the methods of Dingle *et al.* (1955) and Aman (1976).

With regard to the non-myosin nitrogen, the three methods gave almost the same

TABLE 3
PERCENTAGE OF THE DIFFERENT PROTEINS OF THE EDIBLE PORTION OF BOLTI FISH (*Tilapia nilotica*) EXTRACTED BY DIFFERENT METHODS

Reference	*Extraction method*	*Total soluble nitrogen (%)*	*Non-myosin nitrogen (%)*	*Myosin nitrogen (%)*	*Remaining nitrogen (%)*
Dyer *et al.* (1950)	Blending for 3–5 min, then extraction in 5% NaCl, buffered at pH 7–7·5 using 0·02 M sodium bicarbonate	91·6	24·8	66·7	8·5
Dingle *et al.* (1955)	Direct extraction in 5% NaCl, buffered at pH 7–7·5 using 0·02 M sodium bicarbonate	43·8	24·0	19·8	56·2
Aman (1976)	Extraction of the albumins in phosphate buffer of $U = 0{\cdot}05$* at pH 7–7·5, followed by extraction of the globulins with a phosphate buffer of $U = 0{\cdot}5$ at pH 7–7·5	29·2	25·0	4·2	71·0

* U = Ionic strength.

results (24% to 25%). The methods of Dyer *et al.* (1950) and Dingle *et al.* (1955) do not differ except for blending. Hence the superior results could be ascribed to this step. The method of Aman (1976), although utilising the same pH and a phosphate buffer, also involved two extraction steps at different ionic strengths without blending. This method gave the lowest results for myosin nitrogen. As far as non-myosin nitrogen is concerned, neither the type of buffer nor blending is important. However, in myosin nitrogen extraction or determination, both blending and the type of buffer play important roles. Common salt/sodium bicarbonate buffer is superior to phosphate for myosin extraction. This agrees with the previous results of Dyer *et al.* (1950), Suyama (1950), Perry (1953), Bailey (1954), Dyer & Dingle (1961), Cornell (1962), Jebsen (1962) and Partmann (1965) for fish species other than bolti (*Tilapia nilotica*).

Non-protein nitrogen (NPN) of the edible portion

It was found that the edible portion of bolti fish contained 2·99% total nitrogen, 2·43% protein nitrogen and 0·46% non-protein nitrogen. Thus, the non-protein nitrogen accounted for 15·35% of the total nitrogen. This agrees with values previously reported by Hassan (1964) for bolti fish (15·62%) and with the results of Shewan (1951), who reported that NPN was in the range 1–14% in the case of gadoids and flat fishes and 16% to 18% for clupeids. Tarr (1958) gave a range of 9% to 18% in teleosts and 33% to 38% in elasmobranch fishes.

Electrophoretic pattern of bolti fish muscle proteins

The electrophoretic pattern of fresh bolti fish muscle (*T. nilotica*) is shown in Fig. 1. Three bands are distinguished migrating towards the cathode and only one

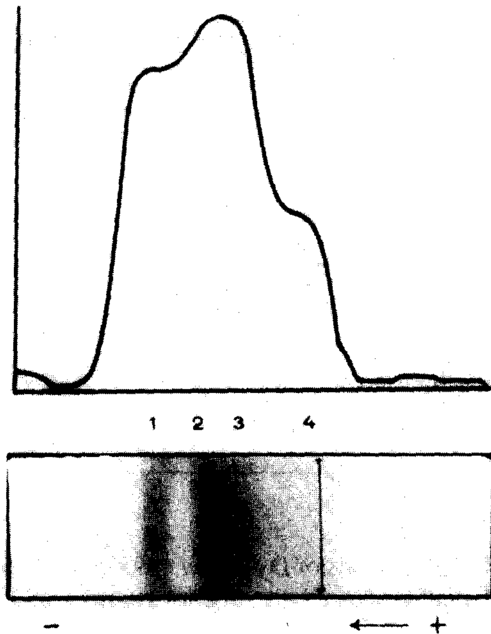

Fig. 1. Electrophoretic pattern of fresh bolti fish (*Tilapia nilotica*) proteins.

migrating towards the anode. Nagib (1963) obtained five bands for the muscle proteins of *Tilapia zillii* while Hassan (1964) obtained seven bands for the same species and six bands for *Tilapia nilotica*. Such differences may be related to varying experimental conditions (length of run and voltage used) but could also be related to differences in the freshness of the fish. Okutani *et al.* (1965) attributed differences in the electrophoretic pattern of a given species to the method of homogenisation, the pH value of the extractant and the size of the electrophoretic cell. Sadek (1975) found that the electrophoretic patterns of bolti fish (*Tilapia nilotica*) and of morgan (*Pagrus* spp.) differ according to their state of freshness.

Qualitative analysis of the protein-bound and free amino acids

Present evidence points to the existence of a fundamental amino acid pattern for the proteins of the myofibril of lower, as well as higher, vertebrates (Bramstedt, 1962; Hamoir, 1962; Cowey, 1965). Bolti fish are no exception, as it can be seen from Fig. 2. All the amino acids are present with the exception of tryptophan, which is probably absent because of its destruction by acid hydrolysis.

Figure 2 also shows the free amino acids found in the fresh muscles of bolti fish. This figure clearly shows that only alanine and glycine are found as free amino acids. Bramstedt (1962) reported a characteristic pattern of free amino acids for different fish species. He also showed that a uniform amino acid pattern made it possible to

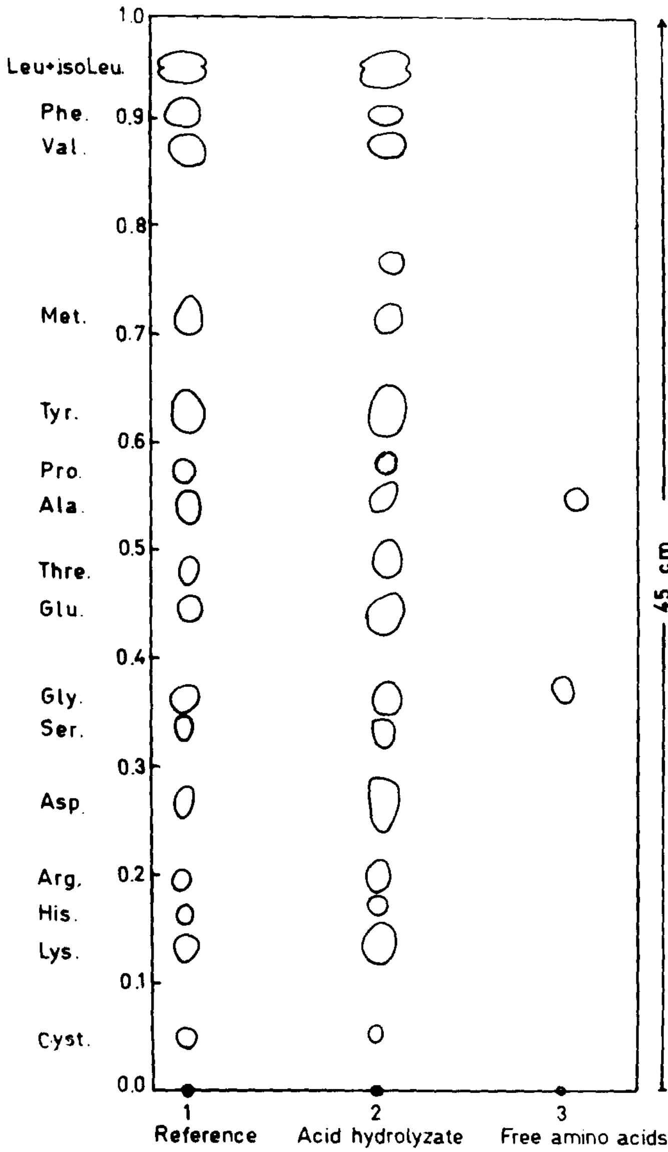

Fig. 2. Chromatographic diagram of amino acids of fresh bolti fish (*Tilapia nilotica*).

readily investigate the influence of factors such as storage, spoilage and processing on the composition of the free amino acids of a given species.

Quantitative determination of essential amino acids of the bolti proteins

Table 4 shows the essential amino acid content of bolti flesh. The values indicate that bolti proteins have a high level of lysine compared with other protein sources. Deas & Tarr (1947) reported that fish protein is a more potent source of lysine than egg protein. Terri *et al.* (1957) also found that the lysine content of fish flesh proteins appeared to be ample, frequently exceeding that found in other animal proteins. Table 4 also shows that bolti proteins are low in methionine. Venkataraman & Chari

TABLE 4
ESSENTIAL AMINO ACIDS IN FAO PROVISIONAL PATTERN—MILK, EGGS AND BOLTI FISH PROTEINS

Essential amino acid	*FAO provisional pattern*[a]	*Cow's milk*[a]	*Human milk*[a]	*Eggs*[a]	*Bolti fish protein*[b]
	Grammes amino acid per 100 g protein				
Leucine/isoleucine	9·0	16·3	15·3	15·8	12·4
Lysine	4·2	7·8	6·3	6·3	10·6
Phenylalanine	2·8	4·9	4·6	6·0	3·3
Methionine	2·2	2·4	2·2	3·1	1·1
Threonine	2·8	4·6	4·6	5·0	3·9
Tryptophan	1·4	1·4	1·6	1·7	—
Valine	4·2	6·9	6·6	7·5	5·4
Histidine	—	—	—	—	1·8
Argenine	—	—	—	—	5·9

[a] After Mayer (1962).
[b] Experimental results.

(1957) and Bose *et al.* (1958) showed that East Indian fishes are also low in methionine. Japanese studies by Tsuchiya (1944), Hatakoshi (1953) and Ogata (1956) generally gave methionine in the range of 2 % to 3·9 % in fish proteins, while Soviet figures ranged between 1·6 % and 2·3 %, as pointed out by Gorozhankina (1955). In spite of the fact that leucine, isoleucine, phenylalanine, threonine and valine are found in bolti fish proteins in smaller amounts than in milk or egg proteins, they are still present in amounts exceeding the FAO provisional pattern.

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BOOK REVIEWS

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This is a unique collection of papers which cover ground well beyond the indication of the title. In 475 pages the subjects range from economics through chemical derivatives to health and dietary implications. The book deals not only with sugar derived from cane and beet but also with glucose, fructose, xylitol and hydrogenated glucose derivatives.

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M. C. BENNETT

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malonate, shikimic acid, the amino acids and finally those derived from mixed precursors. This treatment emphasises relationships between groups of compounds such as the terpenes, carotenoids and steroids, all of which are derived from mevalonate. While most secondary metabolites are of plant origin some, such as the prostaglandins, are found in the animal kingdom and these, too, are discussed.

While the prime value of this book is the provision of a clear picture of the metabolic pathways leading to the major groups of secondary compounds, the selection of examples such as the vitamins D and the alkaloids strychnine and morphine remind us of the important role which many of these compounds play in nutrition, toxicology and medicine.

In the last chapter the author considers the possible ecological roles of secondary metabolites and from the examples given it is clear that the term 'secondary' is itself a misleading one, suggesting as it does that the compound is relatively unimportant to the plant. Even if a compound is not essential for maintaining life in the individual organism in which it occurs it may, nevertheless, be absolutely essential for the survival of the species as a whole in a natural environment.

The provision of problems concerned with the elucidation of biosynthetic pathways affords a useful test of the reader's understanding of the way in which tracer techniques are used in studying secondary metabolism.

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The book is well produced and the chapters well-bibliographed and clearly illustrated. Chapters are written by noted authorities and contain much up to date material of interest to nutritional chemists and other scientists. Despite its top price, this book is invaluable for active research scientists in this area.

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The section on dietary fibre is concisely presented along with the recognition of its role in relation to faecal mass and texture established by British scientists in the last decade. No mention is made, however, of the especial value of wheat bran in this important field.

Each chapter of the book begins with an outline and ends with a summary plus study questions. The book is an introduction to nutrition requiring no university knowledge in the field. However, food chemists will find it most useful as a source of information and its very reasonable cost establishes it as a valuable bargain.

G. G. BIRCH

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G. G. Birch

Food Chemistry 5 (1980) 189–194

OLIGOSACCHARIDE FORMATION DURING HYDROLYSIS OF LACTOSE WITH *SACCHAROMYCES LACTIS* LACTASE (MAXILACT®)—PART 3: DIGESTIBILITY BY HUMAN INTESTINAL ENZYMES *IN VITRO**

A. Burvall, N.-G. Asp & A. Dahlqvist

Department of Applied Nutrition, Chemical Centre, University of Lund, Lund, Sweden

(Received: 15 July, 1978)

ABSTRACT

Practically all the oligosaccharides produced during enzymatic hydrolysis of lactose with Saccharomyces lactis *lactase are formed by attachment of galactosyl residues in* β-*(1 → 6) linkages.*

The activity of human small-intestinal β-*galactosidase on these substrates was less than 10% of the activity on lactose which has its galactosyl moiety in a* β-*(1 → 4) bond. Therefore, ingested disaccharides will pass undigested through the small intestine into the large bowel, where bacterial degradation takes place. This explains the gastrointestinal discomfort after oligosaccharide ingestion described in another paper (unpublished).*

INTRODUCTION

Formation of oligosaccharides has previously been demonstrated to occur when lactose is hydrolysed with *Saccharomyces lactis* lactase (Maxilact®). This formation is due to the transglycosidase activity of the enzyme and occurs particularly in solutions with a high concentration of lactose (Burvall *et al.* 1977). The oligosaccharides have been separated and structural analyses of the quantitatively most important sugars have been performed (Asp *et al.*, 1979).

The human small-intestinal lactose that hydrolyses dietary lactose has a rather strict substrate specificity (Asp & Dahlqvist, 1974) and intestinal discomfort, mainly flatulence, has been demonstrated to occur when healthy subjects are given milk containing large quantities (9 g) of oligosaccharides formed during the hydrolysis of

* Part 1 of this paper appeared in Vol. 4, No. 4, pp. 243–50; Part 2 appeared in Vol. 5, No. 2, pp. 147–53.

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lactose (Asp *et al.*, unpublished data). In this study we investigated the digestibility of these transglycosylation products by human small-intestinal lactase.

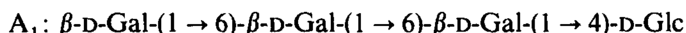
MATERIALS AND METHODS

Human small-intestinal enzyme preparation

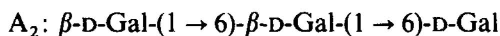
Macroscopically and microscopically normal pieces of human small intestine were obtained during surgical section, chilled and frozen within one hour after removal. The pieces were then homogenised with 4 vol (v/w) of 0·9 % (w/v) NaCl in an Ultra-Turax homogeniser. During the homogenisation the tube was chilled with crushed ice and the homogenate was either used directly or stored frozen at −20 °C until use. The homogenates used had normal activities of lactase and other disaccharidases.

Oligosaccharide preparations

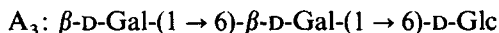
The different fractions, 'A', 'B', 'C' and 'D' described in a previous paper (Burvall *et al.* 1979) were used as substrate. Fraction A contained trisaccharides and a tetrasaccharide:



A_1: β-D-Gal-(1 → 6)-β-D-Gal-(1 → 6)-β-D-Gal-(1 → 4)-D-Glc



A_2: β-D-Gal-(1 → 6)-β-D-Gal-(1 → 6)-D-Gal



A_3: β-D-Gal-(1 → 6)-β-D-Gal-(1 → 6)-D-Glc

Fraction B comprised mainly the trisaccharide B_1: β-D-Gal-(1 → 6)·β-D-Gal-(1 → 4)-D-Glc.

Fraction C contained the disaccharides:

C_1: β-D-Gal-(1 → 6)-D-Gal and C_2: β-D-Gal-(1 → 6)-D-Glc

Fraction D contained by visual judgement approximately 80 % lactose (β-D-Gal-(1 → 4)-D-Glc) but it also contained the disaccharides C_1 and C_2 and, in addition, another disaccharide with a higher mobility than lactose. It should be noted that all major saccharides, except lactose, contained the galactosyl moiety to be split off on enzymic hydrolysis with β-galactosidase in a β-(1 → 6) bond.

All other chemicals used were of analytical grade purity.

Assay of initial reaction velocity

To 50 μlitres of 0·1 M sodium maleate buffer, pH 6·0, containing the substrate (lactose 2 % (w/v), A 3 %, B 3 %, C 2 % or D 2 %) was added 50 μlitres of the intestinal homogenate diluted 1:10 with 0·9 % NaCl. After 60 min at 37 °C the protein was precipitated with 50 μlitres of $Ba(OH)_2$ 0·3 mole/litre and 50 μlitres of $ZnSO_4$ (0·3 mole/litre) according to Somogyi (1945). After centrifugation the supernatant

was used for analysis of glucose with glucose-oxidase (GLOX-novum, Kabi AB, Stockholm, Sweden) and galactose with galactose-dehydrogenase (Dahlqvist & Asp, 1971). Blanks were prepared with the same composition but not incubated.

In addition to the digestive brush border lactase the human small intestinal mucosa also contains two intracellular β-galactosidases with unknown digestive functions. These enzymes account for only about 5% of the total β-galactosidase activity of the homogenates used in this investigation with lactose as substrate. Other substrates can, however, mainly or even entirely be hydrolysed by the intracellular enzymes (Asp & Dahlqvist, 1974). The hydrolysis of oligosaccharides was therefore also determined in the presence of an inhibitor of these enzymes, PCMB, *p*-chloromercury benzoate (obtained from KEBO AB, Stockholm, Sweden). Thirty-six milligrammes of this inhibitor were dissolved in 5 ml of 0·1 M NaOH. Ten microlitres of that stock solution were diluted with 500 μlitres buffer–substrate solution used as described above. The final PCMB concentration obtained (0·1 mmole/litre) inhibits the intracellular β-galactosidases almost completely.

Prolonged incubations

A prolonged incubation with excess intestinal homogenate was also performed with the different substrates. Seventy-five microlitres of the buffer substrate solution described above were diluted with 225 μlitres of 0·1 M sodium maleate pH 6 buffer. To this dilution was added 300 μlitres undiluted intestinal homogenate. The hydrolysis was followed by glucose and galactose analysis (as described above) of 100 μlitre samples taken after 0, 1/2, 1, 2 and 6 h.

RESULTS

Initial rates of oligosaccharide hydrolysis

The initial rates of liberation of glucose and galactose from the different oligosaccharide fractions compared with lactose are shown in Table 1. The

TABLE 1
INITIAL RATES OF HYDROLYSIS OF THE DIFFERENT FRACTIONS AS COMPARED WITH LACTOSE. IN THIS EXPERIMENT NO PCMB WAS ADDED

	Liberation of monosaccharide in per cent of lactose	
Substrate	*Galactose*	*Glucose*
Lactose	100	100
A	9	2
B	5	<2
C	<8	6
D	71	70

liberation of glucose and galactose from lactose was arbitrarily set to 100. The degree of hydrolysis of lactose was less than 5 %.

In fraction D, containing approximately 80 % lactose, the liberation of glucose and galactose was 70 and 71 %, respectively of that in the lactose control. The hydrolysis of the other oligosaccharide fractions proceeded at a much lower rate. Thus, in fraction C the liberation of glucose occurred at 6 % of the rate in the lactose control. The liberation of galactose from this fraction could not be measured accurately due to reaction of unhydrolysed oligosaccharides (presumably galactobiose) with the galactose–dehydrogenase assay system.

Significant liberation of galactose was obtained both in fraction A and in fraction B, containing tri- and tetrasaccharides, respectively, as main components. The glucose liberation registered in fraction B was on the borderline of significance.

TABLE 2

INITIAL RATES OF HYDROLYSIS OF THE DIFFERENT FRACTIONS AS COMPARED WITH LACTOSE. IN THIS EXPERIMENT PCMB WAS ADDED TO INHIBIT INTRACELLULAR ENZYMES WITHOUT KNOWN DIGESTIVE FUNCTION

	Liberation of monosaccharide in per cent of lactose	
Substrate	*Galactose*	*Glucose*
Lactose	100	100
A	2	<2
B	<2	<2
C	<8	5
D	70	67

Table 2 shows initial reaction velocities with PCMB added. In the presence of this inhibitor only the digestive brush border lactase is active. The slow liberation of galactose from fractions A and B was almost completely abolished by PCMB. Thus the intracellular β-galactosidases which, *in vivo*, probably cannot come into contact with ingested oligosaccharides at all, seem responsible for the slow hydrolysis of the tri- and tetrasaccharides demonstrated in the experiments without PCMB.

Prolonged incubation

Prolonged incubations were performed with two different pieces of intestine, both with and without PCMB. A typical experiment without PCMB is shown in Fig. 1. Under the conditions used the hydrolysis of lactose was complete in about two hours. Glucose was liberated somewhat more rapidly than galactose, indicating transglycosylation.

The hydrolysis of fraction D proceeded rapidly until 80 % of the sugar occurred in the form of free monosaccharides. Also with this fraction glucose was liberated more rapidly than galactose. With fraction C as substrate there was a slow continuous

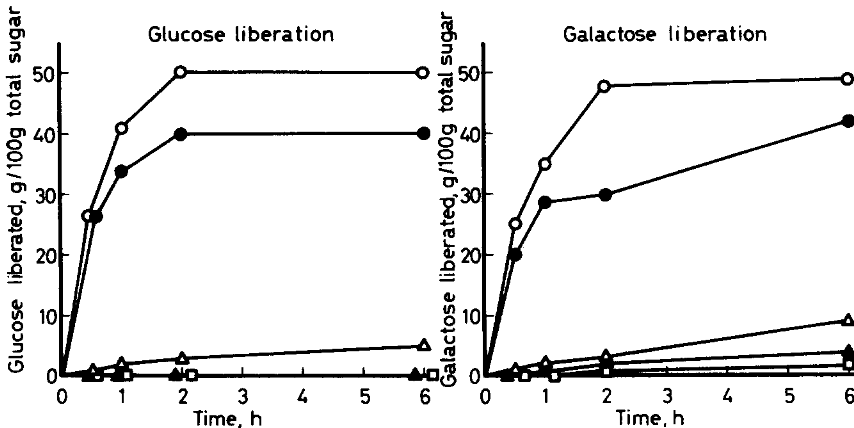


Fig. 1. Liberation of glucose and galactose on prolonged incubation of the different fractions. A (□—□); B (▲—▲); C (△—△); D (●—●) and lactose (○—○). No PCMB was added in this experiment.

liberation of both glucose and galactose with 14% of the sugar as monosaccharides after 6 h. From fractions A and B galactose was liberated slowly, whereas no significant amount of glucose was released even after 6 hours of incubation.

DISCUSSION

In unconcentrated milk or whey the formation of transglycosylation products is low but up to 2–3 g/litre may occur under certain circumstances. During hydrolysis in concentrated milk, however, over 30% of the total sugar content may be in the form of transglycosylation products (Roberts & Pettinati, 1957).

This investigation shows that these oligosaccharides, formed during enzymic hydrolysis of lactose with *Saccharomyces lactis* lactase, cannot be hydrolysed to any practically important extent by the human small-intestinal lactase. The slow liberation of galactose from the tri- and tetrasaccharide-containing fractions demonstrated in the absence of PCMB seemed to be entirely due to intracellular enzymes without known digestive functions.

A preliminary study of the activity of Maxilact® and human intestinal β-galactosidase on lactose and purified allolactose (β-D-Gal-(1 → 6)-D-Glc) has been carried out in our laboratory. Letting V be the initial rate of hydrolysis for the respective sugars, the factor (V lactose/V allolactose) was about 2 for Maxilact® but for human β-galactosidase it was well over ten times larger. The small allolactose hydrolysing activity demonstrated in the human intestinal homogenate was probably due to the intracellular β-galactosidases rather than the digestive brush border lactase.

In vivo, therefore, these sugars can be expected to pass the small intestine without being digested or absorbed and to reach the large bowel unaltered. There they will be subject to bacterial degradation with intestinal disturbances, mainly flatulence, resulting (Asp *et al.*, unpublished data).

ACKNOWLEDGEMENTS

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Food Chemistry 5 (1980) 195–199

DEGRADATION OF MYOFIBRILS AND FORMATION OF PREMEROMYOSIN BY A NEUTRAL PROTEASE PRODUCED BY *PSEUDOMONAS FRAGI*

M. A. PORZIO† & A. M. PEARSON

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(Received: 3 January, 1979)

ABSTRACT

Rabbit muscle myofibrils were treated with a neutral protease (200:1–protein:enzyme) isolated from Pseudomonas fragi (P. fragi) *at 0°C in 100 mM KCl, 5·0 mM $CaCl_2$, 0·2 mM DTT (dithiothreitol) and 20 mM TRIS.HCl (pH 7·50) for 0–60 min. SDS-polyacrylamide disc gel electrophoresis showed extensive fragmentation of the myofibrillar proteins. Three of the major products correspond in molecular weight to heavy meromyosin (HMM), light meromyosin (LMM) and the recently discovered premeromyosin (PMM). Results demonstrated that the neutral protease produced by* P. fragi *is capable of hydrolysing myosin and probably plays an important role in microbial breakdown of the muscle proteins during meat spoilage.*

INTRODUCTION

Pseudomonads have been identified as one of the principal species of microorganisms responsible for spoilage of meat under refrigeration (Ayres, 1960). It has been demonstrated that *Pseudomonas fragi* (*P. fragi*) can break down the sarcoplasmic and urea-soluble proteins from muscle (Hasegawa *et al.*, 1970*a*,*b*), and more recently that they can hydrolyse the salt-soluble proteins (Tarrant *et al.*, 1971) and will specifically degrade G-actin and myosin (Dutson *et al.*, 1971; Tarrant *et al.*, 1973). Porzio & Pearson (1975) have isolated and characterised an extracellular neutral proteinase from cultures of *P. fragi* to obtain 20% yield and 60-fold purification.

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The present study was undertaken to determine the action of the *P. fragi* protease on isolated rabbit myofibrils as a simplified model for following the bacterial spoilage process in muscle.

MATERIALS AND METHODS

The extracellular neutral protease was prepared from actively growing cultures of *P. fragi* according to the procedure described in detail by Porzio & Pearson (1975). The enzyme preparation had a specific activity of 5·5 μmoles of tyrosine equivalents released/mg/min at 35 °C by the casein digestion assay. Frozen stock solutions of the enzyme preparation were thawed and dialysed for 12 h at 0–4 °C against a solution containing 100 mM KCl, 5 mM $CaCl_2$, 0·1 mM DTT and 50 mM TRIS · HCl (pH 7·50). There was no apparent loss of activity during freezer storage or during dialysis. This was verified by comparing its activity with that of the original preparation (Porzio & Pearson, 1975).

Rabbit myofibrils were prepared according to the method of Etlinger *et al.* (1976) using the recent modifications described by Porzio & Pearson (1977*a*). Proteolysis of the myofibrils was initiated at 0 °C by addition of 0·5 mg of the protease (0·5 ml of 5·5 μM/mg, 1·0 mg/ml of stock solution) to 9·5 ml of a myofibril suspension containing 100 mg protein (10 mg/ml) to give a protein to enzyme ratio of 200:1. The control consisted of 0·5 mg of enzyme with 10 mM EDTA in the proteolysis buffer. At 0, 30 and 60 min, 2·0 ml of the proteolysis mixture were removed and added to 10 ml of a quenching-solubilising solution consisting of 2·5 % SDS (sodium dodecyl sulphate), 100 mM TRIS·HCl (pH 8·00), 10 mM DTT and 25 mM EDTA. The sample was heated to 100 °C for 5 min, cooled and alkylated with 10 μM of ICH_2CONH_2 per mg sample and dialysed against sample buffer as described by Porzio & Pearson (1977*a*).

The sample was subjected to SDS-polyacrylamide disc gel electrophoresis. A 10 % gel with an acrylamide to bis-acrylamide ratio of 100:1 (w/w) was used with a continuous buffer system as described by Porzio & Pearson (1977*b*). Molecular weights of the polypeptides are based on standard proteins given in an earlier report (Porzio & Pearson, 1977*b*).

RESULTS AND DISCUSSION

Figure 1 shows SDS-gels of the myofibril samples after incubation with the enzymes at times (t) of 0, 30 and 60 min, with the samples labelled A, B and C, respectively. The upper band, which is labelled myosin, corresponds to the myosin heavy chain with a molecular weight of 200,000. At 30 min, the myosin heavy chain shows a new fragment as a clearly resolved doublet, in addition to numerous secondary

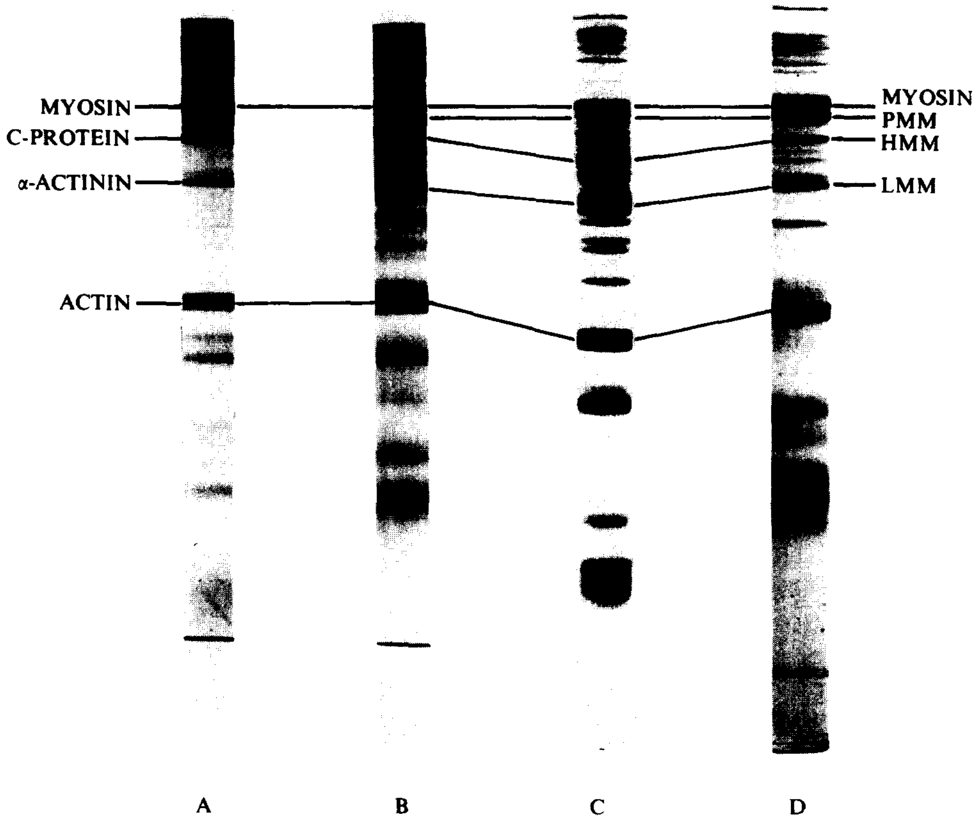

Fig. 1. SDS-polyacrylamide gel analysis of isolated myofibrils subjected to proteolysis by incubation with *P. fragi* protease or with trypsin. Gel A (control) contains 50 μg of myofibril preparation plus 2·5 *P. fragi* enzyme units/100 mg protein after incubation for 0 min at 0 °C. Gel B contains 50 μg of myofibril preparation plus 2·5 *P. fragi* enzyme units/100 mg protein after incubation for 30 min at 0 °C. Gel C contains 70 μg of myofibril preparation plus 2·5 *P. fragi* protease enzyme units/100 mg protein after incubation for 60 min at 0 °C. Gel D contains 55 μg of myofibril preparation plus 225 T.A.M.E. (tosyl-L-arginine-methyl ester) units of trypsin/mg protein after incubation for 10 min at 0 °C.

polypeptide fragments with molecular weights in the 80,000–175,000 dalton range. Among these additional fragments, one can observe two proteins in relatively greater abundance, which show relative mobilities similar to heavy and light meromyosin (Porzio & Pearson, 1977*b*).

Gel D (Fig. 1) shows equivalent proteolysis of rabbit muscle myofibrils treated with trypsin (2000:1 of 225 T.A.M.E. (tosyl-L-arginine methyl ester) units/mg; t = 10 min at 0 °C). This less extensively fragmented sample exhibits the doublet chain of myosin heavy chain and premeromyosin (PMM), which has recently been described as a breakdown product of myosin proteolysis by trypsin (Porzio & Pearson, 1977*a*). In addition, heavy (HMM) and light meromyosin (LMM) are

apparent. Comparison of this gel (D) with gels B and C shows that the neutral proteinase isolated from *P. fragi* causes breakdown of the myosin heavy chain to form not only LMM and HMM but also PMM, the early breakdown product of myosin cleavage (Porzio & Pearson, 1977*a*). The extensive degradation seen in gel C is caused by the *P. fragi* protease. This is not surprising since this protease belongs to the Zn^{2+}-activated-Ca^{2+}-stabilised class of enzymes, which show a high degree of non-specificity (McConn *et al.*, 1967; Morihara *et al.*, 1969; Morihara & Tsuzuki, 1969). The fact that PMM is an initial product indicates that the site of cleavage in the myosin heavy chain is extremely susceptible to proteolysis, even in the case of a psychrophilic bacterial protease which exhibits extremely low catalytic activity.

This study demonstrates that the protease isolated from *P. fragi* is capable of extensive degradation of the myofibrillar proteins, especially of myosin. This confirms earlier work from our laboratory showing that *P. fragi* organisms can break down the myofibrillar proteins during meat spoilage (Dutson *et al.*, 1971; Tarrant *et al.*, 1973). It is also of special interest to note that an early step in the hydrolytic breakdown of myosin by the protease involves formation of PMM—an early degradation product of myosin.

ACKNOWLEDGEMENTS

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MUTUAL INTERFERENCE EFFECTS IN THE COLORIMETRIC METHODS USED TO DETERMINE THE SUGAR COMPOSITION OF DIETARY FIBRE

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ABSTRACT

An examination was made of the mutual interference effects in the colorimetric methods currently used to determine the sugar composition of dietary fibre components. Correction factors for use with these procedures are given.

INTRODUCTION

There is an increasing interest in the physiological effects attributable to the dietary carbohydrates collectively termed dietary fibre. However, this term embraces a wide variety of polysaccharide species and this presents considerable technical difficulties for the analyst. Nonetheless, it is important that any analytical procedure chosen should provide information of such a nature that observed physiological effects may be interpreted. Southgate (1969) has proposed that a detailed chemical analysis of the composition of the constituents separated by physical chemical methods will provide a working basis for the study of the physiological effects of dietary fibre.

This paper presents the results of an examination of the colorimetric methods used in the dietary fibre analytical scheme described by Southgate (1969) and offers correction factors to be used with these methods to compensate for their non-specificity.

EXPERIMENTAL PROCEDURES

(a) *Measurement of hexoses*

Hexoses were determined by measuring absorbance after reaction with anthrone, using the method described by Roe (1955).

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(b) *Measurement of pentoses*

Pentoses were determined by measuring absorbance after reaction with orcinol, using the method described by Albaum & Umbreit (1947).

(c) *Measurement of uronic acids*

Uronic acids were determined by measuring absorbance after reaction with carbazole, using the method described by Bitter & Muir (1962).

(d) *Standards*

(i) *Hexose:* glucose at 20, 100 and 200 μg/ml in saturated benzoic acid.

(ii) *Pentose:* arabinose at 3, 15 and 30 μg/ml in saturated benzoic acid.

(iii) *Uronic acid:* glucuronic acid at 4, 20 and 40 μg/ml in saturated benzoic acid.

(e) *Absorbance measurements*

All absorbance measurements were made with a Pye-Unicam SP30 spectrophotometer fitted with a 1 cm pathlength cuvette.

(f) *Source of materials*

All sugars and reagents used in this work were purchased from British Drug Houses, Poole, Dorset, Great Britain.

RESULTS AND DISCUSSION

Standard solutions of hexose, pentose and uronic acid (see 'Experimental Procedures', section (d)) were carried through each of the colorimetric procedures and the absorbance measurements converted to concentration by the appropriate standard curve. The standard solutions were examined alone and in all combinations.

(a) *Anthrone procedure*

(i) *Pentose alone:* No detectable colour was produced with any of the pentose standard solutions.

(ii) *Uronic acid alone:* No detectable colour was produced with any of the uronic acid standard solutions.

(iii) *Pentose/uronic acid mixtures:* Conversion of the absorbance measurements to concentration yielded values from 1·7 to 7·2 μg (hexose equivalents) per millilitre, but there was no correlation between the magnitude of the absorbance readings and the concentration of either the pentose or the uronic acid.

Although these results vary in an unpredictable manner, it is important to realise that absorbance readings under these conditions will be interpreted in terms of hexose concentration, even when none is present.

(iv) *Hexose/pentose mixtures:* Absorbance measurements on these mixtures, when converted to concentration, gave values close to those predicted for the mixtures containing 100 and 200 μg of hexose per millilitre. However, the mixtures containing only 20 μg of hexose per millilitre gave, on average, 124% of the predicted value.

Clearly, absorbance readings indicating a low concentration of hexose should only be accepted with caution.

(v) *Hexose/uronic acid mixtures:* The measurements obtained with these mixtures were very similar to those obtained with the hexose/pentose mixtures. Again, an average of 124% of the predicted value was found for those mixtures containing 20 μg of hexose per millilitre, and so the same cautionary note applies.

TABLE 1
MEASUREMENT OF SUGAR MIXTURES BY REACTION WITH ANTHRONE

[*Pentose*] (*μg/ml*)	[*Uronic acid*] (*μg/ml*)	% *Expected colour yield* [*Hexose*] (*μg/ml*) 20	100	200
3	4	98	95	99
15	4	101	96	103
30	4	114	104	103
3	20	206	97	104
15	20	138	94	103
30	20	112	98	103
3	40	129	93	93
15	40	123	88	95
30	40	126	85	95

Mixtures of hexose, pentose and uronic acid were made at the concentrations shown in the above Table and carried through the anthrone procedure. The spectrophotometric values were converted to hexose equivalents using the glucose standard curve and these values are given in the Table as percentages of the known hexose concentration.

(iv) *Hexose/pentose/uronic acid mixtures:* The results obtained using mixtures of all three sugars are given in Table 1. As observed for the hexose/pentose and hexose/uronic acid mixtures (see sections (iv) and (v) above), marked deviation from the expected values is found only for those mixtures containing the lowest concentration of hexose (average of 127% of the expected values; *cf.* 124% in sections (iv) and (v)).

(b) *Orcinol procedure*

(i) *Hexose alone:* The hexose standard solutions gave absorbance readings that, when converted to concentration, were equivalent to 4·61% of the known hexose concentration (mean value). This mean value can thus be used to correct for the interference of hexose in this procedure (see 'Conclusions').

(ii) *Uronic acid alone:* Standard solutions containing 4, 10, 20 and 40 μg of glucuronic acid per millilitre gave values that were equivalent to 49·39 % (standard deviation 3·88) of the uronic acid concentration. This mean value can thus be used to correct for the interference of uronic acid in this procedure (see 'Conclusions').

(iii) *Pentose/uronic acid mixtures:* Using the suggested correction factor for uronic acid (section (ii) above), an average of 95·4 % (standard deviation 4·0) of the expected value for pentose was found, suggesting that a correction factor closer to 46 % would have been more appropriate for these mixtures.

(iv) *Hexose/pentose mixtures:* Using the suggested correction factor of 4·61 % for hexose (section (i) above), an average of 100·5 % (standard deviation 5·3) of the expected value was found. Clearly, the correction factor is adequate.

(v) *Hexose/uronic acid mixtures:* Using the correction factors suggested for hexose and uronic acid (sections (i) and (ii)), an average of 102 % (standard deviation 4·5) of the predicted values was found, suggesting that these factors are adequate.

TABLE 2
MEASUREMENT OF SUGAR MIXTURES BY REACTION WITH ORCINOL

[*Hexose*] (*μg/ml*)	[*Uronic acid*] (*μg/ml*)	% *Expected colour yield* 3	[*Pentose*] 15	(*μg/ml*) 30
20	4	107·4	107·3	94·7
100	4	94·5	95·7	100·8
200	4	96·1	102·4	104·8
20	20	93·8	95·8	100·4
100	20	100·1	101·8	102·0
200	20	99·8	100·8	106·2
20	40	93·6	93·0	95·5
100	40	90·6	95·6	94·8
200	40	88·6	90·8	97·2

Mixtures of hexose, pentose and uronic acid were made at the concentrations shown in the above Table and carried through the orcinol procedure. The spectrophotometric values were converted to pentose equivalents using the arabinose standard curve and these values are given in the Table as percentages of the known pentose concentration.

(vi) *Hexose/pentose/uronic acid mixtures:* The results obtained with mixtures of all three sugars are given in Table 2. The results are presented as percentages of the expected values using the correction factors suggested above. The mean of all results was 97·9 % (standard deviation 5·1) of the expected value.

(c) *Carbazole procedure*

(i) *Hexose alone:* Hexose standard solutions gave values equivalent to 16 % of the known hexose concentration. This value can thus be used to correct for the interference of hexose in this procedure (see 'Conclusions').

(ii) *Pentose alone:* No detectable colour was produced with any of the pentose standard solutions.

(iii) *Pentose/uronic acid mixtures:* No interference by the pentose was detected, as expected from the results given in section (ii) above.

(iv) *Hexose/pentose mixtures:* It is to be expected from the results given in sections (i) and (ii) above that the concentration values obtained with these mixtures would be equivalent to 16% of the hexose content. However, the actual values obtained were, on average, 117% of those expected. This effect is not seen when all three sugars are present (see section (vi) below).

(v) *Hexose/uronic acid mixtures:* Using the suggested correction factor for hexose (section (i) above), an average of 102% of the expected values was found.

TABLE 3
MEASUREMENT OF SUGAR MIXTURES BY REACTION WITH CARBAZOLE

[*Hexose*] (*μg/ml*)	[*Pentose*] (*μg/ml*)	% *Expected colour yield* [*Uronic acid*] (*μg/ml*) 4	20	40
20	3	69·4	91·8	101·2
20	15	105·8	97·3	108·5
20	30	75·0	103·2	110·9
100	3	85·9	103·8	97·9
100	15	100·7	102·0	100·6
100	30	96·1	100·0	99·6
200	3	121·1	105·1	100·9
200	15	103·4	104·7	103·9
200	30	108·9	104·6	102·7

Mixtures of hexose, pentose and uronic acid were made at the concentrations shown in the above Table and carried through the carbazole procedure. The spectrophotometric values were converted to uronic acid equivalents using the uronic acid standard curve and these values are given in the Table as percentages of the known uronic acid concentration.

(vi) *Hexose/pentose/uronic acid mixtures:* The results obtained with mixtures of all three sugars are given in Table 3. The values in Table 3 are given as the percentages of those values expected, applying a correction of 16% for the hexose (section (i) above) but making no correction for the pentose. The mean of all results is 100·2% (standard deviation 10·3) of the expected value, suggesting that this correction is adequate.

CONCLUSIONS

Southgate (1969) reported the interference of hexose in the orcinol method at a level of 5% as arabinose (*cf.* 4·61% reported here). At that time he did not examine mixtures containing a high level of uronic acid and so no correction was proposed.

Correction factors similar to those reported here have been applied routinely to the analytical results obtained in this laboratory using these colorimetric methods. The results obtained in this way show good agreement with those obtained by gas–liquid chromatography.

The experiments reported here allow the determination of factors that can be used to correct for the non-specificity of the colorimetric procedures under study.

The factors should be used as follows:

Let the value from the anthrone procedure = h.
Let the value from the carbazole procedure = u.
Let the value from the orcinol procedure = P.

Then:

Hexose concentration = h.

Uronic acid concentration $= u - \frac{16h}{100}$

Pentose concentration =

$$P - \left(\frac{4{\cdot}61h}{100} + 0{\cdot}4939\left(u - \frac{16h}{100}\right)\right)$$

The reader is reminded that the results presented here and the factors derived from them were obtained with single species of hexose, pentose and uronic acid. It may be necessary to establish the validity of these correction factors in circumstances where these particular sugars are known to be only minor components of the material under study.

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THE STRUCTURE OF β-D-FRUCTOFURANOSE IN AQUEOUS SOLUTION

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ABSTRACT

The specific rotation of β-D-fructofuranose in aqueous solution is positive (+78°) and that of methyl β-D-fructofuranoside is negative (−60°). The opposing chir-optical properties of these compounds are attributed to the formation by the former, of a hydrogen bond between the anomeric (OH—2) and C—6 hydroxyl groups to form a bicyclic and dextrorotatory chelate structure. The assignment explains the optical rotatory behaviour of D-fructose in aqueous solution at different temperatures and in aqueous alcohol, urea and borate solutions. It also explains precisely why β-D-fructofuranose is devoid of sweetness.

INTRODUCTION

In the course of our studies on the reactions and properties of sugars in foods, we have encountered unexpected results when dealing with the chemistry of D-fructose in aqueous solutions. Many anomalous aspects of the data obtained now seem to be capable of rationalisation by assigning a bicyclic chelate structure to β-D-fructofuranose. Supporting data and background information leading to this assignment serve as the basis of this paper.

MATERIALS AND METHODS

Preparation of methyl β-D-fructofuranoside

The sucrose methanolysis procedure described by Horvath & Metzenberg (1963) for the preparation of methyl β-D-fructofuranoside was followed without alteration. However, the methyl β-D-fructofuranoside syrup obtained had $[\alpha]_D^{23} = -81 \cdot 4°$ (anhydrous basis) due to the presence of about 10% methyl β-D-fructopyranoside.

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The pyranoside was removed by dissolving the syrup in a minimum amount of water and washing it through a 4·5 × 8 cm Dowex-1 (OH form) column. Evaporation yielded 6·5 g of syrup containing 56 % methyl β-D-fructofuranoside $[\alpha]_D^{23} = -60{\cdot}6°$ (anhydrous basis).

Sucrose hydrolysis procedure

Ten per cent solutions of sucrose $[\alpha]_D^{23} = +66{\cdot}5°$, pH 4·6 (0·2 M acetate buffer) were hydrolysed by adding an equal volume of yeast invertase (0·03–0·5 mg/ml) at 23°.

Optical rotatory measurements

The optical rotation of the various solutions studied was monitored using an automatic polarimeter (Rudolph Autopol) fitted with a recording attachment. All solutions were contained in a two decimetre tube.

Preparation of 'standard' curves

For the purpose of subsequent calculations, the course of the mutarotation of α-D-glucose was monitored using conditions (pH 4·6, invertase present) employed for the sucrose hydrolysis studies. The first-order kinetic plot, which was entirely linear, indicated an initial specific rotation of +112·3° and an equilibrium specific rotation of +53·8°.

A sucrose hydrolysis 'rate' curve was prepared by measuring the generation of free reducing sugars at various times, again using the reaction conditions subsequently employed in various hydrolysis studies using the rotatory technique. The reducing sugars were measured using Nelson's (1944) reagents.

Solvent mixture procedures

Aqueous solutions of anhydrous ethanol and methanol were prepared by adding the alcohols to a given weight of water on the balance to prepare the alcohol–water solutions on a weight/weight basis. These solutions were subsequently used to prepare solutions of the sugars on a weight/volume basis.

Preparation of borate solutions

Sodium tetraborate solutions of varying molarity were prepared by dissolution in citrate–phosphate buffer, 0·2 M, pH 7.

RESULTS AND DISCUSSION

Specific rotation of β-D-fructofuranose

It has recently been shown that β-D-fructofuranose in water solution has an initial specific rotation ($[\alpha]_D$) of at least +64° (Shallenberger *et al.*, 1977) and most

probably +78° (Shallenberger, 1978). Earlier reports on the specific rotation of β-D-fructofuranose (i.e. that form of fructose liberated from sucrose by the action of invertase) set the value at about +15° to +17° (Bailey & Hopkins, 1933) or −4·58° (Andersen & Degn, 1962). Whereas the unwitting error or erroneous assumption in many other studies could be ascertained, the basis for the discrepancy between the sucrose–invertase hydrolysis estimates of the specific rotation of β-D-fructofuranose and our findings seemed, until recently, to be caused by the above-named investigators applying the first-order kinetic equation to a reaction (mutarotation of D-fructose) that obviously is not a first-order reaction in its entirety. However, we have now also been able to obtain values for the specific rotation of β-D-fructofuranose that correspond with those of Bailey and Hopkins and Andersen and Degn by direct calculation of the specific rotation of the 'fructose' form liberated from sucrose by the action of invertase. The source of the discrepancy between their values and our higher estimates has now become apparent.

An equilibrium kinetic plot of the calculated apparent specific rotation of the 'fructose' form liberated from sucrose by the action of invertase at various concentrations is shown in Fig. 1. In this case, the observed rotations at various times were corrected by subtracting the apparent rotatory contribution of the liberated α-D-glucose and the invertase itself from the observed rotations.

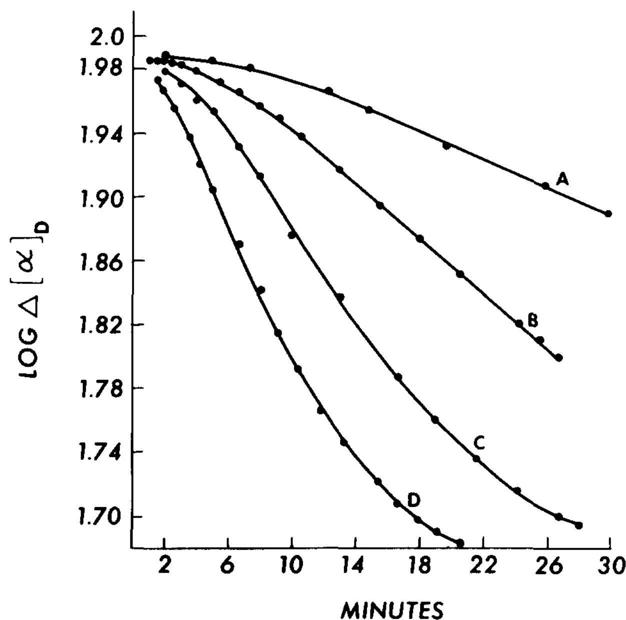


Fig. 1. Change in the apparent specific rotation of the 'fructose component' of sucrose liberated by the action of invertase, at various concentrations, on sucrose. A, invertase at 3 mg/100 ml; B, 6·7 mg/100 ml; C, 12·5 mg/100 ml; D, 25 mg/100 ml.

Extrapolation of the linear portion of these data to zero time and readjustment of the data gave an apparent initial specific rotation for the 'fructose' form liberated from sucrose of $+15{\cdot}5 \pm 0{\cdot}8°$. This value is in excellent agreement with that calculated by Hudson (1909) and seemingly confirmed by Bailey & Hopkins (1933). It needs to be emphasised, however, that the hydrolytic action of invertase, even at the high concentration of 25 milligramme per cent (curve D in Fig. 1) is only 50% complete after three minutes, and the calculated values for D-fructose are in error due to the positive rotatory contribution presented by residual sucrose.

Nevertheless, the deviation of the fructose mutarotation from first-order reaction kinetics is apparent in the data in Fig. 1, especially at increasingly higher enzyme concentrations.

The calculation made by Hudson (1909):

$$(109°)\ (0{\cdot}525) + (X)\ (0{\cdot}525) = +66°,\ X = +17°$$

where $+109°$ and $+66°$ are the specific rotations of α-D-glucose and sucrose, respectively, was justified on the basis that there is not, at the onset of rapid invertase hydrolysis, a striking change in rotation. The error in this premise is that it does not take into account that proportion of the optical rotatory power of sucrose due to the axis of chirality presented by the glycosidic union between the glucose and fructose moieties of sucrose.

When the observed rotations obtained for the data shown in Fig. 1 are corrected for residual sucrose, the invertase and the contribution due to liberated and mutarotating α-D-glucose, the extrapolated value assigned to free 'fructose' is $-4{\cdot}6°$, in apparent excellent agreement with the value reported by Andersen & Degn (1962). Adjustment of the data obtained for curve D in Fig. 1 in this manner is shown in Fig. 2. It would now seem clear that the initial specific rotation of the 'fructose' form liberated from sucrose by invertase hydrolysis does indeed have a value of $-4{\cdot}6°$. It is doubtful, however, that invertase hydrolysis of sucrose procedures is actually capable of revealing an accurate value for the specific rotation of β-D-fructofuranose.

The initial set of events for the invertase inversion of sucrose have long been suspected to be rather complex. Nelson & Bloomfield (1924) were able to detect two distinct stages. One is independent of temperature and the other is not. Evidence obtained by Bacon & Edelman (1950) and also by Blanchard & Albon (1950) showed that the initial products formed by the action of invertase on sucrose are not free glucose and fructose but tri- and higher saccharides which result from transglucosylation. The 'initial specific rotation' of the hydrolysis products would then be the rotatory power of some unknown mixture of transglycosylation products, and not the specific rotation of either free β-D-fructofuranose or α-D-glucopyranose. On the other hand, procedures utilising the invertase hydrolysis of methyl-β-D-fructofuranoside do apparently give more accurate values (Levi & Purves, 1949; Shallenberger, 1978) because transglucosylation possibilities are

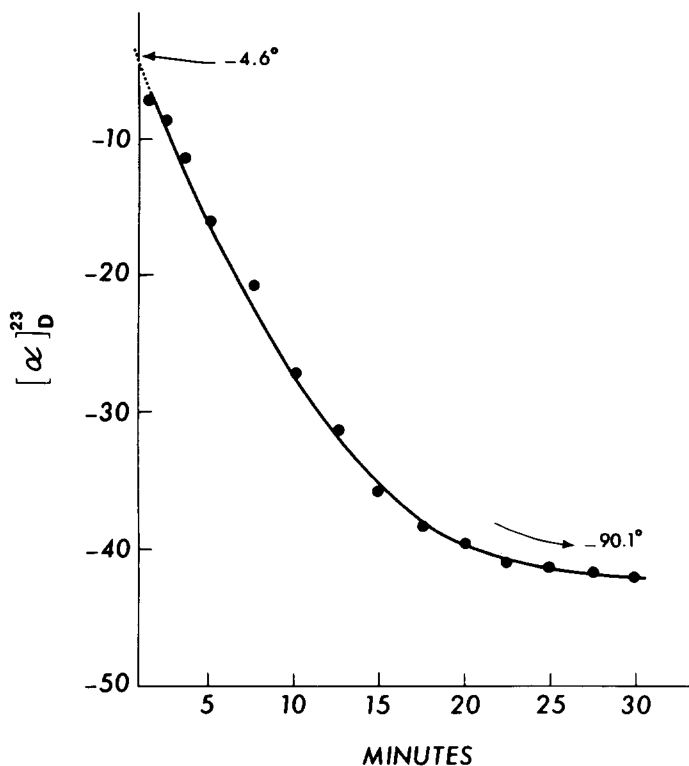
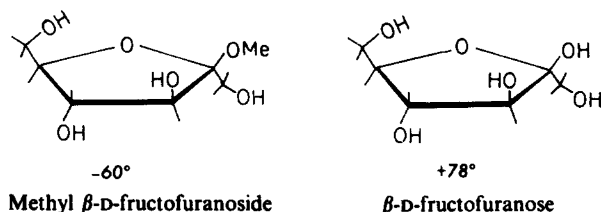


Fig. 2. Curve D from Fig. 1 converted to calculated specific rotations for the 'fructose' form liberated from sucrose by invertase hydrolysis and corrected for the rotatory contribution made by the unhydrolysed sucrose in addition to the correction for the α-D-glucose generated and also for the invertase present.

excluded. Transfructosylation must also not be a problem because of the agreement between thermal mutarotation (Shallenberger *et al.*, 1977; Shallenberger, 1978) and methyl-β-D-fructofuranoside–invertase hydrolysis studies, although certain transfructosylation products have been found (Andersen, 1967) in sucrose–invertase hydrolysis studies.

Structural significance of the positive specific rotation of β-D-fructofuranose

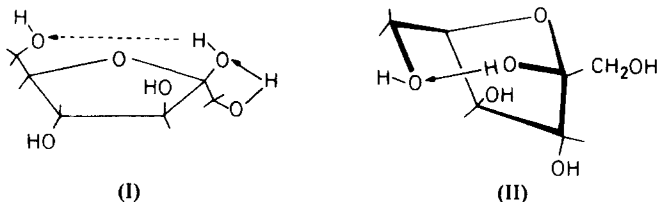
Establishment of the probable specific rotation of β-D-fructofuranose at about +78° in water solution generates a structural problem, the resolution of which serves as the basis of this paper. The problem is posed by the fact that methyl β-D-fructofuranoside has a negative specific rotation (−60°) whereas the free furanose is highly positive. The structural relationship between the two compounds is shown below using Haworth perspective drawings.



Methyl β-D-fructofuranoside β-D-fructofuranose

It would seem unlikely that the mere substitution of a proton for a methyl group at the anomeric centre of a compound would cause a reversal of rotatory sign (actually a change in the chir-optical properties) unless the structure that contributes significantly to those rotatory properties for one of the compounds is not represented by the conventional Haworth–Hirst furanose formula. Since OH—2 of the fructofuranose structure is obviously involved in the change in chir-optical properties of methyl-β-D-fructofuranoside, and yet configuration about that chiral centre is unchanged during the transformation, it was reasoned that the chir-optical transformation must involve the formation of a secondary ring structure that is not only optically active but is strongly dextrorotatory.

Among the sugar structures known for D-glucose and D-fructose, it can be stated that, in order of increasing number of hydrophobic sites, the following ranking can be developed: α- and β-D-glucopyranose < β-D-fructopyranose < β-D-fructofuranose. The two glucose anomers have only one unambiguous hydrophobic site (the methylene carbon atom at C-6). β-D-fructopyranose has two hydrophobic sites (the methylene carbon atom at C-1 and the pyranose ring methylene carbon atom at C-6). α- and β-D-fructofuranose have three hydrophobic sites, the C-1 and C-6 methylene carbon atoms and the ring —CH_2— structure at C-5. With three hydrophobic sites in its structure—and consequently a relatively high degree of lipophilicity—it would seem that in a polar environment, e.g. in solution in water, β-D-fructofuranose would tend to (intrinsically) create its own hydrophobic environment. Furthermore, those polar (OH) substituents bonded single covalently to the hydrophobic sites would have an enhanced tendency to hydrogen bond each other. In aqueous solution, therefore, a probable structure for β-D-fructofuranose that seems to explain its highly positive dextrorotatory character is the internal hydrogen bonded chelate structure (I) shown below and redrawn in (II) to emphasise that the chelate ring generated is dextrorotatory. It therefore makes the major contribution to the rotatory power of β-D-fructofuranose in an aqueous environment.



(I) (II)

Justification for structure (II) is that the proton of OH-2 is activated by virtue of the hydrogen bond in structure (I) created between the proton of OH-1, in a hydrophobic environment of its own, and the oxygen atom of OH-2 (the 'Lemieux effect'—Lemieux & Brewer, 1973; Shallenberger, 1978). The subsequent hydrogen bonding (dotted line), across the furanose ring, to the oxygen atom of OH-6 is, in structure (II), a neat example of 'co-operative' hydrogen bonding (Jeffrey & Lewis, 1978). The Lemieux effect is not shown in structure (II) for the sake of clarity. The new ring structure thus formed is, of course, chiral and also dextrorotatory.

This structural interpretation of the chir-optical anomaly presented by the specific rotations of methyl β-D-fructofuranoside and β-D-fructofuranose in water solution seems to be applicable to understanding and predicting the optical rotatory behaviour of aqueous fructose solutions at different temperatures, in solvents of different polarity, in solutions of urea and in borate solutions of varying concentrations. The structure also explains why β-D-fructofuranose is devoid of sweet taste.

Effect of temperature on the equilibrium specific rotation of D-fructose

As the temperature of equilibrium D-fructose in aqueous solutions is increased, the equilibrium shifts markedly to favour the formation of furanoses, with a rapidly increasing ratio of α-furanose to β-furanose (as is to be expected by the severing of an H-bonded chelate structure for β-D-fructofuranose). There is also a corresponding increase in the positive rotation of the system (Shallenberger *et al.*, 1977). A partial explanation of this phenomenon lies in the fact that the polarity of water decreases markedly with the increasing temperature to thus favour the generation of the more intrinsically hydrophobic fructose forms. By the same token, increasing temperatures have only a slight effect on shifting the α-D-, β-D-glucopyranose equilibrium.

Effect of increasing concentrations of alcohol in water–alcohol mixtures on the equilibrium specific rotation of D-fructose

It was also reasoned that increasing the alcohol content of D-fructose–water solutions, while decreasing the polarity of the solvent mixture system, would, if the H-bonded chelate structure for β-D-fructofuranose is correct, be reflected in an increase in the positive rotation of the solutions, again because the chelate structural form would be favoured. An early study by Hudson & Yanovsky (1917) indicated that this is indeed the case. Moreover, increasing concentrations of ethanol should be more effective in this respect than methanol. The data that we have obtained to substantiate these ideas are shown in Fig. 3.

Effect of urea on the equilibrium specific rotation of D-fructose in aqueous solution

Because of its ability to sever H-bonds, it was reasoned that urea also should alter the equilibrium specific rotation of D-fructose. In this case, however, the effect of increasing concentrations of urea should be to increase the laevorotatory power of

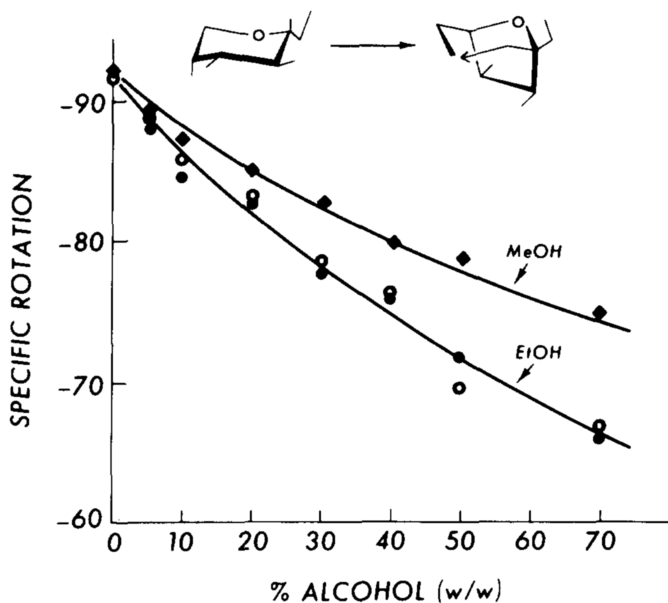


Fig. 3. Effect of increasing concentrations of ethanol and methanol on the equilibrium specific rotation of D-fructose.

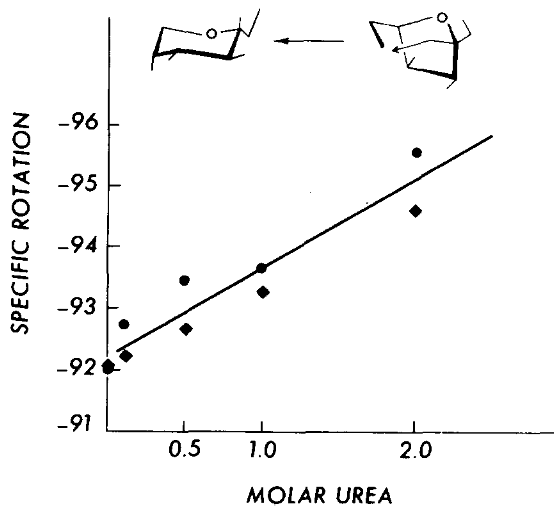


Fig. 4. Effect of increasing concentrations of urea on the equilibrium specific rotation of D-fructose.

the fructose solutions, since formation of non-hydrogen bonded β-D-fructofuranose and the β-D-pyranose structure (specific rotation, −132°) is favoured. The data obtained to support this contention are shown in Fig. 4. The data also confirm an earlier report of Brown & Zerban (1941).

Effect of borate on the equilibrium specific rotation of D-*fructose in buffered aqueous solutions*

The reaction of D-fructose solutions with borate (Böeseken, 1949) is very rapid, extensive and complex. Nevertheless, the most likely candidate for explaining these observations is the chelate structure proposed as the water solution structure for β-D-fructofuranose. If β-D-fructofuranose were to form the stable tridentate borate complex (Angyal & Mchugh, 1957), the extent of the optical rotatory change of equilibrium fructose in borate solution would be large and it would be positive. As shown in Fig. 5, the shift in specific rotation is large (+48°), probably due to an

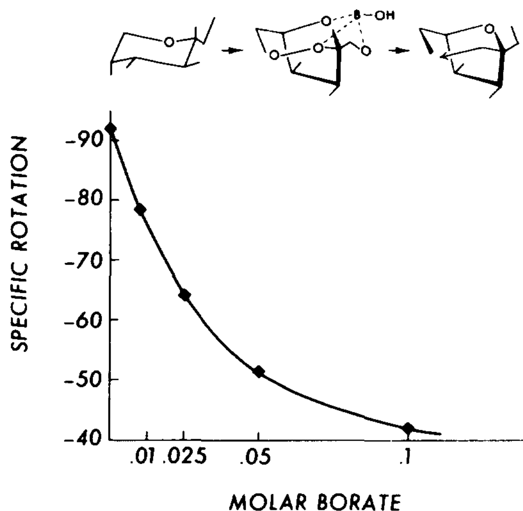


Fig. 5. Effect of increasing concentrations of borate on the equilibrium specific rotation of D-fructose.

increased specific rotation of the borate complex itself, plus the favouring of the formation of free β-D-fructofuranose. Although the data obtained and shown in Fig. 1 employed buffered solutions, there was a slight drop in pH due to the acidic nature of the borate–fructofuranose adduct. There is, however, no change in the specific rotation of aqueous fructose solutions at widely different pH values (3–8).

*Taste properties of the bicyclic-chelate structure of β-*D-*fructofuranose*

The intramolecularly H-bonded chelate structure explains best why β-D-fructofuranose is devoid of sweetness and why fructose sweetness is so variable. This

particular fructose structure has the primary fructose AH unit (OH-2), which is a prerequisite for sweetness (see Shallenberger & Lindley, 1977), hydrogen bonded intramolecularly. The structure is therefore incapable of participating in the initial chemical interaction prerequisite for sweet taste. Any change in the fructose environment which alters the position of the fructopyranose–furanose equilibrium will then have a corresponding effect on fructose sweetness.

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A MODIFIED METHOD FOR THE ISOLATION AND QUANTIFICATION OF OIL SEED PHOSPHOLIPIDS

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ABSTRACT

A modified method for the isolation and accurate quantification of oil seed phospholipids is described. Castor bean and sunflower were taken to represent seeds of high neutral lipid and low phospholipid content.

Preparative thin layer chromatography (TLC) on silica gel was used for the separation of the phospholid class from the total lipid extract which was prepared by a modified method. An eluant composed of methanol:acetic acid:water, 94:1:5, which gave 95 ± 3% phospholipid recovery was used. The phospholipids were fractionated by one-dimensional TLC and identified by a series of colour reagents as well as by comparison with a natural reference which contained all the known phospholipid fractions and was prepared from rat liver. The fractionated phospholipids on TL chromatographic plates were quantified by phosphorus determination.

INTRODUCTION

It is well known that the quantification of phospholipids† in a tissue requires a series of analytical steps which begin with the extraction of the lipids from the tissue, followed by the chromatographic fractionation of phospholipids, identification and, finally, quantitative determination of each fraction (Spaner, 1973).

The most successful method for total lipid extraction is that devised by Folch *et al.*, (1957) using chloroform: methanol (2:1) which was originally used for the

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† Abbreviations: Ps—Phosphatidyl serine; LPC—Lysophosphatidyl choline; PI—Phosphatidyl inositol; SL—Sphingolipid; PC—Phosphatidyl choline; PE—Phosphatidyl ethanolamine; PG—Phosphatidyl glycerol; PA—Phosphatidic acid.

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extraction of brain tissue lipids. Numerous modifications were introduced by Wren & Mitchell (1959), Dawson *et al.* (1960) and Privett *et al.* (1973) to make it more suitable for specific tissues.

The simplest and most efficient procedure for separating phosphatides from neutral lipids is the acetone precipitation technique which depends on the general insolubility of phospholipids and the solubility of glycerides in cold acetone. It has been used for the purification of the lipids of egg yolk (Rhodes & Lee, 1957) and heart muscle (Hoevet *et al.*, 1968). Kates (1972) pointed out that this procedure worked best with animals and microbial lipids, less well with leaf lipids and was not effective with either seed lipids or mixtures containing high proportions of neutral lipids and low amounts of phosphatides. Borgstrom (1952), Vorbeck & Marinetti, (1965) and Rouser *et al.* (1963) used a silicic acid column to separate neutral lipids from phospholipids. Mangold (1961) gave an extensive review of the TLC methods for phospholipids up to 1961. Harrocks (1963) and Bunn *et al.* (1969) modified the silica gel used in TLC by the addition of borate buffer at pH 8 which improved the resolution of phospholipids with less streaking and diffusion.

No detailed method is available in the literature for the rapid and accurate quantification of plant phospholipids which overcomes the different problems stemming from the fact that plant lipids, especially those of seed origin, contain higher proportions of neutral lipids and have a low phospholipid content (Eckey, 1954, Hitchcock, 1971 and Kates, 1972).

The present work was conducted primarily to devise a complete and detailed method for the resolution and quantification of seed phospholipids.

MATERIALS AND METHODS

Materials

Albino rat lipid extract was prepared by the modified method described below and used as a natural standard for phospholipids.

Egg lecithin (B. D. H., Great Britain) was used for the elution experiments.

Blends of varieties of castor beans (*Ricinus communis*) and Miak of sunflower seeds (*Helianthus annus*) were dehulled by hand before lipid extraction.

All the chemicals and solvents were reagent grade and the solvents were redistilled, as described by Vogel (1964), before use. Silica gel G (E. Merck) was used for preparative and analytical TLC.

Apparatus

Centrifuge: Type 'T15' Heinz Janck, Leipzig, Germany.

Homogeniser: Unipan Universal Laboratory Aid Type 309 (Poland).

Rotary film evaporator: Unipan Laboratory Aid Type 309 was used for the evaporation of solvents under reduced pressure.

Spectrocolorimeter: Type Spekol Carl-Zeiss Jena, Germany.

TLC equipment consisted of standard glass plates (10 × 20 and 20 × 20), racks, jars lined on three sides with filter paper, a template and a manual adjustable Camag applicator (Camag, Muttenz, Switzerland).

Methods

Preparation of total lipid extract free of non-lipid contaminants: The preparation of the total lipid extract was carried out according to the methods of Folch *et al.* (1957) and Privett *et al.* (1973), with some modifications. Two to four gramme samples were extracted three times by homogenisation under nitrogen at 14,000 rpm for 3 min with 20-fold their weight of chloroform:methanol, 2:1 (v/v). The miscella were collected by filtration under reduced pressure using a G_2 sintered glass funnel. The combined filtrates were diluted by the addition of 100 volumes of the chloroform:methanol mixture for each gramme of sample, washed with one-fifth their volume of 0·04 % $MgCl_2$ to remove the non-lipid contaminants and the mixture was allowed to stand overnight at 4 °C to permit phase separation. The chloroform phase containing the lipid extract was concentrated under reduced pressure at 35 °C with a rotary film evaporator, diluted with benzene or ethyl alcohol and reconcentrated. The addition of benzene or ethyl alcohol was repeated several times to remove any traces of water present. The concentrated lipid extract was cleared by filtration and the filtrate evaporated to dryness. The residue was immediately dissolved in chloroform, transferred to volumetric flasks (10 or 25 ml), stored in glass vials with screw caps, flushed with nitrogen and kept at −14 °C. Accurate determination of the total lipids in the stock solution was carried out as described by Kates (1972). A suitable aliquot of the stock solution was evaporated in a weighing bottle in a stream of nitrogen and then dried in a desiccator over potassium hydroxide pellets in an atmosphere of nitrogen to constant weight.

Isolation and concentration of phospholipids from total lipid extract: The method of Braddock & Dugan (1972) was followed with some modification. A suitable aliquot of the stock solution of the total lipid extract was pipetted into a 15-ml graduated centrifuge tube, then concentrated by solvent evaporation under a stream of nitrogen to 200–400 μg of lipid phosphorus per millilitre. The concentrated solution was applied as streaks on three 1 mm thick preparative thin layer plates. The plates were developed in a solvent system composed of petroleum ether:diethyl ether: acetic acid (70:30:2 v/v). The phospholipid class, which was still adsorbed on the silica gel at the origin, was scraped off and the silica gel was transferred to a 1 × 15 cm glass column from which the phospholipids were eluted by a suitable eluant which gave maximum recovery. The elution process was accomplished under a nitrogen pressure of 0·4 kg/cm^2. The eluant was collected in a 50-ml volumetric flask and the recovery of phospholipids was calculated after phosphorus determination. A suitable aliquot of the collected eluant (30–40 ml) was evaporated to dryness, immediately redissolved in a suitable volume of chloroform and filtered through a 5-ml glass syringe fitted with a small plug of previously defatted cotton. The cotton

plug was then washed several times with small amounts of chloroform. The combined filtrates were again concentrated under reduced pressure and transferred to a 10-ml volumetric flask, then flushed with nitrogen gas and stored at −14 °C, serving as a stock solution of the phospholipid class.

Fractionation of phospholipid class by thin layer chromatography (TLC): Silica gel G mixed with 0·1 M borate buffer at pH 8 (1:2 w/v), spread on 0·5 mm thick thin layer plates was used for the fractionation of the phospholipid class according to the method of Bunn *et al.* (1969). The plates were air dried and activated at 100 °C for 1 h. A suitable aliquot of the phospholipid class solution was concentrated, as previously described, in a stream of nitrogen to give the final stock solution which should contain 1–1·5 μg phosphorus per microlitre. An aliquot containing 20–30 μg phospholipid phosphorus was applied to the thin layer plates which were developed using the solvent system of chloroform:methanol:water (65:25:4). The chromatograms were dried and sprayed with the charring reagent of Rouser *et al.* (1970) which was composed of three volumes of 37 % formaldehyde and 97 volumes of 98 % sulphuric acid.

Identification of individual phospholipids: A sample of rat liver lipid extract with a known pattern of phospholipid composition was used as a reference (Aman *et al.*, 1970). The identification of individual phospholipids was accomplished using the following specific detection reagents. The reagent of Vasovsky & Kostetsky (1968) was used as a general spray for phospholipids, the ninhydrin stain of Marinetti (1964) was employed for phospholipids having free amino groups such as PE and PS, the Dragendroff reagent of Beiss (1964) was used for choline-containing lipids, the α-naphthol stain of Siakotos & Rouser (1965) for glycolipids, the stain of Bischel & Austin (1965) for sphingolipids and the Nile blue reagent of Spaner (1973) for phospholipids containing inositol.

Determination of phospholipid phosphorus: A modification of the micro method described by Rouser *et al.* (1970) was used for this determination. The total phospholipid phosphorus was determined in the lipid extract using an aliquot of the stock solution containing 0·5 to 10 μg of phospholipid phosphorus. The stock solution was transferred to a 15-ml pyrex tube. The solvent was evaporated to dryness then 0·9 ml of perchloric acid (70 %) was added. The tubes, plugged with small pieces of pre-washed glass wool, were digested by heating on a direct flame for complete carbonisation and were then transferred to a sand bath controlled thermostatically at 180 °C until the digest was clear and colourless. The cooled digest was diluted to 5 ml with distilled water and the method was completed as described by Rouser *et al.* (1970). One millilitre of ammonium molybdate (2·5 %) was added and mixed, followed by the addition of 1 ml of ascorbic acid (10 %) and further mixing. The walls of each tube were then washed with 2 ml of distilled water. The tubes were mixed again, stoppered with aluminium foil covered plugs and placed for 5 min in a boiling water bath to allow maximum development of molybdenum blue colour. At the end of the heating period, the tubes were cooled and the absorbance of

the stable colour was measured after 15 min at 780 nm in 1 cm cells. A calibration curve was prepared using dipotassium hydrogen orthophosphate solutions of 1 to 10 μg phosphorus concentration in a final volume of 9 ml. This modified method was also used for phosphorus determination of the individual phospholipid fractions separated as spots on the thin layer plates. After the chromatograms had been charred, each spot was scraped off and transferred to a 15-ml pyrex tube. The method followed was as described for phospholipid phosphorus determination in lipid extract(s). After the colour development, the suspension was centrifuged at 4000 rpm for 5 min and the colour of the supernatant was determined.

RESULTS AND DISCUSSION

Isolation and concentration of phospholipids from total lipid extracts

Preliminary experiments on the chromatographic fractionation of phospholipids from rat liver, along with castor bean and sunflower lipid extracts, revealed the following results. (1) On applying TLC chromatographic spots of rat liver lipid extract containing suitable phospholipid concentrations under suitable conditions, the neutral lipids moved with the solvent front and satisfactory fractionation of phospho- and glycolipids to their individual fractions occurred. (2) On the other hand, when the lipid extracts of either castor bean or sunflower were chromatographed in the same manner, poor or no resolution of the phospholipids resulted.

Therefore, in order to obtain effective fractionation of the phospholipids in the samples either rich in neutral lipids or poor in phospholipids, as in the case of oil seeds, it was necessary to concentrate the phospholipids by elimination of neutral lipids. The phospholipids were separated from the lipid extract and made as free as possible of most of the neutral lipid. Usually this step is achieved by column chromatographic methods. The less strongly adsorbed neutral lipid classes are eluted first and finally the more tightly adsorbed phospholipid class is eluted. The elution process requires different solvent systems in order to elute the different lipid classes. These methods are laborious and call for enormous amounts of solvents. It was found that the technique of preparative TLC recommended by Braddock & Dugan (1972) was the least time consuming and most economically sound and effective in the separation of the phospholipid class from the other lipid classes using small volumes of solvent system. The solvent system used (petroleum ether:diethyl ether:acetic acid (70:30:2)), which has a low polarity, separated the non-polar lipids from those of higher polarity. It must be observed, as shown in Fig. 1, that the most polar phospholipid class remained tightly adsorbed on the silica gel at the baseline of the chromatoplates. The other less polar classes of lipids, including glycerides, sterols, hydrocarbons and pigments, migrated towards the front of the plate in decreasing R_F values according to their polarity. This modified method overcame the problem of the low concentrations of phospholipids in plant lipid extracts.

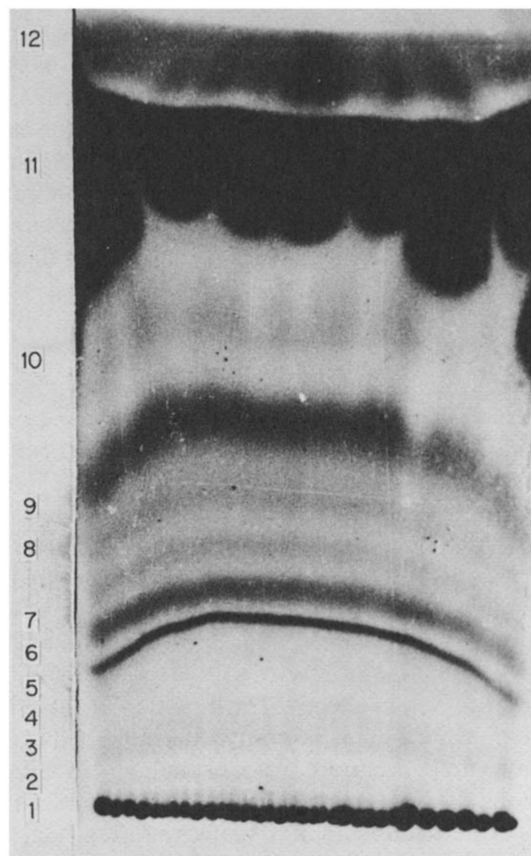

Fig. 1. Preparative TL chromatogram showing the separation of the phospholipid class from the lipids of Castor bean.

Assay conditions:
Aliquot volume: 0·2–0·4 ml.
Aliquot amount: 100–500 μg phosphorus.
Solvent system—petroleum ether:diethyl ether:acetic acid (70:30:2).
Visualisation: Sulphuric acid/formaldehyde reagent.
Fraction No. 1 refers to phospholipid class, fraction Nos 2 to 12 refer to other lipid classes.

Elution and recovery of separated phospholipid class

The major difficulties encountered in the quantitative determination of chromatographically separated lipid classes of differing polarities lies in obtaining quantitative recovery from the thin layer plates and in ensuring that no selective loss of the lipid occurs in any of the subsequent procedures (Christie *et al.*, 1970). Thus, a preliminary study was carried out to test the efficiency of the various eluants

proposed by some workers in order to select the most suitable which would give the highest recovery of phospholipids and require the shortest evaporation time under reduced pressure. The elution and recovery experiments were carried out using egg lecithin as a source of phospholipids.

The elution of phospholipids from the scraped off silica gel was carried out using two methods—centrifugation and column elution. The scraped off phospholipids were transferred either to a centrifuge tube in the first method, or to a glass column in the second, and eluted with a suitable solvent mixture.

Table 1 shows the phospholipid recoveries from preparative thin layer chromatoplates, as affected by eluant type and elution method. It was found more practical to use the column method and the No. 5 eluant, composed of methanol:acetic acid:water, (94:1:5) which gave comparatively good recovery and required less time in evaporation. The percentage of phospholipid recovery obtained

TABLE 1
RECOVERIES OF CHROMATOGRAMMED PHOSPHOLIPIDS AS AFFECTED BY ELUANT TYPE AND METHOD USED[a]

No.	*Eluant (v/v)*	*Reference*	*Phospholipid recovery by*[b]	
			Centrifugation[c]	*Column*
1	$CHCl_3$:MeOH, (4:1)	Braddock & Dugan, (1972)	8	—
2	$CHCl_3$:MeOH, (1:2)	Aman *et al.* (1970)	34	54
3	$CHCl_3$:MeOH:water (65:40:5)	Privett *et al.* (1965)	55	77
4	Methanol	—	55	77
5	MeOH:acetic acid: water (94:1:5)	Kyriakides & Balint (1968)	78	94
6	MeOH:ETOH:water: acetic acid (30:100:20:2)	Biezenski (1967)	81	99
7	$CHCl_3$:MeOH:water: acetic acid (30:100:20:2)	—	78	95
8	$CHCl_3$:MeOH:water (30:100:10:2)	—	29	46
9	$CHCl_3$:MeOH:water: formic acid (97:97:4:2)	Cuzner & Davison (1967)	56	77
10	$CHCl_3$:MeOH:water (20·3:44·7:1)	—	77	90
11	$CHCl_3$:MeOH:water (37·8:27·2:1)	—	62	88

[a] Egg lecithin dissolved in chloroform. The sample, containing 200–400 μg phospholipid phosphorus, was applied to TLC plates.
[b] Each value is an average of four determinations.
[c] Centrifugation at 4000 rpm for 15 min with 150 ml of eluant.

by this eluant was 95 % ± 3 in six estimations. No data concerning the recovery of phospholipid was found in the literature.

Fractionation and identification of phospholipids

Owing to the high cost and scarcity of standard phospholipid references, a lipid extract prepared from albino rat liver, served as a reference mixture (Aman *et al.* 1970). It contained a known composition of the major phospholipid fractions. Aliquots of rat liver extract, each containing 20–25 μg of phospholipid phosphorus, were fractionated. After development, the chromatograms were

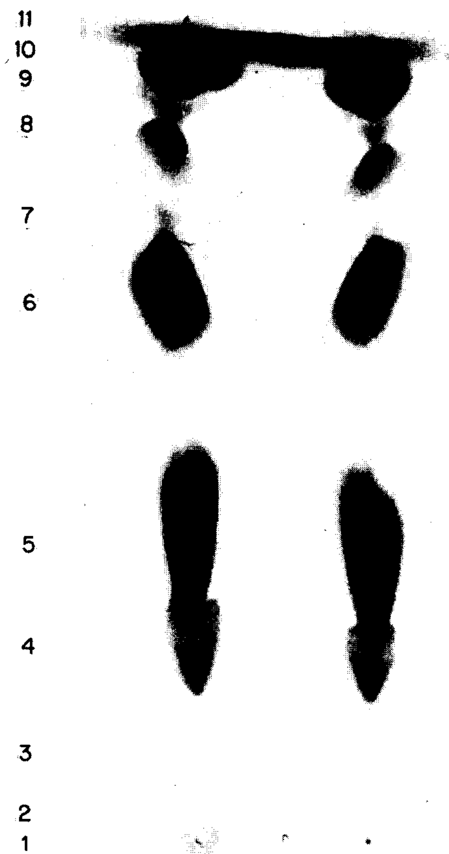

Fig. 2. Thin-layer chromatogram of rat liver phospholipids. 1 = PS, 2 = LPC, 3 = PI, 4 = SL, 5 = PC, 6 = PE, 7 = PA, 8 = Cardiolipin and glycerophosphatides, 9 = Glycolipid, 10 = sterols, 11 = neutral lipids.

Assay conditions:
Solvent system chloroform: methanol:water (65:25:4).
Spray reagent: Sulphuric acid formaldehyde reagent of Rouser *et al.* (1970).

TABLE 2

TENTATIVE IDENTIFICATION OF LIPID CLASSES FRACTIONATED BY THIN LAYER CHROMATOGRAPHY[a]

Fraction number	*Sulphuric acid*	*Iodine vapour*	*Phosphate*	*Dragendroff*	*Ninhydrin*	*Sphingolipid*	*Nile blue*	*Napthol*	*Phospholipid phosphorus (P) as percentage of total lipid (P)*	*As μg*	*Lipid fractions*[b]	R_F *values* (× *1000*)
1	+	+	+	−	+	+	−		0·5	0·1	Phosphatidyl serine	36
2	+	+	+	+	−	−	−		2·5	0·5	Lysophosphatidyl choline	94
3	+	+	+	−	−	−	+		10·3	2·1	Phosphatidyl inositol	160
4	+	+	+	−	−	+	−		53·6	10·9	Sphingomyelin	269
5	+	+	+	+	−	−	+		26·6	5·4	Phosphatidyl choline	380
6	+	+	+	−	+	+	−		3·5	0·7	Phosphatidyl ethanolanine	500
7	+	+	+	−	−	−	+		2·0	0·4	Phosphatidic acid	690
8	+	+	+	−	−	−	−		1·0	0·2	Cardiolipin and glycerophosphatide	730
9	+	+	−	−	−	−	−	+			Glycolipid	870
10	+	+	−	−	−	−	−	+			Sterols	920
11	+	+	−	−	−	−	−	−			Neutral lipids	950

[a] Using buffered borate gel G plates, solvent system—chloroform:methanol:water (65:25:4).

[b] Present work with albino rat liver phospholipids.

Applied phospholipid phosphorus 21 μg.

Recovery: 97%.

sprayed with different reagents, each of which was capable of detecting one or more of the phospholipid fractions. Figure 2 and Table 2 summarise the results. It is clear from these that rat liver lipid extract was separated into eleven fractions with R_F values ranging from 0·03 to 0·95. From these fractions eight were identified as phospholipids by the corresponding specific spray reagent. These phospholipid fractions are the first eight fractions, starting from the baseline. The sequence of phospholipid separation coincided with that reported by Stahl (1969).

Quantification of the fractionated phospholipids

Egg lecithin: Egg lecithin was concentrated, eluted, fractionated and quantified by the methods previously described. Table 3 gives the percentages of individual phospholipid fractions of egg lecithin compared with those found in the literature. The results indicate that the adopted modified technique is quite satisfactory.

TABLE 3
PHOSPHOLIPIDS OF EGG LECITHIN EXPRESSED AS PERCENTAGES OF THE TOTAL LIPID PHOSPHORUS

Fraction number	*Phospholipid fraction*	*Phospholipid phosphorus (P)*			
		In μg (present work)	*As percentage of total lipid*		
			(present work[a])	*(Rhodes & Lee (1957))*	*(Parkinson (1966))*
1	PS	0·5	1·6	—	—
2	LPC	1·7	5·1	5·8	—
3	PI	Traces	Traces	0·6	Traces
4	SL	0·9	2·9	2·5	2·5
5	PC	21·0	62·9	72·8	79·0[b]
6	PE	8·6	25·8	17·9	17·0
7	PG	0·5	1·6	—	—
8	PA	Traces	Traces	—	—

[a] Applied phospholipid phosphorus = 33·8 μg.
Total phospholipid recovery = 99 %.
[b] PC + LPC.

Oil seeds: The optimum conditions previously established were adopted to chromatographically study the qualitative and quantitative distribution of phospholipids in two oil seeds. Castor beans and sunflower were selected because of their high content of neutral lipids (Eckey, 1954). The data presented in Tables 4 and 5 indicate that the phospholipid contents of castor beans and sunflower are very low compared with that of total lipids. Fractionation of phospholipids in the samples was undertaken using the stock solutions of the phospholipid class previously separated from the total lipid extracts.

The phosphorus content of each phospholipid fraction was quantified and expressed as a percentage of the total lipid phosphorus. The recovery of the chromatographic process was computed by comparing the amount of the applied

TABLE 4
PHOSPHOLIPIDS OF CASTOR BEAN[a] LIPID EXTRACT EXPRESSED AS PERCENTAGES OF THE TOTAL LIPID PHOSPHORUS

Fraction number	*Phospholipid fraction*	*Phospholipid phosphorus (P)*		
		In μg (present work)	*As percentage of total lipid*	
			(present work)	*(Paulose* et al. *(1966))*
1	PS	1·1	12·8	—
2	LPC	Traces	Traces	—
3	PI	1·0	11·6	Traces
4	SL	Traces	Traces	—
5	PC	4·2	48·8	13
6	PE	2·3	26·7	83
7	PG	Traces	Traces	—
8	PA	Traces	Traces	—

[a] Kernel per cent, total lipid extract and phospholipid phosphorus were 66·5, 45·7 and 0·01 %, respectively of the whole seed (blend of varieties).
Applied phospholipid phosphorus = 9·4 μg.
Total phospholipid recovery = 92 %

lipid phosphorus with the total quantified phospholipid phosphorus. The data indicate that the procedure adopted succeeded in the concentration of the phospholipid class of castor beans and sunflower to the extent that eight phospholipid fractions were separated and quantified. The data given by Paulose *et al.* (1966) and Galliard (1973) indicate poor resolution for phospholipid fractions separated from castor beans and sunflower seeds.

TABLE 5
PHOSPHOLIPIDS OF SUNFLOWER[a] LIPID EXTRACT EXPRESSED AS PERCENTAGES OF THE TOTAL LIPID PHOSPHORUS

Fraction number	*Phospholipid fraction*	*Phospholipid phosphorus (P)*		
		In μg (present work)	*As percentage of total lipid*	
			(Present work)	*(Galliard (1973))*
1	PS	0·3	1·8	—
2	LPC	Traces	Traces	—
3	PI	1·2	7·4	22
4	SL	2·3	14·2	—
5	PC	9·1	56·2	51
6	PE	1·9	11·7	23
7	PG	0·7	4·3	4
8	PA	0·7	4·3	—

[a] Kernel per cent, total lipid extract and phospholipid phosphorus were 71·8, 35·9 and 0·02 % in the whole seed (Miak variety).
Applied phospholipid phosphorus = 17·6 μg.
Total phospholipid recovery = 92 %.

CONCLUSIONS

The following conclusions can be drawn on the basis of the results obtained in the course of this study.

(1) Preparative TLC proved to be quite satisfactory for obtaining effective separation of the phospholipid class from the other neutral lipid classes.

(2) Continuous elution using the column method was found to be more practical and efficient than the batchwise method for the elution of the phospholipid class.

(3) Rat liver lipid extract, along with various detection reagents, can serve as a good reference material for the identification of individual phospholipid fractions. The phospholipid phosphorous of fractions isolated after TLC can be used to establish the phospholipid composition of the samples.

(4) The methods described above were found to be successful in giving high recoveries of both total lipids and phospholipids. Moreover, better fractionation and quantification of eight phospholipid fractions were obtained when this procedure was applied to castor beans and sunflower seeds which are rich in neutral lipids and poor in phospholipids.

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THE NUTRITIVE VALUE OF SOME NIGERIAN LEAFY GREEN VEGETABLES—PART 2: THE DISTRIBUTION OF PROTEIN, CARBOHYDRATES (INCLUDING ETHANOL-SOLUBLE SIMPLE SUGARS), CRUDE FAT, FIBRE AND ASH*

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ABSTRACT

The crude protein contents of ten leafy vegetables commonly eaten by the peasant population of the Cross River State of Nigeria ranged between 17·2% and 28·4% dry matter. The ranges of values for their crude fat, fibre and ash contents were 2·7–8·1%, 8·5–20·9% and 9·7–18·6% dry matter, respectively. The fructose, glucose, sucrose and maltose contents in 70% ethanol extracts of these vegetables were 0·6–1·6%, 0·6–1·8%, 0·8–2·6% and 0·3–2·3% dry matter, respectively. The dry matter in the vegetables ranged between 7·7% and 24·7% of the fresh weight. The high nutritive potentials of these leafy vegetables justify their wide consumption.

INTRODUCTION

The scarcity of documented information on the nutritive potentials of most of the foodstuffs eaten in Nigeria has greatly contributed towards the ineffectiveness of food production programmes in meeting the nutritional needs of a vast segment of the Nigerian population.

This has been reflected in the lack of adequate food composition data which would serve as a guide in the selective cultivation and consumption of foods that could supply specific nutrients. Deficiency syndromes caused by lack of such nutrients have been reported (Ekpo, 1970) among the low-income class in parts of the country.

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Leafy green vegetables have gained widespread acceptance as a dietary constituent among most Nigerians. Reported studies on Nigerian leafy vegetables (Oyenuga, 1968; Oke, 1968*a*,*b*; Bassir & Fafunso, 1975) are scanty and leave much to be desired when compared with the wide varieties of vegetables commonly consumed by peasant Nigerians. In our earlier study (Ifon & Bassir, 1979) we investigated the vitamin and mineral contents of some of the leafy green vegetables commonly eaten in the Cross River State of Nigeria. The present study covers an investigation of the proximate contents of these varieties of leafy vegetables.

EXPERIMENTAL

The leafy green vegetables

The vegetables used were ten varieties widely consumed by many Nigerian peasants. They were cultivated and processed as reported previously (Ifon & Bassir, 1979).

The processed vegetable samples were used for analysis, except for dry matter determination for which farm-fresh samples were used. All determinations were carried out in triplicate.

The dry matter content in each sample was determined by drying known weights of fresh material to constant weight at 60 °C. The nitrogen determinations on the samples (5 g each) were carried out by the semi-micro Kjeldahl method using a commercial mercury-based catalyst (Hopkin and Williams, Essex, Great Britain).

The crude protein values were obtained by multiplying the nitrogen values by 6·25. The crude fat in each sample (2 g) was determined after about 7 h of Soxhlet extraction with petroleum ether (boiling point, 40 °C). The residue, after fat extraction, was used for crude fibre determination (AOAC, 1970). The total ash content was determined by heating the samples (5 g) in a muffle furnace at about 600 °C until they were completely ashed. Total carbohydrate content was found by difference. This was done by subtracting the percentages of crude protein, crude fat and total ash from 100.

The ethanol-soluble simple sugars were estimated by the methods of Trevelyan *et al.* (1950) and Nalewaja & Smith (1963). This involved paper chromatographic separation of the sugars in 70 % ethanol extracts of the samples, followed by the phenol–sulphuric acid estimation of the eluted sugars.

RESULTS AND DISCUSSION

The proximate contents of the vegetables are shown in Table 1, while the ethanol-soluble sugar contents are presented in Table 2. The dry matter contents of the vegetables, ranging between 7·7 % (*Talinum triangulare*) and 24·7 % (*Abelmoschus*

TABLE 1
THE PROXIMATE CONTENTS OF SOME NIGERIAN LEAFY GREEN VEGETABLES

Vegetables		%	% dry matter[a]				
Local name	*Botanical name*	*fresh weight + dry matter*	*Crude fat*	*Crude fibre*	*Ash*	*Crude protein*	*Total carbo-hydrate*
Ikongetighi	*Abelmoschus esculentus*	24·7 ±1·6	4·1	11·3 ±0·1	11·9 ±0·1	26·4 ±0·8	57·6 ±0·9
Etinyung	*Corchorus olitorius*	23·7 ±1·3	4·2 ±0·1	8·5	12·6 ±0·1	27·7	55·5 ±0·2
Mmongmmongikong	*Talinum triangulare*	7·7 ±1·4	5·4 ±0·1	11·1 ±0·1	18·6 ±0·1	22·5 ±0·2	53·5 ±0·5
Inyang afia	*Amaranthus hybridus*	18·9 ±0·6	4·8	11·4	17·2	27·03 ±0·7	51·0 ±0·7
Etinkene	*Piper guineense*	13·8 ±0·6	8·1 ±0·1	20·9 ±0·1	15·5	17·2 ±0·1	59·2 ±0·4
Ntong	*Ocimum basillicum*	20·6 ±0·4	4·5 ±0·1	11·9 ±0·1	12·7	21·8 ±0·1	60·9 ±0·3
Ikong nnangi	*Cucurbita pepo*	23·4 ±0·9	4·0	10·8 ±0·1	15·0	28·4	52·7 ±0·2
Etidot	*Vernonia amygdalina*	21·6 ±0·8	2·7 ±0·1	10·9 ±0·1	10·8	22·2	64·4 ±0·2
Ikongubong	*Telfairia occidentalis*	14·0 ±1·0	6·5	15·0	11·0	22·0 ±0·1	60·4 ±0·1
Utasi	*Marsdenia latifolia*	17·8 ±0·4	5·9	14·7	9·7	18·4 ±0·1	66·1 ±0·1

[a] Mean values ± Standard deviations.

esculentus) of fresh weight, show that about 5 to 14 times the amounts of vegetables on a dry weight basis would be required to provide equivalent amounts of nutrients on a fresh weight basis. This observation is important as fresh leafy vegetables are commonly used in the preparation of stews and soups. However, recent evidence (Epenhuijsen, 1974) has indicated an increasing use by some Nigerians of dry vegetable powders in dietary preparations.

TABLE 2
ETHANOL-SOLUBLE SUGAR CONTENTS OF THE VEGETABLES

	% Dry matter			
Vegetables	*Fructose*	*Glucose*	*Sucrose*	*Maltose*
Abelmoschus esculentus	1·2	1·3	1·9	0·5
Corchorus olitorius	1·5	1·7	1·6	2·3
Talinum triangulare	0·9	1·0	1·5	0·4
Amaranthus hybridus	1·0	1·1	1·6	0·3
Piper guineense	1·6	1·7	2·6	0·3
Ocimum basillicum	1·6	1·8	2·6	0·7
Cucurbita pepo	0·6	0·6	0·8	1·0
Vernonia amygdalina	0·7	0·8	1·0	1·0
Telfairia occidentalis	1·0	1·1	1·5	0·8
Marsdenia latifolia	1·2	1·3	1·9	0·9

The crude protein contents in the vegetables ranged between 17 % and 28·5 % dry matter. These values compare quite favourably with previous reports on the protein contents of some Nigerian leafy vegetables (Bassir & Fafunso, 1975). These results confirm that the vegetables are an important source of dietary protein and, if consumed in sufficiently large quantities, could meet a large proportion of an individual's daily protein requirements. The studies of Fafunso (1972) have shown that the proteins in Nigerian leafy vegetables (including some of those used for our studies) have high nutritional qualities judging from their amino acid profiles. This high nutritive value is further enhanced by the high protein:fibre ratios (computed from Table 1) observed in most of the vegetables we studied. The high contents of good quality protein, coupled with the low fibre contents, suggest a possibility of using most of these leafy vegetables in the preparation of leaf protein concentrates. Such preparations have been reported to support good growth in children (Waterlow, 1962) and Kwashiorkor patients (Olatunbosun *et al.*, 1974).

The fibre contents of the leafy vegetables, ranging between 8·5 % (*Corchorus olitorius*) and 20·9 % (*Piper guineense*) are important dietary constituents. Recent reports (Anon., 1973; Eastwood, 1974) have claimed the existence of a causal relationship between the absence of fibre in the diet and the incidence of a wide range of disease in man, notably colon diverticulitis, diabetes mellitus, obesity and coronary artery disease.

The crude fat content of 5·9 % to 8·1 % dry matter in *Marsdenia latifolia*, *Telfairia occidentalis* and *Piper guineense* are appreciably high. The major constituents of leafy vegetable crude fat have been reported to include carotenoids (provitamin A), vitamins E and K and some polyunsaturated fatty acids (Hudson & Karis, 1974; Davidson *et al.*, 1973). Apart from palm oil, leafy vegetables form a major source of vitamin A in the diets of Nigerian peasants. In addition to its role in curing night blindness, vitamin A has been implicated in protein synthesis (Bauernfeind, 1972). Davidson *et al.* (1973) have reported that polyunsaturated fatty acids probably influence plasma cholesterol by affecting its catabolism in the liver. This is important as the same authors have associated ischaemic heart disease with increased plasma cholesterol. The high ash contents in most of the vegetables reflect their mineral contents. Our previous studies (Ifon & Bassir, 1979) showed that most of the vegetables were rich in a wide range of mineral elements including potassium, calcium, iron and phosphorus.

The leafy vegetables may not be considered as important sources of carbohydrate (and hence energy) as the vegetable stews and soups are usually consumed along with predominantly carbohydrate preparations. However, the simple sugars in these vegetables (Table 2) may be of considerable importance as these specific nutrients have been implicated, directly or indirectly, in various metabolic and pathologic processes in the body (Yudkin, 1968; Amine & Hegsted, 1971). Our studies have shown that fructose, glucose, sucrose and maltose contents of the vegetables were 0·6–1·6 %, 0·6–1·8 %, 0·8–2·6 % and 0·3–2·3 % dry matter, respectively.

The results showed that *Ocimum basillicum* had the highest contents of glucose, sucrose and fructose, while *Cucurbita pepo* contained the least glucose and sucrose. The proportions of fructose and glucose were almost the same and represented about 66% of the sucrose contents in most of the vegetables. The contents of the simple sugars are generally low but could be important in the diets of certain types of patient.

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CARBOHYDRATE CONTENT AND CALORIC VALUES OF CARBONATED SOFT DRINKS

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ABSTRACT

The concentrations of glucose, fructose and sucrose in selected carbonated soft drinks were determined by high pressure liquid chromatography (HPLC). Sugar-free drinks did not contain glucose, fructose or sucrose; two calorie-reduced drinks were sweetened mainly with fructose; one regular drink contained mainly sucrose and the remaining beverages were sweetened mainly by equal amounts of glucose and fructose. HPLC provides a rapid and reproducible means of determining carbohydrates in soft drinks.

Bomb calorimetry data revealed one calorie-reduced soft drink that contained more than 50% of the calories normally found in the corresponding regular drink. Caloric values calculated from total sugar content were within 10% of values obtained by combustion.

INTRODUCTION

One of the most remarkable trends in the sugar industry in the last twenty years has been the shift in the use of sugar from the home to the industrial user (Mitchell, 1974). Indeed, at present, food products and beverages (as opposed to direct consumer use) account for more than 66% of the refined sugar consumed in the United States. Furthermore, soft drink beverages now comprise the largest single industrial use of refined sugar accounting for more than 20% of the total refined sugar in the American diet (Page and Friend, 1974).

In Canada, the recent banning of saccharin prompted soft drink manufacturers to place more emphasis on calorie-reduced drinks in lieu of sugar-free drinks which previously contained this artificial sweetener. In order to conform to Canadian Food and Drug regulations (B.24.004), a calorie-reduced beverage must not contain

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more than 50% of the calories found in the corresponding regular product. A method was therefore required to monitor compliance of producers with this regulation.

The objectives of the present study were to: (i) determine amounts of glucose, fructose and sucrose in carbonated soft drinks using recently developed high pressure liquid chromatographic (HPLC) techniques and (ii) develop a suitable method by which to monitor compliance with Canadian Food and Drug regulations regarding calorie-reduced beverages.

MATERIALS AND METHODS

Origin of samples

Regular and calorie-reduced soft drinks were purchased from local food stores on three separate occasions. Caloric values were determined for groups 1 and 2 while carbohydrate determinations were performed on the third group from October to December of 1977.

Bomb calorimetry

Samples to be combusted in the bomb calorimeter were degassed and a portion was then weighed, frozen and lyophilised (Virtis freeze-drier model 10-MR-TR, Virtis Co., Gardiner, NY). A 1–2 g portion of the lyophilised sample was pelleted for analysis in a model 1241 adiabatic bomb calorimeter (Parr Instrument Co., Moline, IL). Fourteen centimetres of nickel–chromium fuse wire were attached to the electrodes of the bomb. The bomb was sealed and placed into a water bucket containing exactly 2000 g distilled water. The temperature of the water was recorded and combustion was carried out as described in the Parr Instrument Co. manual 153. The final temperature of the water was recorded and heat of combustion was calculated from the following formula:

$$H = \frac{[TW] - E_1 - E_2}{M}$$

where: H = heat of combustion in calories per gramme; T = change in temperature; E_1 = ml of alkali used for titration × calories per ml; E_2 = cm of wire burned × 2·3 calories per cm; M = mass of sample in grammes and W = energy equivalent of the calorimeter in calories per °C.

Analysis of sugars by HPLC

Soft drinks to be analysed using HPLC were degassed and then filtered through a 0·45 μm membrane filter using a Swinnex apparatus (Millipore Corp., Bedford, MA).

Chromatography of degassed, filtered soft drinks was performed using a Spectra Physics model SP 8000 high pressure liquid chromatograph (Santa Clara, CA). The method used was essentially that of Conrad & Palmer (1976). A 10 μl sample was

injected onto a Waters Associates (Milford, MA) carbohydrate analysis column (3·9 mm × 30 cm) which was maintained at a constant temperature of 25 °C. The helium-degassed liquid phase consisted of acetonitrile (Caledon Laboratories Ltd, Georgetown, Ont.) and water at a ratio of 79:21. Sugars were detected with a Waters Associates refractive index detector with attenuation set at 16 × .

The microprocessor-equipped chromatograph was programmed for the external standard method which permitted the concentration of a sample component to be determined by comparing its peak area to that of the corresponding component of known concentration in the standard chromatogram. The standard solution contained glucose, fructose and sucrose (Sigma Chemical Co., St Louis, MO) dissolved in distilled water.

RESULTS AND DISCUSSION

Sugar content of soft drinks by HPLC

A typical chromatogram showing the separation of glucose, fructose and sucrose is presented in Fig. 1.

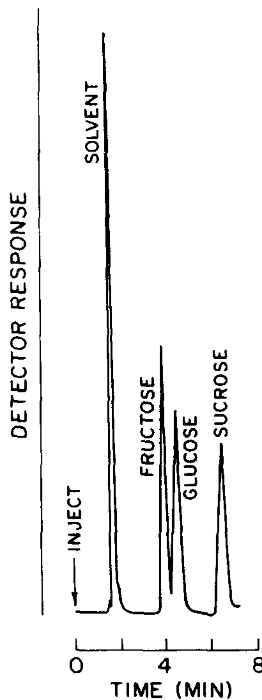

Fig. 1. HPLC separation of carbohydrates in carbonated soft drink. A 10 μl sample of degassed, filtered soft drink was chromatographed under the following conditions: acetonitrile/water (79/21), 2·0 ml/min, R.I. attenuation 16 ×, 25 °C.

In regular soft drinks, the total sugars content (glucose + fructose + sucrose) ranged from 91·7 to 128·6 mg/ml (mean of samples 1 and 2, Table 1). Beverage G contained the largest amount of sucrose while beverage B contained no sucrose.

The calorie-reduced drinks contained 44·1 to 58·1 mg/ml total sugars (mean of samples 1 and 2, Table 1) with each having less than 50% of the total sugars of the corresponding regular drink. A discrepancy was apparent with respect to beverage G and its calorie-reduced counterpart. The caloric values calculated from the total sugar data showed that beverage G-reduced contained less than 50% of the calories in the corresponding regular drink. However, the bomb calorimetry data showed the reverse (Table 2). The most likely explanation is a variation in the batch examined since caloric determinations were performed on groups 1 and 2 whereas sugar determinations were carried out on group 3.

Two of the calorie-reduced soft drinks, B-reduced and D-reduced, contained mainly fructose, reflecting the change in formulations prompted by the banning of saccharin and the ready availability of high fructose corn syrups. With the exception of beverage G, the remaining sugar-containing soft drinks derived sweetness mainly from equal amounts of glucose and fructose which may have originated from inversion of sucrose during production and storage.

Since beverages account for such a large proportion of sugar consumed, we must be aware of possible adverse effects on health which may derive from various component sugars. The metabolic response varies with the type of carbohydrate consumed, and certain individuals in the population with hypertriglyceridemia, hyperuricemia or diabetes may be more susceptible to effects of different sugars in the diet (see Reiser, 1975).

High pressure liquid chromatography provides a rapid and reproducible means of determining carbohydrates in beverages and other foods (Conrad & Palmer, 1976; Hunt *et al.*, 1977).

Caloric values

Caloric values obtained by bomb calorimetry (Table 2) ranged from 30·8 to 46·9 Calories/100 g for regular drinks and 15·8 to 20·7 Calories/100 g for calorie-reduced drinks. With one exception, G-reduced, the calorie-reduced beverages all contained less than 50% of the calories found in the corresponding regular drinks. Although the sugar-free drinks did not contain glucose, fructose or sucrose, there was a small amount of combustible material present (see Table 2).

With the exception of beverage G, caloric values calculated from the mean total sugars concentration (Table 1) were well within 10% of the values obtained by combusting the lyophilised soft drink material in the bomb calorimeter (Table 2). Thus, although combustion is the more accurate of the two methods, a reasonable approximation of the caloric value can also be obtained from a knowledge of the total carbohydrate content (see also Bender, 1968).

It has been pointed out by Jackson & Davis (1977) that bomb calorimetry may

TABLE 1

CARBOHYDRATE CONTENT (mg/ml) OF CARBONATED SOFT DRINKS[a]

Sample	Fructose 1	Fructose 2	%[c]	Glucose 1	Glucose 2	%[c]	Sucrose 1	Sucrose 2	%[c]	Total sugars[d] 1	Total sugars[d] 2
regular											
A	[b]59·3±0·8	61·5±0·6	47·0	61·3±2·3	65·4±1·0	49·3	6·3±5·5	3·4±0·9	3·7	126·9±8·6	130·3±2·5
B	60·4±0·5	61·4±0·9	49·5	59·8±0·8	64·4±2·2	50·5	0	0	0	120·2±1·3	125·8±3·0
C	57·2±0·3	59·4±0·4	46·1	57·3±0·5	62·6±1·1	47·5	10·5±1·7	6·8±0·3	6·4	125·0±2·5	128·8±1·8
D	40·2±1·2	39·9±0·2	35·7	40·0±1·1	43·2±0·3	37·0	31·7±1·1	27·5±0·9	27·3	111·9±3·4	110·6±1·4
E	24·2±0·1	25·9±0·7	27·4	24·1±0·6	27·3±0·8	28·0	41·4±1·9	40·4±2·9	44·6	89·7±2·6	93·6±4·4
F	43·1±0·5	44·7±0·2	44·3	43·0±1·0	44·7±0·3	44·3	12·9±2·9	9·5±2·1	11·4	99·0±4·4	98·9±2·6
G	18·4±0·1	19·9±0·3	15·5	18·3±0·3	20·3±0·1	15·5	90·2±10·0	81·3±1·4	69·0	126·9±10·4	121·5±1·8
calorie-reduced											
Z	12·0±0·5	12·5±0	21·5	11·7±0·6	12·3±0·1	20·9	34·0±0·6	32·0±0·3	57·6	53·7±1·7	55·0±0·4
B-reduced	45·7±2·7	44·8±3·1	97·0	0	1·6±0·1	1·7	0	1·2±2·0	1·3	45·7±2·7	47·6±5·2
D-reduced	44·6±0·1	46·9±0·4	96·6	0	2·3±0·0	2·4	0	0·7±1·1	1·0	44·6±0·1	49·9±1·5
F-reduced	13·6±1·1	14·5±1·6	31·9	13·6±0·9	14·8±0·4	32·1	15·9±0·7	15·8±1·2	36·0	43·1±2·7	45·1±3·2
G-reduced	15·7±0·2	15·9±0·4	27·1	15·5±0·5	16·2±0·8	27·3	26·3±1·2	26·7±1·6	45·6	57·5±1·9	58·8±2·8

[a] The samples used for these determinations were separate from those used for calorimetry.
[b] Mean of three determinations ± standard deviation.
[c] Mean of six determinations expressed as a percent of total sugar.
[d] Glucose + fructose + sucrose.

TABLE 2
CALORIC VALUES OF SELECTED CARBONATED SOFT DRINKS

Beverage	*Density (g/ml)*	*Caloric value by calorimetry (Calories/100 g)*	*Calculated[a] caloric value (Calories/100 g)*
regular			
A	1·039	[b]43·0 ± 0·3 (8)[c]	46·4
B	1·058	42·4 ± 1·6 (8)	43·6
C	1·045	46·9 ± 0·8 (8)	45·4
D	1·037	38·3 ± 0·3 (8)	40·6
E	1·042	30·8 ± 0·2 (8)	33·0
F	1·035	33·5 ± 0·9 (8)	35·9
G	1·039	38·4 ± 1·3 (8)	44·9
calorie-reduced			
Z	1·019	20·6 ± 0·2 (8)	20·0
B-reduced	1·028	18·2 ± 0·1 (8)	17·0
D-reduced	1·019	18·4 ± 0·3 (8)	17·4
F-reduced	1·016	15·8 ± 0·2 (8)	16·3
G-reduced	1·016	20·7 ± 0·1 (8)	21·5
sugar-free			
X	1·000	0·4 ± 0·1 (4)	0
Y	1·001	0·5 ± 0·0 (3)	0

[a] Mean of total sugar × 1·00/density × 3·75, where 3·75 is the energy conversion factor for carbohydrates (Bender, 1968).
[b] Mean ± standard deviation.
[c] Number of determinations.

misrepresent the available energy of carbohydrates. They suggest that energy values for carbohydrates be related to the weight ingested and the free energy change associated with metabolism to a common point for fat accumulation or conversion to physical energy. Although we agree this would be a consideration when determining metabolic energy, it must be emphasised here that for purposes of comparing one beverage to another, caloric values obtained by bomb calorimetry are quite satisfactory. Thus, bomb calorimetry is the method of choice for monitoring compliance with Food and Drug regulations regarding calorie-reduced soft drinks.

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VITAMIN C LOSSES IN COOKED FRESH LEAFY VEGETABLES

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ABSTRACT

The total vitamin C levels (AA and DAA) in six freshly harvested vegetables ranged from 482 to 582 mg/100 g dry weight. Vitamin C is present as L*-xyloascorbic acid (AA) in all the vegetables investigated except* Corchorus olitorus *where some partially oxidised form (DAA) was detected at a level of 1·8% of the total vitamin C. The total vitamin C losses of blanched vegetable leaves varied from 62·2 to 93·1%. DAA accounted for 3·4 to 19·4% of the total vitamin C left in blanched leaves and for between 0 and 2·8% of the total vitamin C extracted into the blanching water. During the blanching of vegetables, enzymic oxidation of vitamin C is more important than non-enzymic oxidation by oxygen, catalysed by traces of metals such as copper and iron.*

INTRODUCTION

Leafy green vegetables constitute a very important source of the vitamin C dietary requirements of Nigerian families. The leaves are consumed cooked in various dishes, especially soups and stews (Oke, 1968; Cadwell, 1972). Blanching usually precedes cooking and takes one of several forms. With the older generation, blanching takes the form of cooking in boiling water for not less than 10 min. Younger people either blanch the vegetable leaves in boiling water for a maximum of 10 min or soak them in freshly boiled water in a covered pot for a maximum of 10 min. A less common method is to steam heat the washed vegetables for 10 min. The blanched vegetable is then cooked with other ingredients, after most of the water in it has been squeezed out by hand pressing. Cooking can last for between 15 and 30 min.

Nutritionists have been worried about the loss of vitamin C from vegetables cooked as described above (Hartzler and Guerrant, 1952; Hughes, 1977). Losses up

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to between 63 and 76% have been reported (Oke, 1967; Fafunso & Bassir, 1977). These studies compared the level of L-xyloascorbic acid (AA) in fresh and blanched vegetables. The loss of vitamin C from the leaves may be due to the extraction of the water-soluble vitamin from the leaves into the blanching water or to partial oxidation to dehydroascorbic acid, DAA (which also has vitamin C activity), or complete oxidation to gulonic acid and oxalic acid, both of which have no vitamin C activity.

Available data are more or less silent on the mode of loss and the impression is created that the solubility of vitamin C in water accounts for a large proportion of the loss. It is expected that oxidation to the inactive forms will be limited during blanching in boiling water or steam since the supply of dissolved oxygen required for the oxidation becomes inadequate. Nevertheless, a confirmation of these observations may be worth while.

EXPERIMENTAL

Samples and sample treatment

Fafunso & Bassir (1976) reported a decrease in the vitamin C content of leafy vegetables as a result of wilting. For this reason, freshly harvested vegetables were used. These were obtained from plotted plants which had been growing for 5 to 6 weeks and the harvesting was carried out early in the morning. The fresh weights of the vegetables were taken as the weight after drying to constant weight at 30 °C (average ambient temperature in Nigeria) in a moisture extraction oven.

Six edible leafy vegetables—Tete (*Amaranthus hybridus*, Soko (*Celocia argentea*), Ewedu (*Corchorus olitorus*), Ilasa (*Hibiscus esculentus*), Osun (*Solanum* sp.) and Gbure (*Talinum triangulàre*)—commonly eaten in Nigeria were used for this study.

Dry matter determination and blanching

Approximately 20 g (fresh weight) samples of each vegetable were dried to constant weight at 100 °C in a moisture extraction oven. Continuous drying for 24 h was found to give satisfactory results.

The blanching procedure described by Fafunso & Bassir (1977) was adopted. This involves boiling a 2 g (fresh weight) portion of the sample for 5 min in 10 cm^3 of distilled water in an uncovered 50 cm^3 boiling tube. The tube was cooled in running tap water immediately after boiling.

Determination of total vitamin C (AA and DAA) and of AA only

Fresh leaves: About 2 g of the fresh leaves were covered with 30 ml of extracting solution (3% metaphosphoric acid in 8% glacial acetic acid) in a mortar. Acid-washed sand, of about the same weight as the sample, was added and the mixture was

ground with a pestle. The macerate was filtered through glass wool into a 100 cm³ volumetric flask. The mortar and pestle, sand and glass wool were rinsed with the extractant and the washings added to the flask. The latter was made to the mark with the extractant. Twenty cubic centimetre aliquots of this filtrate were assayed colorimetrically with 2,6-dichlorophenol indophenol for the reduced form of vitamin C (AA) according to the methods of Twomey & Goodchild (1970). For the determination of total vitamin C, hydrogen sulphide gas was bubbled through another 20 cm³ aliquot of the filtrate for 15 min to reduce any dehydro ascorbic acid (DAA) to AA according to the method of Kellie & Zilva (1936). Nitrogen gas was bubbled through the solution for 30 min to remove residual hydrogen sulphide gas. The solution was then analysed for AA as described above. A blank determination was carried out with 20 cm³ aliquots of distilled water, treated in the same manner as the sample aliquots.

Blanched vegetables and blanching water: The blanched vegetable leaves were immediately covered with 30 cm³ of the extractant after the blanching water had been decanted into a 100 cm³ volumetric flask which was later made to the mark with the extractant. The blanched vegetables were ground with acid-washed sand in a mortar. The macerate was filtered through glass wool into a 100 cm³ volumetric flask. The flask was made to the mark with the extractant and 20 cm³ aliquots of the solution were assayed for AA and DAA. The blanching water was also assayed for AA and DAA.

RESULTS AND DISCUSSION

The results obtained during this study are summarised in Table 1.

The total vitamin C levels in the fresh samples ranged from 482 mg/100 g dry weight in *Hibiscus esculentus* to 582 mg/100 g dry weight in *Talinum triangulare*. Except in two cases (*Celocia argentea* and *Amaranthus hybridus*), our results for total vitamin C in the fresh vegetables are much higher than those obtained by Fafunso & Bassir (1977) but fall within the range reported by Cadwell & Gim-Sai

TABLE 1

THE EFFECT OF COOKING ON THE TOTAL VITAMIN C (AA AND DAA) CONTENT OF FRESH LEAFY VEGETABLES

Local name	*Botanical name*	*% Dry matter*	*Vitamin C content (mg/100 g dry weight)*								
			Fresh leaves			*Blanched leaves*			*Blanching water*		
			AA	*DAA*	*Total*	*AA*	*DAA*	*Total*	*AA*	*DAA*	*Total*
Tete	*Amaranthus hybridus*	17·9	560	—	560	220	8	228	345	2	256
Soko	*Celocia argentea*	11·4	553	—	553	186	45	231	359	11	370
Ewedu	*Corchorus olitorius*	17·6	577	11	588	114	26	140	519	15	534
Ilasa	*Hibiscus esculentus*	15·6	482	—	482	72	19	91	415	4	419
Osun	*Solanum* sp.	14·5	523	—	523	79	25	104	487	—	487
Gbure	*Talinum triangulare*	7·2	582	—	582	190	18	208	362	—	362

(1973) for some Malaysian edible leaves. In comparing these results, we may note here that variations of up to ± 10 mg AA/100 g dry weight may result from an error of ± 1 % in dry matter determination.

Vitamin C is present as AA in all of the fresh vegetables except *Corchorus olitorus* where some partially oxidised form (DAA) was detected at a level of about 2 % of the total vitamin C. DAA accounted for between 3·4 and 19·4 % of the total vitamin C left in blanched leaves and for between 0·0 and 2·8 % of the total vitamin C extracted into the blanching water. This seems to suggest that, under the blanching conditions, enzymic oxidation of vitamin C (by ascorbic acid oxidase present in the leaves) is more important than non-enzymic oxidation by oxygen, catalysed by traces of Cu^{2+} and Fe^{2+}. The latter, if significant, will lead to high levels of DAA in the blanching water. Oxidation does not seem to go beyond the DAA stage; that is, no significant loss by oxidation to vitamin C inactive gulonic and oxalic acid products seems to occur during blanching.

TABLE 2

DISTRIBUTION OF VITAMIN C* (AA AND DAA) BETWEEN THE AQUEOUS AND SOLID PHASES AFTER BLANCHING

% Total vitamin C in blanched leaves	*% Total vitamin C in blanching water*	*Total vitamin C*	*Net increase (%)*	*% DAA in blanched leaves*	*% DAA in blanching water*
40·7	63·5	104·2	+4·2	1·4	0·4
41·7	66·9	108·6	+8·6	8·1	2·0
23·8	90·8	114·6	+14·6	4·4	2·6
18·9	86·9	105·8	+5·5	3·9	0·8
19·9	93·1	113·0	+13·0	4·9	0·0
35·7	62·2	97·9	−2·1	3·1	0·0

* All % expressed relative to total vitamin C in the fresh vegetable.

Total vitamin C in blanched leaves relative to fresh leaves varied from 18·9 to 40·7 %, corresponding to an apparent loss of between 59 and 81 % (Table 2). The loss is essentially by extraction into the blanching water, in which the level of total vitamin C relative to that in fresh leaves varied from 62·2 to 93·1 % (Table 2). A net increase in total available vitamin C was noticed in almost all cases (average increase 7·4 %). This may be attributed to the softening of the plant tissue by boiling water, a process which releases otherwise unavailable vitamin C.

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CHANGES IN SOME CHEMICAL CONSTITUENTS DURING THE FERMENTATION OF CASSAVA TUBERS (*MANIHOT ESCULENTA*, CRANTZ)

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ABSTRACT

Cassava tuber, Manihot esculenta, *Crantz, was processed, after five days of fermentation, into 'gari' and 'lafun'. The pH fell from 6·5 to 4·3 in the former. Titratable acidity increased during the course of fermentation. No cyanide residue was detected in gari by fluorimetry although low cyanide levels were detected in some samples of lafun. Thin layer chromatography (TLC) of extracts from gari confirmed the absence of cyanogenetic glycosides.*

INTRODUCTION

Cassava is grown extensively in the tropics. In Nigeria the production of cassava was 3·47 million tons in 1963/64. Its plantation covers 792·4 thousand square meters. There are three main ways in which cassava is eaten. The sweet variety only is boiled and eaten as a vegetable. Both the 'sweet' and 'bitter' varieties may be processed into gari or cassava flour (lafun). The sweet cultivars commonly grown in Nigeria are the Red Local and the improved 60444 cultivars with cyanide contents of less than 60 mg/kg. The bitter types are 53101 and the improved cultivars 60506 and 60447. 53101 contains more prussic acid (138–203 mg HCN/kg) than the improved cultivars 60506 and 60447 (77–116 mg HCN per kilogramme of peeled tuber) (Obigbesan, 1977).

The production of gari

The cassava tuber is harvested, peeled and washed, grated and packed into coarsely knit bags. A weight is put on the bag to express some of the juice. It is then left to undergo natural fermentation for several days. The grated cassava, after sieving to remove any coarse lumps and impurities, is heated by means of constant

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turning over a heated steel pan. This process has been termed 'garifying'. The surface temperature of the oven is about 120 °C (Akinrele *et al.*, 1962). During the process, the grated cassava is dried to about 10 % moisture content and the starch is probably partially dextrinised (Akinrele *et al.*, 1962). At this stage a little palm oil may be added to give it colour; the final product is gari. When it is soaked in water the grains swell to several times their original size. Microscopic examination shows that each grain is composed of thousands of gelatinised and ungelatinised granules bound together.

It is generally known that the cassava tuber is highly toxic. Collard & Levi (1959) studied the fermentation process. They isolated the glucosides and stabilised them with potassium hydroxide; the sugars were removed with Fehling's solution and the glucoside was crystallised from alcohol. These workers isolated two micro-organisms—*Corynebacterium* and *Geotrichum candida*—from the ferment. According to these authors fermentation occurs in two stages. In the first, *Corynebacterium* attacks the starch, producing various organic acids and lowering the pH. This causes spontaneous hydrolysis of the cyanophoric glycoside with the release of hydrocyanic acid. In the second stage, from the organic acids, including lactic acid, *Geotrichum candida* produces aldehydes and esters which give gari its characteristic aroma.

Wood (1965) isolated and purified the glycoside but found that this compound is stable to acid at normal temperatures and at 100 °C. He suggested that the fall in pH accompanying the fermentation process must be regarded as producing a favourable condition for the action of endogenous linamarase as opposed to the spontaneous hydrolysis of linamarin occurring as a result of low pH. He contends that the preparations of Collard and Levi were contaminated with small amounts of linamarase.

In later work, Wood (1966) purified linamarase and found that its optimum pH is 3·5 which coincides with the pH of the cassava ferment.

Akinrele (1964) reported that the optimum fermentation temperature is 35 °C and that the pH is lowered to 4·25 in four days.

The production of cassava flour

Another product made from the cassava tuber is flour 'lafun'. This is prepared by soaking whole tubers in water—usually running water. It is left for a few days, during which the flesh becomes soft. During soaking, the tuber undergoes natural fermentation. It is then peeled and the tubers are broken up into small chips which are dried on hot rocks (an oven) until it becomes dry (with about 13 % moisture content). It is then ground into flour and sieved to remove coarse fibre particles.

Some important minor constituents of cassava

Cassava contains two cyanophoric glycosides, linamarin and lotaustralin (Jones, 1959). The chronic toxicity of cassava products due to cyanide residues has received

attention from several workers (Osuntokun *et al.*, 1969). The present study was undertaken to observe the changes in the levels of these and other constituents during the course of fermentation.

MATERIALS AND METHODS

Plant materials

The cassava tubers used in this study were supplied by the University of Ife farm. They were the local white variety which is a bitter type.

Preparation of fermenting cassava mash for the study of changes in pH, total reducing sugars and titratable acidity: About 5 kg of cassava tubers were peeled, grated and placed in a small, loosely woven bag. Some weight was placed on the bag to express the juice. Samples were drawn out for analysis every 24 h. The experiment was carried out in duplicate.

Preparation of gari for the determination of pH, titratable acidity and hydrocyanic acid: About 400 kg of cassava tubers were purchased from the University of Ife farm. The tubers were hand peeled, washed and grated. They were mixed together thoroughly and divided into eight portions. Each portion was placed into a loosely knitted bag. Some weights were placed on the bag to express the juice. It was then allowed to undergo natural fermentation and processed into gari at the end of 1, 2, 3 and 4 days, respectively. The pH, reducing sugar content and titratable acidity were determined in all the prepared gari samples. Hydrocyanic acid was determined in gari samples made from the portion fermented for four days. The mean and standard deviations of the hydrocyanic acid values were recorded for four determinations.

Purchased gari and lafun samples

Five gari samples, A–E, and three lafun samples, F–H, were purchased from different sellers in Ile-Ife market. The mean of two determinations was recorded for each sample.

Determination of moisture content: The moisture content was determined by the air-oven method (AOAC, 1960).

Measurement of pH: Ten grammes of cassava product were blended with a hundred millilitres of distilled water in a Waring blender. The pH of the decanted supernatant was measured using a pH meter.

Determination of titratable acidity: Two grammes of gari were stirred with distilled water for 30 min and then filtered. For cassava tuber or mash, 2 g of the gari sample was blended with 50 ml distilled water at high speed. The filtrate was titrated with 0·1N NaOH using phenolphthalein as an indicator.

Determination of potential hydrocyanic acid content: Crude linamarase extract was prepared from the parenchymal tissue of fresh cassava tubers (peeled tubers)

(Wood, 1966). To remove glycosides the extract was dialysed for 5 h against cold distilled water. Ten grammes of gari or lafun were incubated with the crude enzyme for five hours. Liberated HCN was steam distilled into 0·2N NaOH. This was reacted with pyridoxal HCl and measured in a fluorimeter with the excitation wavelength at 365 nm and the secondary wavelength at 432 nm (Shigeru & Zengo, 1970). Standard curves were prepared with KCN dissolved in sodium hydroxide.

Thin layer chromatography (TLC) of cyanogenic glycosides: Cyanogenic glycosides and sugars were extracted by refluxing 5 grammes of gari or lafun with 50 ml of 80% ethanol for 1 h. Eighty per cent ethanolic extracts from cassava peel were used as a standard. Some 50 to 100 μl of the extracts were separated on TLC plates coated with silica gel G. The plates were developed with chloroform–methanol, 5:1 (v/v) (Clapp *et al.*, 1966). The glycoside spots were visualised by spraying the developed plates with 0·2% α-naphthol solution in methanol/water, 1:1 (v/v), followed by light spraying with concentrated H_2SO_4. On heating the plates sugars and cyanogenic glycosides yield purple spots.

Determination of reducing sugars: The sugars were extracted with 80% ethyl alcohol clarified with neutral lead acetate. The excess lead was removed with potassium oxalate. Reducing sugars were determined by a colorimetric method using 3,5-dinitrosalicylic acid.

RESULTS AND DISCUSSION

Changes in pH, titratable acidity and reducing sugars

The results are shown in Table 1. In the fermenting mash, the pH decreased to 4 in 3 days. The level of reducing sugars was doubled after one day of fermentation but this was followed by a decrease on subsequent days of fermentation. The titratable acidity increased about threefold after three days of fermentation. The increase in titratable acidity is due to the production of organic acids from sugars carried out by micro-organisms (Collard & Levi, 1959). The increase in reducing sugars after the first day of fermentation could be due to the breakdown of starch by starch splitting enzymes. Phosphorylase and amylases have been identified in cassava by Viswanathan & Krishnan (1966). The hydrolysis of linamarin and lotaustralin by linamarase would also contribute to the reducing sugars as hydrolysis of the

TABLE 1
CHANGES IN pH, TITRATABLE ACIDITY AND REDUCING SUGARS IN GARI FERMENTING MASH

	pH	*Reducing sugars (mg/g)*	*Titratable acidity as % lactic acid*
Fresh tuber	6·2	3·1	0·27
1 day	5·5	6·2	0·36
2 days	4·5	4·4	0·49
3 days	4·0	2·8	0·77

TABLE 2
EFFECT OF FERMENTATION PERIOD ON pH AND TITRATABLE ACIDITY IN GARI

	pH	*Titratable acidity as % lactic acid*
1 day	4·5	0·42
2 days	4·1	0·58
3 days	4·0	0·85
4 days	3·9	0·82

glycoside yields glucose, hydrocyanic acid and acetone (methyl ethyl ketone) in the case of lotaustralin.

Table 2 shows the pH and titratable acidity in finished gari. The pH decreased with increasing days of fermentation. Titratable acidity increased to a high level of 0·82 %. This high acidity contributes in large measure to the flavour of gari (Collard & Levi, 1959; Akinrele *et al.*, 1962). The dough prepared from gari is called 'eba'. It keeps well for a few days at room temperature. In contrast, the dough from pounded yam is only stable for a few hours. The high acidity of gari is largely responsible for the difference in stability between these two products.

Levels of HCN in gari and lafun

As can be seen from Table 3, lafun prepared from cassava tubers cubed, dried slowly and ground into flour, had a low cyanide content, but that obtained from

TABLE 3
LEVELS OF HCN RESIDUES IN CASSAVA PRODUCTS

		mg HCN/kg	*mg HCN/kg (mfb)*[a]
Fresh cassava tuber	Mean	78	130
	SD	1·5	
Flour (from unfermented cassava tuber)	Mean	13·4	15·2
	SD	0·6	
Flour (from tuber fermented for 4 days, i.e. lafun)		—	—
Gari sample prepared experimentally (four days of fermentation)		—	—
Gari samples obtained from the local market			
A		0·13	0·15
B		—	—
C		—	—
D		0·04	0·04
E		—	—
Lafun samples obtained from the local market			
F		0·48	0·55
G		0·50	0·57
H		0·46	0·52

[a] mfb = Moisture-free basis.
[b] SD = Standard deviation.

tubers fermented for four days was free of cyanide. Gari samples purchased at random from the local market in Ile-Ife contained no cyanide. Lafun, however, contained about 0·5 mg HCN per kilogramme. The results of the thin layer chromatography showed that cyanogenic glycosides were not present in gari. A very faint spot having the mobility of linamarin was obtained from extracts of lafun. The peel extract contained a high level of linamarin (R_f 0·32) as judged by the intensity of the spot. These observations are in agreement with the results of chemical analysis (Table 3). All the extracts analysed have compounds having the mobilities of standard glucose, fructose and maltose.

Several workers (Osuntokun *et al.*, 1969) have associated certain chronic neurological disorders with dependence on food from cassava derivatives because of cyanide residues. The present work shows that gari processed properly contained no cyanide residue. Lafuns as obtained from the local market do contain low levels of cyanide which may not be safe. Figures as high as 12 mg/kg have been quoted by some authors for gari (Oke, 1966; Wood, 1965). The method used by Oke (the AOAC titration method) is not specific for cyanide. It is thus conceivable that other products of fermentation may have interfered with his result and these could account for the apparent high cyanide content. Wood did not describe the way in which gari is prepared in Ghana where he carried out his work. The product may not be the same as the gari prepared in Nigeria.

CONCLUSIONS

Titratable acidity increased and reducing sugars decreased during the course of cassava fermentation. Acidity increased during the course of fermentation. The present study shows that gari, a widely eaten food in Nigeria and elsewhere, if processed properly by fermenting the mashed tubers for four days or longer, is completely free from cyanide or any cyanide-yielding glycoside. Other cassava products (except starch) contain some cyanide residues, indicating that these products should be given longer fermentation periods followed by a higher degree of heat treatment.

ACKNOWLEDGEMENTS

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Food Chemistry 5 (1980) 257–261

BOOK REVIEWS

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This interesting little book begins with a thoroughly researched historical treatment of the development of the sucrose industry and the use of sucrose as a foodstuff, as a sweetening agent and as a raw material for the manufacture of other industrial products. Some political aspects of sucrose production and a rational discussion of the health implications of sucrose are also presented.

Chapter 2 discusses the production of glucose and fructose syrups from various sources (including inulin); Chapter 3, the polyhydric alcohols; Chapter 4 the protein sweeteners and Chapter 5 the peptide-based sweeteners whilst Chapter 6 is a survey of the 'less common sweeteners', such as steveoside and glycyrrhizin. The final chapter is a discussion of the most recent attempts to formulate theories of sweetness in terms of structure–activity relationships with emphasis on the problem of sweetness intensity.

There seem to be very few errors other than an occasional trivalent carbon atom, and I believe that the chemist who first prepared xylitol was Emil and not Erich Fischer (p. 77). The methanol-like aftertaste described on p. 148 probably should be changed to read 'menthol-like'.

R. S. Shallenberger

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Contrary to the enjoyment of who-dunnits, readers of this book are well advised to start at the back where Professor Nursten's paper gives an excellent review of all the papers, an update of reference material and an initial appraisal of flavour research, present and in the future.

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MALCOLM W. KEARSLEY

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The book is divided into seven chapters, each describing a federal agency which has some responsibility for enforcing food laws, and each chapter contains (usually) a description of the agency and its role in establishing food standards and in food inspection. The agency's responsibilities, its relevant publications, food standards and future trends are described and references where the reader can obtain original material are given.

A problem with this type of book on legislation is that changes in food laws occur constantly and an update of the book every year at least is usually necessary. This book avoids this to a large extent by not providing too much specific information but giving a more general picture which is much less affected by changes in basic legislation.

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The rapid reproduction technique inevitably results in some loss of style consistency. Different authors seem to adopt different formats for their presentations, especially in regard to references. These vary also in number from paper to paper and are, in fact, absent in some. Nevertheless, the book is full of valuable and up-to-date information and is therefore highly recommendable for its chemical and technological content.

G. G. Birch

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As the editor indicates in the preface, this book presents material regarding the effects of heating on foodstuffs in a single text, the material otherwise being scattered in isolated research reports and other textbooks. The book is another volume from Applied Science, dealing with various aspects of foods and their components.

The first four chapters deal with the effects of heat on the basic components of foodstuffs—that is, proteins, carbohydrates, pigments and vitamins—whilst lipids are conspicuous only by their absence. Subsequent chapters consider meat, eggs, fish, fruit and vegetables and milk products. There is a final useful chapter on starch products.

Of particular interest is the chapter on animal and plant pigments, some of which are used as natural colouring materials in foods. This chapter combines easy reading and sufficient detail to be useful to a wide range of research workers.

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Many of the chapters provide long lists of references which are undoubtedly of great value. However, the chapters on vitamins (200+ references), fruit (300+) and vegetables (350+) do tend to be very difficult to read in places where every other word appears to be a reference.

Greenwood and Munro, on the other hand, in their chapters on carbohydrates, offer only six references for further reading, apparently to mention much of the work carried out in Great Britain and in the United States.

The two chapters on fruit and vegetables deal alphabetically with a wide range of products and are set out as structure and composition followed by effects of heat and this enables the problems associated with the heat processing of a particular fruit or vegetable to be located very quickly.

This book will undoubtedly appeal to a certain section of the scientific community and mainly libraries, although the greatest source of potential sales could have been to students of food chemistry. At £24, however, it would require a very devoted student to consider purchasing the book.

MALCOLM W. KEARSLEY

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ROLE OF TRIGLYCERIDES AND PHOSPHOLIPIDS ON DEVELOPMENT OF RANCIDITY IN MODEL MEAT SYSTEMS DURING FROZEN STORAGE

J. O. IGENE,† A. M. PEARSON, L. R. DUGAN, JR. & J. F. PRICE

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(Received: 9 May, 1979)

ABSTRACT

The effect of triglycerides, phospholipids and total lipids on development of rancidity during frozen storage at −18°C for 8 months was studied using lipid-free muscle fibres in combination with added triglycerides, phospholipids and total lipids. Results showed that added phospholipids greatly increased TBA values. Oxidation took place in two stages. Phospholipids were the first to oxidise, with their rate of oxidation decreasing with time. Oxidation of the triglycerides began only after a prolonged induction period. Results demonstrated that both triglycerides and phospholipids contribute to development of rancidity, although phospholipids make the greatest contribution. The influence of triglycerides on the development of rancidity was shown to depend upon the degree of unsaturation and the length of time in frozen storage. The relationship between oxidation of the polyunsaturated fatty acids (PUFAS) of the phospholipids and development of rancidity was confirmed.

INTRODUCTION

It is often assumed that deteriorative changes in frozen meat are generally minimal. The storage stability and quality of frozen meat depend essentially on the composition of constituent lipids, and especially on the degree of unsaturation (Watts, 1954; Greene, 1969; Igene *et al.*, 1976). Some researchers (Sulzbacher & Gaddis, 1968; Bratzler *et al.*, 1977) have concluded that autoxidation of the triglycerides, principally in the adipose tissue, is responsible for the development of rancidity in raw frozen meats. This view is contrary to that held by others (Cadwell *et al.*, 1960; Watts, 1962; Greene, 1969) who have concluded that oxidative changes in

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tissue lipids are primarily due to autoxidation of the phospholipids. Thus, the principal objective of this research was to determine the role of meat triglycerides, total lipids and phospholipids in the development of rancidity in frozen beef, chicken dark meat and white meat model systems.

EXPERIMENTAL

Preparation of model meat system

Lipid free muscle fibres were used as the matrix for the model system. Fresh raw beef, chicken dark meat or white meat were each ground once through a $\frac{3}{16}$ in plate using a Hobart meat grinder. Total lipids in the ground meat were extracted by the method of Folch *et al.* (1957). The phospholipids were separated from total lipids using the procedure of Choudhury *et al.* (1960). The solvent was removed from the residue (protein matrix) by drying under vacuum and later in a stream of nitrogen gas at room temperature, and the model systems were then used immediately. Preparation of the model system is described in greater detail by Igene & Pearson (1979) and is shown schematically in Fig. 1.

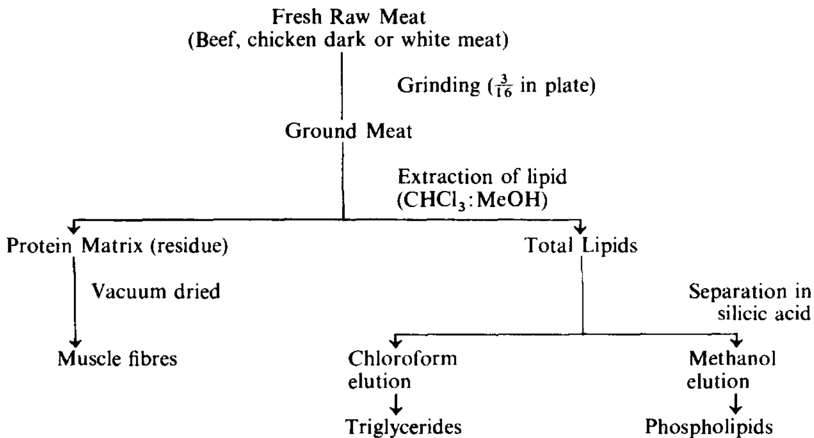

Fig. 1. Preparation of model meat system.

Design of experimental treatments

The design of the study is shown in Table 1. The levels of total lipids, triglycerides and phospholipids added back to the model systems closely corresponded to those removed during the extraction process. Total phospholipids were used in the beef and chicken meat model systems at levels of 0·8 and 0·7 %, respectively. Total lipids

TABLE 1
DESIGN OF MODEL MEAT EXPERIMENTAL TREATMENTS[a]

Meat source	*Code number*	*Composition of model meat systems*
Beef	B_1	0·8% beef phospholipids in beef muscle fibres
	B_2	9·2% beef triglycerides in beef muscle fibres
	B_3	10·0% beef total lipids in beef muscle fibres
	B_4	Beef muscle fibres only (control)
Chicken dark meat	D_1	0·7% dark meat phospholipids in dark meat muscle fibres
	D_2	4·3% dark meat triglycerides in dark meat muscle fibres
	D_3	5·0% dark meat total lipids in dark meat muscle fibres
	D_4	Dark meat muscle fibres only (control)
Chicken white meat	W_1	0·7% white meat phospholipids in white meat muscle fibres
	W_2	4·3% white meat triglycerides in white meat muscle fibres
	W_3	5·0% white meat total lipids in white meat muscle fibres
	W_4	White meat muscle fibres only (control)

[a] Muscle fibres without added lipids served as controls. Each experiment was replicated 4 times. The same amount of lipids were applied to chicken dark meat and white meat fibres in order to eliminate variations due to lipid levels.

and triglycerides were added to the beef model system at levels of 10·0 and 9·2%, respectively. Similarly, total lipids and triglycerides were added to the chicken dark and white meat model systems at concentrations of 5·0 and 4·3%, respectively.

The experimental model systems (Table 1) were stored in polyethylene bags at −18 °C for 8 months. Prior to frozen storage, the initial TBA values and the fatty acid composition of the fresh lipids in the samples were determined. TBA values of the samples were also measured at 1, 4 and 8 months of frozen storage. At the end of 8 months storage, fatty acid analyses of the triglycerides and phospholipids, including that of PC (phosphatidyl choline) and PE (phosphatidyl ethanolamine) were also carried out.

TBA test: The TBA (2-thiobarbituric acid) distillation test of Tarladgis *et al.* (1960) was utilised to measure development of rancidity. TBA numbers were expressed as mg malonaldehyde/kg meat.

Separation of phospholipid components: Total phospholipids were separated into their components by one dimensional TLC (thin layer chromatography) using 20 × 20 cm precoated silica Gel G (500 μm thickness) glass plates (Fisher Scientific Co.). Exactly 50 mg/ml of phospholipids (equivalent to 0·475 mg phospholipid/spot) were spotted on each plate under a stream of nitrogen gas. A standard mixture of authentic phospholipids (Supelco, Inc.) was simultaneously spotted on the left side of the plate. Separation was accomplished using chloroform–methanol–water (65:25:4, v/v). The spots were identified by spraying the plates with iodine vapours. Spots containing PC and PE were immediately recovered and eluted with chloroform–methanol (4:1, v/v), evaporated to dryness under nitrogen gas and redissolved in chloroform. PC and PE were checked for purity by TLC before converting them to methyl esters.

Preparation of methyl esters: Triglycerides, phospholipids, PC and PE were converted to methyl esters by the boron-trifluoride/methanol procedure as described by Morrison & Smith (1964).

GLC (gas liquid chromatography) analysis of fatty acid methyl esters was performed using a Perkin–Elmer, model 900 gas chromatograph equipped with a hydrogen flame ionisation detector. The column, 1·83 m × 2 mm (i.d.) stainless steel, was packed with 10%(w/w) diethylene glycol succinate (DEGS) on Supelcoport (Supelco, Inc.). The column temperature was set at 185 °C, the injection port at 220 °C and the detector at 250 °C. The carrier gas was helium and the flow rate was maintained at 30–40 ml/min, while hydrogen gas and air flow were adjusted to 30 and 285 ml/min, respectively. Peaks were identified by comparison with retention times for standard mixtures of known fatty acid methyl esters (Applied Science Lab., Inc.; Supelco, Inc.). Peak areas were calculated quantitatively as the product of peak height and width at half height. Results were expressed as percentage of the total area.

Statistical methods: Analysis of variance for TBA values was calculated using a Control Data Corporation 6500 computer. The significance between treatments was determined using Tukey's test for multiple comparisons.

RESULTS AND DISCUSSION

Beef lipids

The TBA values presented in Table 2 show the effects of beef lipids on development of rancidity during frozen storage. In the control samples, the TBA numbers were only 0·40, 0·42, 0·89 and 1·05 at 0, 1, 4 and 8 months of frozen storage, respectively. The low TBA values indicate that the control samples, which were

TABLE 2
THE EFFECT OF ADDING BEEF TRIGLYCERIDES, TOTAL PHOSPHOLIPIDS AND TOTAL LIPIDS ON TBA VALUES OF MODEL BEEF MEAT SYSTEMS STORED AT −18 °C[a]

Time in frozen storage (months)	*Experimental treatments*			
	Control (B_4)	*Triglycerides (B_2)*	*Total phospholipids (B_1)*	*Total lipids (B_3)*
0	0·40[b]	0·38[b]	0·65[c]	0·66[c]
1	0·42[b]	0·82[c]	13·01[h]	20·84[m]
4	0·89[d]	6·33[f]	15·14[i]	21·52[l]
8	1·05[e]	8·31[g]	15·74[k]	20·30[k]

[a] TBA numbers = mg malonaldehyde/kg meat. B_1 = 0·8% phospholipids; B_2 = 9·2% triglycerides; B_3 = 10·0% total lipids; B_4 = muscle fibres only.
Values within and between experimental treatments bearing the same superscript are not significantly different at the 5% level.

composed of the extracted meat fibres, did not undergo appreciable oxidation. Thus, the validity of using the lipid extracted muscle fibres as the basis of the meat model system was verified. In the samples containing added triglycerides, the TBA values were 0·38, 0·82, 6·33 and 8·31 at 0, 1, 4 and 8 months, respectively. The TBA numbers of the triglycerides increased most rapidly between 1 and 4 months of freezer storage and then more slowly between 4 and 8 months, indicating a slowing down in the rate of lipid oxidation.

The TBA values for the samples containing added phospholipids were 0·65, 13·01, 15·14 and 15·74 at 0, 1, 4 and 8 months of freezer storage, respectively. On the other hand, a combination of triglycerides and total phospholipids (total lipids) gave TBA values of 0·66, 20·84, 21·52 and 20·38 at 0, 1, 4 and 8 months of freezer storage, respectively. Thus, both phospholipids and triglycerides contribute to oxidation, with their combined effects being approximately additive. Results show that phospholipids make the greatest contribution to rancidity, but triglycerides also contribute to development of rancidity, either alone or in combination with the phospholipids.

The increase in TBA numbers during freezer storage suggests that unlike the triglycerides, total lipids and phospholipids do not exhibit a noticeable induction period before lipid oxidation. This is consistent with data reported by El-Gharbawi & Dugan (1965) and Chipault & Hawkins (1971).

Analysis of variance indicates that differences between treatments, storage periods and interactions between treatments and length of storage time were all highly significant ($p < 0{\cdot}001$). This further verifies the importance of phospholipids and triglycerides in development of rancidity during freezer storage of meat, and indicates that their combined effect as total lipids is additive. Furthermore, results clearly indicate that phospholipids contribute more to the development of off-flavours in frozen meat than the triglycerides, which supports the conclusions of Cadwell *et al.* (1960), Watts (1962) and Greene (1969). The role of beef triglycerides in the development of rancidity may not be important when meat is stored frozen for a short period of time. However, beef triglycerides could play an important role when meat is stored frozen for longer periods.

Chicken dark meat lipids

The TBA values for samples containing chicken dark meat triglycerides, total lipids and phospholipids are presented in Table 3. The control samples (muscle fibres alone) showed consistently greater TBA values than control beef samples. The higher TBA values for chicken dark meat indicate that the extracted muscle fibres probably contained some unextracted bound lipids.

The samples containing triglycerides had TBA values of 1·40, 5·52, 10·75 and 10·36 at 0, 1, 4 and 8 months of freezer storage, respectively. This indicates that added triglycerides contributed considerably to lipid oxidation. Chicken dark meat triglycerides did not exhibit the long induction period that was observed for beef

triglycerides. This could be related to the higher degree of unsaturation in chicken triglycerides (Katz *et al.*, 1966).

When phospholipids were added back to the model system and frozen, the TBA values were 1·63, 11·52, 12·20 and 13·33 at 0, 1, 4 and 8 months, respectively. In the samples containing total lipids, on the other hand, TBA numbers were 2·05, 15·05, 16·86 and 17·33 at 0, 1, 4 and 8 months of freezer storage, respectively. Thus, phospholipids and total lipids from chicken dark meat behaved in a similar way to those of beef. Analysis of variance for TBA values shows that treatments, length of time in frozen storage and the interaction of treatment × length of storage were all highly significant ($p < 0{\cdot}001$).

These results indicate that triglycerides, total lipids and phospholipids are all important in development of rancidity in chicken dark meat. The results also demonstrate that triglycerides contribute almost as much to development of rancidity as phospholipids during prolonged frozen storage of meat. Hornstein *et al.*

TABLE 3
EFFECT OF ADDING CHICKEN DARK MEAT TRIGLYCERIDES, TOTAL PHOSPHOLIPIDS AND TOTAL LIPIDS ON TBA VALUES OF CHICKEN DARK MEAT MODEL MEAT SYSTEMS STORED AT −18°C[a]

Time in frozen storage (months)	*Experimental treatments*			
	Control (D_4)	*Triglycerides (D_2)*	*Total phospholipids (D_1)*	*Total lipids (D_3)*
0	$1{\cdot}13^{b}$	$1{\cdot}40^{c}$	$1{\cdot}63^{d}$	$2{\cdot}05^{e}$
1	$3{\cdot}08^{f}$	$5{\cdot}52^{i}$	$11{\cdot}52^{m}$	$15{\cdot}05^{o}$
4	$3{\cdot}23^{f}$	$10{\cdot}75^{k}$	$12{\cdot}20^{m}$	$16{\cdot}86^{p}$
8	$3{\cdot}48^{h}$	$10{\cdot}36^{j}$	$13{\cdot}33^{n}$	$17{\cdot}33^{q}$

[a] TBA numbers = mg malonaldehyde/kg meat. D_1 = 0·7% phospholipids; D_2 = 4·3% triglycerides; D_3 = 5·0% total lipids; D_4 = muscle fibres only. Values within and between experimental treatments bearing the same superscript are not significantly different at 5% level.

(1961) observed that the phospholipid fraction of total lipids from pork and beef became rancid quickly when exposed to air. The triglyceride fraction developed off-flavours less readily, leading them to conclude that phospholipids make the greatest contribution to off-flavour development.

Chicken white meat lipids

TBA numbers for the chicken white meat model samples containing triglycerides, total lipids and phospholipids are presented in Table 4. TBA numbers for the control samples were 0·42, 0·57, 1·39 and 1·78 at 0, 1, 4 and 8 months of frozen storage. Although the samples containing added triglycerides showed very low levels of TBA numbers, they were significantly different from the control. Similarly, TBA values for samples containing added phospholipids and total lipids were generally low. Thus, lipids from chicken white meat were more stable to autoxidative

degradation during frozen storage than those from beef or chicken dark meat. The greater stability exhibited by chicken white meat lipids was unexpected and is more dramatic in view of the fact that the phospholipid and triglyceride levels were the same as those for chicken dark meat. It is postulated that the greater amount of oxidation in beef and chicken dark meat when compared to chicken white meat may be due to their higher levels of residual meat pigments in the muscle fibres. However, the low TBA values observed in the chicken white meat samples are closely related and supported by the observed greater stability of chicken white meat triglycerides and phospholipids, including PC and PE as will be discussed later.

Changes in fatty acid composition due to storage

It has been demonstrated by Lea (1953) and by Keller & Kinsella (1973) that changes in the fatty acid composition of lipids provide an indirect measure of susceptibility to lipid oxidation. Thus, the composition of fatty acids in the

TABLE 4

EFFECT OF ADDING CHICKEN WHITE MEAT TRIGLYCERIDES, TOTAL PHOSPHOLIPIDS AND TOTAL LIPIDS ON TBA VALUES OF CHICKEN WHITE MEAT MODEL MEAT SYSTEMS STORED AT −18°C[a]

Time in frozen storage (months)	*Experimental treatments*			
	Control (W_4)	*Triglycerides* (W_2)	*Total phospholipids* (W_1)	*Total lipids* (W_3)
0	$0{\cdot}42^b$	$0{\cdot}70^d$	$0{\cdot}78^e$	$0{\cdot}95^g$
1	$0{\cdot}57^c$	$0{\cdot}83^f$	$0{\cdot}94^g$	$1{\cdot}15^h$
4	$1{\cdot}39^j$	$1{\cdot}30^i$	$1{\cdot}98^m$	$1{\cdot}48^k$
8	$1{\cdot}78^l$	$1{\cdot}46^k$	$1{\cdot}95^m$	$1{\cdot}74^l$

[a] TBA numbers = mg malonaldehyde/kg meat. W_1 = 0·7% total phospholipids; W_2 = 4·3% triglycerides; W_3 = 5·0% total lipids; W_4 = muscle fibres only. Values within and between experimental treatments bearing the same superscript are not significantly different at the 5% level.

triglycerides, total phospholipids and individual phospholipids (PC and PE) was determined prior to frozen storage and after 8 months in freezer storage.

Beef triglycerides: The composition of fatty acids in beef triglycerides is presented in Table 5. Prior to frozen storage the levels of saturated, mono-, di- and polyunsaturated fatty acids were 52·07, 45·81, 1·27 and 0·85%, respectively. These values are in good agreement with the data presented by Hornstein *et al.* (1967). Some changes in the fatty acid profile of the triglycerides were observed at the end of 8 months freezer storage. The dienoic acids increased from 1·27 to 4·31%, while the polyenoic fatty acids increased from 0·85 to 1·43% during freezer storage. Consequently, there was a corresponding increase of 4·5% in total unsaturation from the original value at the end of frozen storage. Thus, beef triglycerides were relatively stable during frozen storage. It is known that beef triglycerides are highly saturated, and as such are slow to oxidise (El-Gharbawi & Dugan, 1965; Chipault &

TABLE 5

CHANGES IN THE FATTY ACID COMPOSITION OF TRIGLYCERIDES DURING FROZEN (−18 °C) STORAGE OF MODEL MEAT SYSTEMS (0–8 MONTHS)[a]

Fatty acid	*Beef*		*Chicken dark meat*		*Chicken white meat*	
	Prior to storage	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*
12:0	—	—	—	—	—	—
14:0	3·15	2·63	0·54	0·98	0·84	0·82
14:1	—	—	—	—	—	—
15:0	—	0·33	—	—	0·80	—
16:0	25·26	24·06	22·46	24·80	25·14	21·94
16:1	3·62	4·34	3·70	6·15	2·77	3·27
16:2	—	1·68	—	—	—	0·39
17:0	1·02	0·89	—	1·18	—	0·45
18:0	22·64	22·08	7·57	8·96	8·30	8·65
18:1	42·19	39·93	39·07	42·91	34·32	39·07
18:2	1·27	2·63	25·03	14·27	24·93	21·52
18:3ω6	—	0·13	—	—	—	0·34
18:3ω3	0·85	1·30	1·63	0·74	2·90	1·20
20:1	—	—	—	—	—	—
20:2	—	—	—	—	—	0·22
20:3	—	—	—	—	—	0·17
20:4	—	—	—	—	—	1·96
% Saturated	52·07	49·99	30·57	35·92	35·08	31·86
% Monoenoic	45·81	44·27	42·77	49·06	37·09	42·34
% Dienoic	1·27	4·31	25·03	14·27	24·93	21·91
% Polyenoic	0·85	1·43	1·63	0·74	2·90	3·89
Total unsaturation	47·93	50·01	69·43	64·07	64·92	68·14

[a] As percent of total fatty acids.

Hawkins, 1971). This probably explains the long induction period observed in beef samples containing added triglycerides.

Chicken dark and white meat triglycerides: The initial levels of saturated, mono-, di- and poly-unsaturated fatty acids (Table 5) in chicken dark meat triglycerides were 30·57, 42·77, 25·03 and 1·63%, respectively. The corresponding levels of the same fatty acids in chicken white meat were 35·08, 37·09, 24·93 and 2·90%, respectively. These results are in good agreement with those reported by Katz *et al.* (1966).

Unlike the beef triglycerides, $C_{18:2}$ and $C_{18:3}$ fatty acids decreased in chicken dark meat and white meat triglycerides during frozen storage. The dienoic acids (principally $C_{18:2}$) decreased from the original value by 43 and 14% in chicken dark and white meat triglycerides, respectively. In addition, polyenoic fatty acids decreased from the original value by 56% in the dark meat but increased by 34% in the white meat triglycerides during frozen storage. At the end of frozen storage, total unsaturation had decreased from the original value by 7·72% in the dark meat, but

increased by nearly 5% in the white meat. Since the unsaturated fatty acids in chicken white meat triglycerides were stable during frozen storage, they probably accounted for the low TBA numbers for samples containing added white meat triglycerides. The observed decrease in di- and polyenoic fatty acids in chicken dark meat triglycerides, on the other hand, reflects the high TBA values (Table 3) observed in the samples containing added triglycerides.

Stability of fatty acids of phospholipids

The fatty acid composition of phospholipids was measured prior to frozen storage and again at the end of storage, and is presented in Table 6. The initial levels of saturated, mono-, di- and polyenoic fatty acids in beef phospholipids were 34·98,

TABLE 6
CHANGES IN THE FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS DURING FROZEN STORAGE (−18 °C) OF MODEL MEAT SYSTEMS (0–8 MONTHS)[a]

Fatty acid	*Beef*		*Chicken dark meat*		*Chicken white meat*	
	Prior to storage	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*
12:0	—	—	—	—	—	—
14:0	1·46	1·03	0·14	1·63	1·54	0·70
14:1	0·52	0·67	—	0·45	—	0·16
15:0	1·16	2·80	0·37	0·24	1·94	0·20
16:0	18·49	26·15	15·60	24·93	21·94	16·55
16:1	3·61	3·83	1·05	3·66	1·49	2·21
16:2	0·74	1·09	—	1·22	—	0·20
17:0	0·92	3·47	—	2·44	—	0·86
18:0	12·95	14·41	18·16	18·93	10·25	15·29
18:1	33·44	34·05	20·43	35·82	25·62	27·01
18:2	10·52	7·60	21·51	6·92	20·32	21·54
18:3ω6	0·37	0·36	0·37	0·37	—	0·24
18:3ω3	1·29	1·95	0·41	0·98	0·57	0·70
20:1	—	0·24	—	0·24	—	—
20:2	0·69	—	—	—	0·61	0·30
20:3	2·77	0·64	0·53	0·20	1·20	0·72
20:4	8·51	1·22	17·41	1·97	11·26	9·67
20:5	0·76	0·49	—	—	0·43	0·18
22:3	—	—	—	—	—	—
22:4	0·88	—	1·23	—	—	1·37
22:5ω6	—	—	—	—	2·22	0·54
22:5ω3	0·92	—	—	—	0·60	0·54
22:6	—	—	2·79	—	—	1·02
% Saturated	34·98	47·86	34·27	48·17	35·67	33·60
% Monoenoic	37·57	38·79	21·48	40·17	27·11	29·38
% Dienoic	11·95	8·69	21·51	8·14	20·93	22·04
% Polyenoic	15·50	4·66	22·74	3·52	16·28	14·98
Total unsaturation	65·02	52·14	65·73	51·83	64·32	66·40

[a] As percent of total fatty acids.

37·57, 11·95 and 15·50 %, respectively. These values are in close agreement with the data presented by Hornstein *et al.* (1967). The initial values of the saturated, mono-, di- and polyenoic fatty acids obtained in chicken dark meat phospholipids were 34·27, 21·48, 21·51 and 22·74 %, respectively. The initial values for the corresponding fatty acids in the chicken white meat samples were 35·67, 27·11, 20·93 and 16·28 %, respectively. These values generally agree with the data reported by Katz *et al.* (1966).

Significant losses were observed in the PUFAS associated with the phospholipids (Table 6). During frozen storage of beef and chicken dark meat samples containing added phospholipids, the $C_{18:2}$ fatty acid decreased by 28·0 and 68·0 % from the original values, respectively. On the other hand, it increased by 6·0 % in chicken white meat. Arachidonic acid ($C_{20:4}$) decreased from the original value by 86 % in beef, 89 % in chicken dark meat, but by only 14 % in chicken white meat. Total PUFAS decreased from the original values by 69·94, 84·52 and 7·98 % in beef, chicken dark meat and white meat, respectively. Consequently, the levels of total unsaturation at the end of 8 months freezer storage were 52·14, 51·83 and 66·40 % in beef, chicken dark meat and white meat, respectively. These results verify the stability of the white meat samples (Table 4) during freezer storage and support the observed instability of beef and chicken dark meat samples. Thus, the changes observed in the fatty acid profiles of the phospholipids provide good evidence for the involvement of PUFAS in the development of rancidity.

Lea (1953, 1957), Younathan & Watts (1960), Hornstein *et al.* (1961) and Keller & Kinsella (1973) have concluded that changes in PUFAS of the phospholipids, and especially of arachidonic acid, result in serious flavour problems. Thus, the results of this study provide convincing evidence for the involvement of phospholipids in development of rancid flavour during frozen storage of meat.

Changes in the fatty acid profiles of PC and PE

In studying the mechanisms of autoxidative degradation of lipids during frozen storage of meat, the compositions of the fatty acid profiles of PC and PE in phospholipids were also determined before and following 8 months of frozen storage and are presented in Tables 7 and 8, respectively.

The most remarkable changes in the fatty acids of PC and PE occurred in the polyenes, in general, and in the $C_{20:4}$ and $C_{22:4}$ fatty acids in particular. The levels of $C_{20:4}$ in PC and PE for beef declined from 20·34 to 0·24 % and from 32·75 to 1·23 %, respectively. Similarly, $C_{20:4}$ in the PC and PE of chicken dark meat phospholipids decreased from the original value by 96 and 74 %, respectively, for chicken white meat, the levels of $C_{20:4}$ in PC and PE also declined during frozen storage, the decreases being 76·0 and 50·23 % from the original values, respectively. In the beef samples, the levels of PUFAS declined by 59·22 and 64·04 % from the original values in PC and PE during freezer storage, respectively. PUFAS also decreased in the

TABLE 7
CHANGES IN THE FATTY ACID COMPOSITION OF PC DURING FROZEN STORAGE (−18 °C) OF MODEL MEAT SYSTEMS (0–8 MONTHS)[a]

Fatty acid	*Beef*		*Chicken dark meat*		*Chicken white meat*	
	Prior to storage	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*
12:0	6·05	—	—	—	—	—
14:0	5·14	0·36	—	—	0·34	—
14:1	—	0·16	—	—	1·38	—
15:0	1·00	1·49	0·93	0·43	1·55	—
15:1	—	0·52	—	1·28	—	2·10
16:0	21·97	26·39	19·70	26·04	17·84	18·76
16:1	0·73	1·90	—	1·03	—	1·35
16:2	—	0·79	—	0·51	—	0·36
17:0	1·94	1·43	3·35	0·69	1·72	1·45
18:0	10·53	10·46	17·14	18·50	12·07	17·79
18:1	23·73	23·78	15·45	25·70	14·94	18·76
18:2	4·48	7·77	10·78	10·79	10·11	11·54
18:3ω6	—	0·48	0·42	0·90	0·52	—
18:3ω3	0·27	1·39	0·35	1·03	0·40	2·40
20:1	—	0·28	—	—	—	—
20:2	0·78	15·03	2·76	3·43	5·52	3·00
20:3	—	—	0·24	—	—	0·84
20:4	20·34	0·24	17·84	0·72	16·66	3·97
20:5	—	7·13	0·28	8·95	11·89	17·67
22:3	—	—	—	—	—	—
22:4	3·03	—	10·78	—	5·06	—
22:5ω6	—	—	—	—	—	—
22:5ω3	—	0·40	—	—	—	—
22:6	—	—	—	—	—	—
% Saturated	46·63	40·13	41·12	45·66	33·52	38·00
% Monoenoic	24·46	26·36	15·45	28·01	16·32	22·21
% Dienoic	5·26	23·87	13·54	14·73	15·63	14·90
% Polyenoic	23·64	9·64	29·91	11·60	34·53	24·88
Total unsaturation	53·36	59·87	58·90	54·34	66·48	61·99

[a] As percent of total fatty acids; PC = phosphatidyl choline.

chicken dark meat, the levels being 61·22 and 29·0 % in PC and PE, respectively. Only moderate losses occurred in the PUFAS of PC and PE associated with chicken white meat phospholipids.

Examination of the data shows that the changes in unsaturation of PC (Table 7) and PE (Table 8) during freezer storage were less in chicken dark meat than in beef or chicken white meat. This was not surprising in the case of the beef model system, which contained more total lipids (10 %) in comparison to both chicken model systems (5 %). However, the greater magnitude of changes in the chicken white meat system as compared to the chicken dark meat system cannot be explained on the

TABLE 8
CHANGES IN THE FATTY ACID COMPOSITION OF PE DURING FROZEN STORAGE (−18°C) OF MODEL MEAT SYSTEMS (0–8 MONTHS)[a]

Fatty acid	*Beef*		*Chicken dark meat*		*Chicken white meat*	
	Prior to storage	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*	*Prior to storage*	*Frozen storage (8 months)*
12:0	0·48	—	—	—	—	—
14:0	1·55	1·23	0·25	0·49	1·80	0·34
14:1	—	0·34	0·33	0·16	1·56	0·14
15:0	0·33	0·45	1·60	0·65	4·14	0·63
15:1	—	0·92	—	0·72	—	4·62
16:0	13·96	21·48	15·73	13·28	13·85	16·60
16:1	1·65	2·01	—	0·65	—	1·44
16:2	—	1·68	—	1·23	—	0·58
17:0	1·49	2·24	2·70	1·08	2·25	2·98
18:0	14·89	17·01	23·23	22·68	13·64	21·36
18:1	19·50	24·84	16·39	22·88	15·92	21·17
18:2	4·80	8·39	11·84	12·82	11·08	10·97
18:3ω6	—	—	0·33	1·15	0·28	0·07
18:3ω3	0·37	1·96	0·28	1·32	0·33	0·96
20:1	—	—	—	0·26	—	0·58
20:2	1·06	4·92	1·06	3·94	5·97	1·56
20:3	0·37	—	0·23	0·79	0·28	0·38
20:4	32·75	1·23	18·68	4·83	17·52	8·72
20:5	—	10·74	—	9·73	7·58	3·46
22:3	—	0·56	0·35	—	—	—
22:4	6·80	—	5·94	0·99	3·79	1·44
22:5ω6	—	—	—	—	—	0·36
22:5ω3	—	—	0·57	—	—	0·58
22:6	—	—	0·49	0·35	—	1·06
% Saturated	32·70	42·41	43·51	38·18	35·68	41·91
% Monoenoic	21·15	28·11	16·72	24·67	17·48	27·95
% Dienoic	5·86	14·99	12·90	17·99	17·05	13·11
% Polyenoic	40·29	14·49	26·87	19·16	29·78	17·03
Total unsaturated	67·30	57·59	56·49	61·82	64·31	58·09

[a] As percent of total fatty acids; PE = phosphatidyl ethanolamine.

basis of lipid content, since both model systems contained 4·3% triglycerides and 0·7% phospholipids. Nevertheless, PE consistently changed more in unsaturation during freezer storage than PC, supporting the concept that PE was more susceptible to autoxidation.

Results clearly show that polyenoic fatty acids are not stable and undergo autoxidative degradation. Lea (1953) reported that unsaturation rapidly disappears at advanced stages of autoxidation. Therefore, results strongly suggest that the PUFAS appear to be principally responsible for development of rancidity, which is in agreement with earlier reports by Lea (1953, 1957), Hornstein *et al.* (1961) and Keller & Kinsella (1973). Results also indicate that PC and PE may play an important role in the development of rancidity during frozen storage of meat and

meat products. Data presented for TBA values (Tables 2 and 3) and changes in unsaturation of the PUFAS (Tables 6, 7 and 8) seem to confirm the positive relationship between rancidity and oxidation of fatty acids. It is well known that PUFAS are extremely reactive, and through oxidative degradation give rise to a number of carbonyl compounds, which greatly influence oxidised flavour (Lea, 1953, 1957; Younathan & Watts, 1960).

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EFFECT OF PROCESSING CONDITIONS ON THE OIL CONTENT OF PARBOILED-RICE BRAN

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ABSTRACT

The oil content of residual milled kernel was consistently lower and of bran higher in parboiled as compared to raw rice at all degrees of milling (d.m.), showing that the rice oil migrated outwards upon parboiling. However, the d.m.-oil content curves for the residual kernel as well as the bran were largely unaffected by varying conditions of parboiling. The total oil content of the grain was also unchanged after parboiling. When the data for the fraction of the total grain oil coming into the bran were plotted against the d.m. for a variety of parboiling conditions, they again fell into a single line. These results showed that, contrary to many earlier claims, the processing condition during parboiling had no effect on the redistribution of the fat in the grain.

INTRODUCTION

It is well known in the trade, confirmed by analysis of commercial samples (Louden & Kinsella, 1939; Kumar David *et al.*, 1964), that bran milled from parboiled rice contains more oil than that from raw rice.‡ The most plausible explanation is provided by the classical study of Subrahmanyan *et al.* (1938), who noted that under a variety of parboiling conditions the content of residual fat in the milled kernel was always lower in parboiled than in raw rice, from which they postulated that as the water-soluble nutrients migrated inwards during parboiling, fat moved outwards. This postulate is supported by data from many subsequent studies. Raghavendra Rao *et al.* (1965), in a laboratory milling study, not only confirmed that parboiled-

* Work carried out in part fulfilment of the M.Sc. (Food Technology) Degree of the University of Mysore in 1976 and 1978 respectively.

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‡ In rice milling, paddy (rice in husk) is first shelled (dehusked) to give husk and brown rice, which when milled (whitened, pearled) yields milled rice and bran. Paddy which has been soaked, drained, steamed and dried is called parboiled paddy; paddy not so-treated is called raw paddy.

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rice bran contained more oil, but also showed that the highest concentration of fat occurred in a more outer layer in the parboiled than in the raw grain. Bhattacharya *et al.* (1972) found that the *total* oil content was lower in parboiled than in raw milled rice at all degrees of milling from 0–10%, but that the oil content on the kernel *surface* was quite the reverse. The histological observations of Mahadevappa & Desikachar (1968) that the discrete oil globules located in the aleurone layer in the raw grain lost their identity after parboiling and were converted into a band adjacent to the bran layer, explain most of the above observations. However, Raghavendra Rao *et al.* (1965) suggested two other possible explanations for the above phenomenon: (a) less admixture of bran with endosperm particles while milling the hard parboiled grain, and (b) better extractability of oil from parboiled than from raw bran by fat solvents. Padua & Juliano (1974) suggested that the higher fat content of parboiled bran was due to more efficient removal of aleurone and germ layers from the parboiled grain during milling.

Whatever the explanation, there have been suggestions, in view of the economic importance of the subject, that the oil content of parboiled-rice bran can be increased by suitable adjustment of the parboiling conditions. The oil content of the bran was thought to increase with increasing temperature (Chakravarty & Ghose, 1966) and time (Vasan *et al.*, 1971) of soaking, and with greater pressure of steaming (Padua & Juliano, 1974) of paddy during parboiling. However, the degree of milling, which profoundly influences the oil content of the milled products, was generally not strictly controlled in the above studies, and the complete oil balance in the grain after parboiling was never determined.

A detailed study of the effect of processing conditions during parboiling on the distribution of fat in the rice grain was therefore carried out.

MATERIALS AND METHODS

Jenugudu paddy, a high-amylose local tall *indica* variety, available in laboratory stock, was used.

For parboiling, paddy was soaked at room temperature (RT: 23–28 °C) and various other temperatures for different time intervals, or in warm water (starting at about 70 °C and leaving in a covered vessel at RT, designated here as apparent room temperature, RT′) overnight, steamed for different time intervals under different pressures, and dried in the shade (Bhattacharya & Subba Rao, 1966; Bhattacharya & Indudhara Swamy, 1967).

The paddy was shelled in a laboratory Satake rubber-roller sheller and the brown rice was milled to different degrees of milling (d.m.) in a modified McGill miller No. 1 (Bhattacharya & Sowbhagya, 1972).

Fat content

In view of the very large number of samples, it was not practicable to dry them in vacuum before solvent extraction as prescribed by the standard AACC (1969)

procedure. After a number of preliminary investigations, the following abridged procedure was adopted which gave results reasonably close to the standard method. Bran, or rice ground in a Buhler disc mill to 40 mesh, was weighed into a thimble, which was then dried in an oven at 80 °C for 3 h, cooled in a desiccator and extracted with petroleum ether (60–80 °C) in a Soxhlet apparatus for 10–16 h.

The moisture content of all the samples was reasonably constant at about 9 % (wet basis). In view of this, and since the analysis was mainly based on comparison of percentage of total oil removed into bran, no correction for moisture was applied to any of the data.

RESULTS AND DISCUSSION

A very large number of parboiling conditions were tested.

The curves of d.m. *vs* oil content of both milled rice (Fig. 1) and bran (Fig. 2) were distinctly different for raw and parboiled rice. However, the data of all the parboiled samples fell reasonably close to a single line each, suggesting that the processing condition had not much effect. There was undoubtedly some scatter in the results,

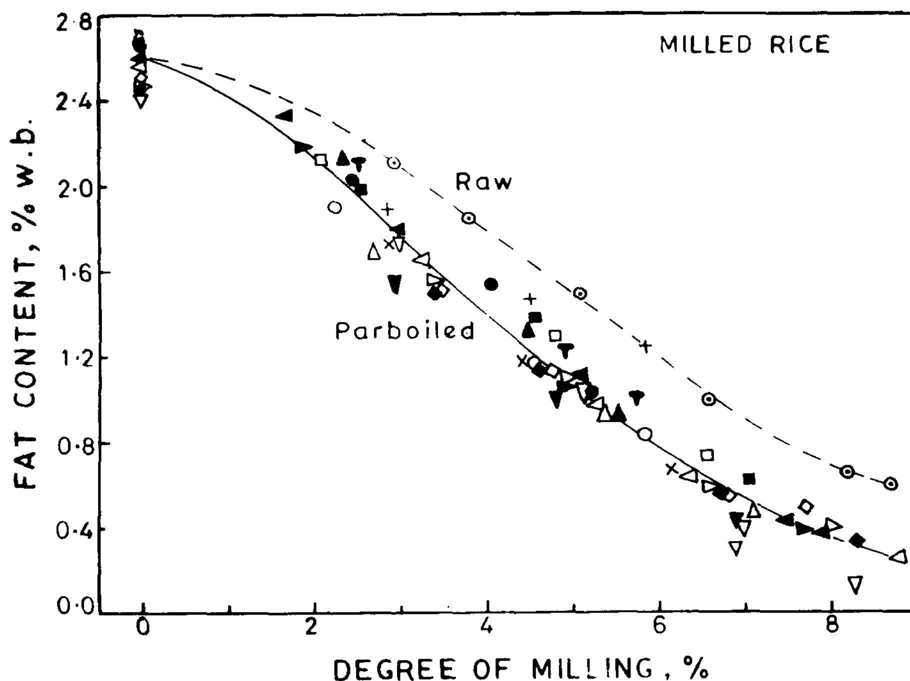

Fig. 1. Fat content of residual raw- and parboiled-rice kernels at different degrees of milling. Different symbols represent different conditions of parboiling (see Fig. 3).

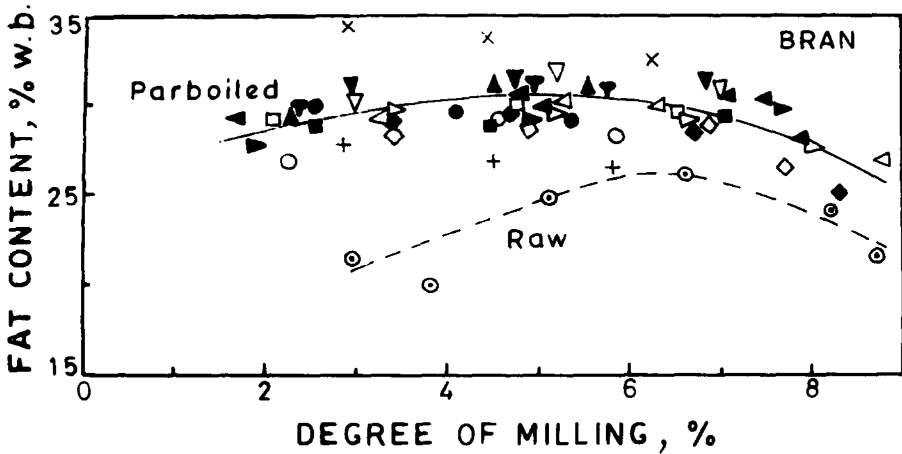

Fig. 2. Fat content of raw- and parboiled-rice bran at different degrees of milling. For explanation of symbols, see Fig. 3.

but the variation was entirely random without any suggestion of a trend.

To get a clearer idea, the complete oil balance was calculated in each case. The calculated total oil content in each pair of milled rice plus its corresponding bran was reasonably constant in all the samples, being in the range of 2·40–2·72% (wet basis). When the amount of oil entering the bran was expressed as a percentage of the corresponding total (oil in milled rice + bran), the data for all parboiled samples again fell in a single line (Fig. 3). Clearly the parboiling condition seemed to have no effect on the results.

It will be noted that (a) the total oil content of the grain remained reasonably constant after all conditions of parboiling, (b) the fat of residual milled rice decreased correspondingly as that of the bran increased after parboiling, and (c) the difference in bran oil between the raw and parboiled grain was shown even at very low degrees of milling where significant admixture of bran with endosperm particles is most unlikely. Taken together, these data show that the increased oil content of parboiled-rice bran was not due to better oil extractability or to lesser admixture with endosperm particles or to more efficient removal of aleurone and germ layers (Raghavendra Rao *et al.*, 1965; Padua & Juliano, 1974), but due to outward migration of fat (Subrahmanyan *et al.*, 1938). This is evidently related to the rupture of oil globules in the grain during parboiling (Mahadevappa & Desikachar, 1968). Figure 3 also suggests that, contrary to many earlier claims, the parboiling condition has no effect on the extent of this outward migration of fat. Apparently, the oil globules are either ruptured or not, and once ruptured, the fat cannot be further expelled by increasing the severity of the parboiling process.

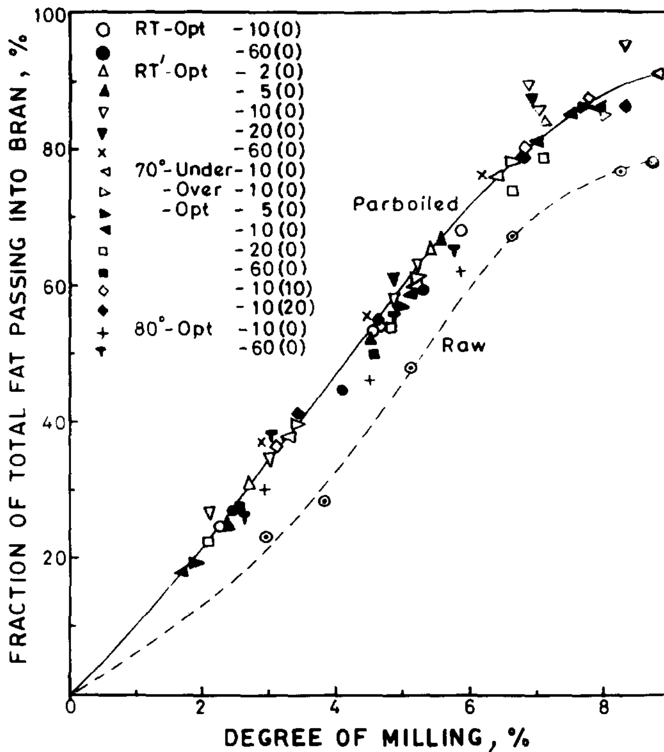

Fig. 3. Fraction of total grain fat passing into the bran at different degrees of milling in raw and parboiled rice. Abbreviations of the parboiling conditions, shown in inset, stand for (in the order shown): (i) temperature of soaking (°C), (ii) time of soaking (under, optimum and over, for the respective temperature), (iii) time of steaming (min), and (iv) pressure of steaming (p.s.i.).

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THE EVOLUTION OF PANARY FERMENTATION AND DOUGH DEVELOPMENT—A REVIEW

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ABSTRACT

An understanding of the changes taking place during breadmaking and the practical implementation of this knowledge is outlined from the Egyptian era to the present day. A summary is given of the principles and applications of the major commercial breadmaking procedures used in the developed world during the present century. Several types of more modern processes are discussed in greater detail, particular attention being given to those employing mechanical means for developing dough. Theoretical considerations used to account for the changes taking place during mechanical dough development are also discussed. Finally, an attempt is made to predict possible future trends in the baking industry.

INTRODUCTION

Apart from wheat flour, salt and water, the only ingredient which has been indispensable to the production of traditional forms of leavened bread throughout the ages, although only relatively recently recognised as a separate, living entity, is yeast.

No one knows with certainty when yeast was first used in breadmaking. It is generally accepted, however, that it owes its discovery to the Egyptians and Babylonians, who no doubt observed that when they baked sour dough, the resultant product possessed properties totally different from the unleavened material, the bread being rendered soft and porous and more easily assimilated.

The leavening effect of yeast in bread, therefore, was highly desirable. To maintain a supply of yeast, it was recognised that there was no need to obtain yeast from the air since a piece of old sour dough could be retained in order to 'implant' the

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leavening in the new dough. This was the origin of the reproductive sour dough, which was sacredly preserved in Egyptian households for fear that they lose the precious primal material of baking—that which raised the dough. Saint Paul mentioned in the New Testament: 'Know ye not that a little leaven leaveneth the whole lump.'

Dough preparation was undoubtedly carried out in a manner resembling current breadmaking operations—salting of the dough for taste, kneading to incorporate the granular salt and forming the dough into a convenient shape. To prevent the dough piece from adhering to the base of the oven during baking, a layer of bran was spread over the bottom. The art of this breadmaking was passed from the Egyptians to the Jews, thence to the Greeks, and was introduced into northern Europe by the Roman armies.

'The wise priests of Egypt', wrote Plutarch in his essay to Isis and Osiris, 'call the black earth *chemia*. Just as unknown substances are mingled in this black earth that was once the gift of the Nile, to form soil of ultimate fertility, so known substances are coupled within the oven according to an unknown law.'

PANARY FERMENTATION

The word *fermentation* is derived from the Latin *fermento*, representing the act of boiling, which is the first impression gained from a vigorously fermenting liquid. The English word *yeast* appears to be related to brewing, being derived from Old and Middle English *gist* or *giest* which are thought to originate from the Indo-European *yes*, representing a seething or boiling action. The Dutch *gist* has a similar origin. In some other European languages, however, the equivalents of *yeast* are more closely related to breadmaking, the French *levure* being derived from the Latin verb *levâre* and the German *hefen* from *heben*, both verbs meaning 'to raise'. It is also of interest to note that *enzyme* is derived from the Greek *ζύμη*, denoting leaven. *Bread* is derived. from the Middle English *breed*, bit or piece, the Teutonic form surviving as *loaf*.

Advances in the technology of yeast for breadmaking have been greatly influenced by the results of studies concerned with beer and wine fermentation. Some of this work is discussed below but, for a more comprehensive treatment of the development of the yeast industry, the reader is referred to Frey (1930, 1957).

Although Leeuwenhoek, in 1680, actually observed living yeast cells in a fermenting liquid, it was not until 200 years later that their role in fermentation was universally recognised. Until the time of Pasteur, most of the progress in the utilisation of yeast had originated from brewers and bakers themselves rather than from scientists.

In the mid-17th century it was proposed by Kircher that 'animalculae' were responsible for putrefaction and decay, a theory which was further developed by Plenciz who, in 1762, stated that decay only occurred when minute wormlike bodies were present.

Various others believed that fermentation was brought about by an unstable substance which transmitted its vibrations to other materials present. The idea originated with Willis in 1659 and was strongly supported two centuries later by Liebig (1803–73) who, because of the respect he commanded, probably had a considerable adverse effect on the scientific study of yeast at a critical period. Even the theory of spontaneous generation, advanced by Needham in 1745, received the backing of Joly and Pouchet as late as 1865.

Despite the confusion in scientific circles regarding the nature and action of yeast, much practical experience had been accumulated and, in 1775, an encyclopaedia on yeast was produced by Krunitz. The main products of fermentation, alcohol, carbon dioxide and acid, were known in 1789, as a result of the work of Lavoisier. In 1819, Hermstadt differentiated between top and bottom yeasts and Tebenhoff, in 1833, concluded that top yeast or distillers' yeast was better for breadmaking.

The first to assert that yeast is a vegetative organism, whose vital function is to ferment, appears to be a German baker, Erxleben, in 1818, although his views were not widely publicised. Between 1830 and 1870 was the most active period of controversy concerning the nature of yeast. In 1835–37 several workers, notably Schwann, were adamant that beer fermentation was a result of the action of living organisms. Quevenne stated in 1838 that yeast was an organised body and was not a chemical compound and Blondeau, in 1846, considered that each fermentation was caused by a specific organism. However, there were many bitter opponents to the vitalistic theories, particularly Liebig. In an attempt to reconcile the opposing ideas of Liebig and Pasteur, Traube, in 1858, proposed that ferments (enzymes) were responsible for fermentation but that these were produced by the living cell. It was Buchner and Meisenheimer, however, in 1896, who demonstrated that enzymes could actually be separated from yeast cells and ferment sugar by themselves.

The question of the role of yeast in fermentation was only resolved by the work of Pasteur in the 1870s, which finally disproved the theory of spontaneous generation. His work laid the foundation for progress in the culture and manufacture of yeast as well as the understanding of its nature and action.

The production of compressed yeast on a commercial scale started in Europe around 1800, although yields were low. In 1879, the aeration method developed by Marquardt, based on the work of Pasteur, led to great increases in yield and, in 1910, methods employing higher aeration were introduced. It was clearly recognised that the success of the yeast and bread industries were largely interdependent, and this has proved to be the case up to the present day.

The history of yeast in bread can be divided into three eras:

(a) Ancient times, when sour dough or leaven was used;
(b) The period of barms (about 2000 years, up to the beginning of this century);
(c) The present era, about 80 years old, which has seen the universal acceptance of compressed yeast and, to a lesser extent, active dry yeast.

The use of yeast as a separate ingredient in traditional breadmaking was first

documented around the 1900s. Simmons (1903) mentions that bakers obtained their yeast from brewers and distillers, and that it was widely used to supplement barms, which are discussed below. No mention is made of bakers' compressed yeast, which is surprising since its production had been established around 1800 (Freund, 1961). Jago & Jago (1911) list several methods in which the use of decoctions is recommended to initiate fermentation. It would appear, therefore, that up to the turn of this century, before the introduction of more efficient yeast production plants, bakers' compressed yeast was not readily available in England and traditional barm-making still predominated as the most popular means of yeast propagation.

In barm-making a watery medium was prepared containing the necessary nutrients for yeast growth. It was common practice to add a small quantity of sour dough or the previous batch of barm to act as a starter. Popular ingredients for barm were crushed barley malt, malt flour, potatoes and scalded flour (Bennion, 1967). The addition of hops or hop extract to the medium inhibited spoilage organisms, 'keeping the mash clean'. To speed up fermentation it was not unusual to add cold stout or porter to the wort. The barm would be left open to the atmosphere for 18–24 h at about 26 °C, during which time fermentation would become well established. The liquor was then strained and used as a starter in a wheaten bread dough. One gallon of barm is said to have been equivalent to 3–4 oz bakers' compressed yeast (Banfield, 1947).

Although there were many types of barm, formulation and method often being handed down from generation to generation, these variations appeared to be mainly regional preferences and were all essentially alike. Terminology in barm-making, often unclear, mentions *Spons* or *spontaneous* barm, *Virgin*, *Parisian* and *compound* barms. Parisian barm, popular in Scotland, was made essentially from potatoes. Virgin barm referred to the fresh liquor to which no old barm had been added which, in the case of barm made from potatoes, became a Parisian barm on the addition of starter. Since these relied on contamination by yeast from the surrounding atmosphere, the process was rather slow, usually taking 4–5 days in preparation as opposed to 24 h for the other types. The most widely used were compound barms, a typical recipe for this, using crushed barley malt, being:

Formulation for Compound Barm (Daniels, 1963)

	kg	%
Water	18·2	79·1
Crushed barley malt	4·5	19·6
Hops	0·09	0·04
Salt	0·03	1·3
Total mass	23	

Although the use of compressed yeast became widespread during the early part of this century it was not universally accepted even at the end of World War II, as

Banfield (1947) wrote: 'Still a few bakers remain, mostly in outlandish places, who make their own barm. The age of barms is truly passed.'

In breadmaking, yeast has three major functions. It is primarily a leavening agent, producing the gas which is trapped as bubbles in the dough resulting in the porous nature of the end product. It also contributes to the characteristic flavour of bread due to the various aromatic products of its metabolism. The third function is that of assisting dough development, which is discussed below.

DOUGH DEVELOPMENT

Since different breadmaking procedures vary mainly in their method of dough development, an explanation of this term is necessary.

When a dough is mature or ripe it is considered by the baker to be in its optimum physical condition for handling and baking. Dough maturity is achieved by the process known as development. This complex process encompasses both physical and chemical changes, principally gluten formation, modification of organic constituents by yeast and enzymes, effects of mixing (work hardening) and the effects of expansion due to fermentation. However, since the main change involved in dough development is the formation and modification of the gluten complex, this is discussed in more detail.

Gluten does not pre-exist in wheat flour but is formed when the protein fractions, gliadin and glutenin, become hydrated on the addition of water. Mechanical handling or mixing is necessary to 'develop' the gluten, forming a matrix in which the other dough components are embedded and which is responsible for the gas-retaining ability of the dough. As a result of the changes taking place during dough development, the product exhibits both elasticity and extensibility, properties which are highly desirable in breadmaking since the dough is required to withstand mechanical abuse and be capable of expanding and retaining evolved gas. Doughs having the correct physical attributes are essential if bread of high volume and good texture is to be produced. These attributes are dependent both on the quality of the wheat flour and the control of the development process.

On mixing a dough, its appearance gradually changes from a wet, lumpy material to a more coherent, elastic mass. Each dough has its optimum mixing requirements depending on the flour properties, dough formulation and type and speed of the mixer. Departure from this optimum results in inferior dough. In the case of immature or 'green' doughs, insufficient development occurs so that the elastic properties of the dough are not fully realised, with the result that the final loaf has a low volume since it cannot expand fully and retain sufficient gas. When a dough is mixed for too long, however, it loses its elastic properties and becomes highly extensible, a phenomenon usually referred to as 'breakdown'. An overmature dough, having a more fluid character, also results in a low-volume loaf.

In addition to the effects of mixing, dough development is encouraged by the action of yeast during the fermentation period. Despite the importance of this effect in breadmaking procedures employing biological dough development, its mechanism is not understood. According to Bloksma (1971): 'It is not precisely known how the disappearance of fermentable sugars, the reduction of the oxygen pressure, the formation of fermentation by-products and the stretching of the dough by expanding gas cells during fermentation affect dough properties; their effects are superimposed on the changes that proceed in unyeasted dough.' In breadmaking methods employing mechanical dough development there is generally only a short fermentation period, and the effect of yeast action on development is insignificant.

About 85% of the proteins in wheat flour consist of the gluten components, gliadin and glutenin (Holmes, 1966). These fractions differ markedly in their physical properties. Gliadin (m.wt 25–100,000) is extensible and inelastic whereas glutenin (m.wt $> 100{,}000$) is elastic but relatively inextensible. The gluten of a good breadmaking flour contains gliadin and glutenin in approximately equal proportions.

Flour strength refers to wheat or flour protein and encompasses both its quality and quantity. On the basis of flour strength, wheats can be classified into two major groups, namely hard (strong) or soft (weak). Hard wheat flours have a relatively high protein content, high water absorption, and form an elastic gluten with good gas-retaining properties. Such flours yield good bread over a wide range of mixing and fermentation conditions and, of particular importance in highly automated operations, have good resistance to mechanical abuse. Soft wheat flours, on the other hand, have a lower protein content and yield a gluten which is less elastic. Such flours are generally better suited for other baked goods such as cakes, biscuits and crackers.

BREADMAKING METHODS OF COMMERCIAL IMPORTANCE

Breadmaking methods of commercial significance only are discussed below. Throughout the world there are a great number of other procedures, particularly traditional methods practised on a small scale. Many of these are described by Pomeranz & Shellenberger (1971).

Processes employing biological dough development

In these processes the effect of yeast fermentation on dough development is significant.

Straight-dough process: Prior to the introduction of mechanical dough development systems, the majority of countries producing English-type breads utilised the straight-dough process of breadmaking. This method is so-called

because the dough is prepared by incorporating all the ingredients in a single stage and fermentation is carried out in bulk.

At the completion of mixing, when the dough has reached its optimum elasticity, it is allowed to stand for a predetermined time in order to achieve maturity by the action of yeast. Three- to four-hour doughs are generally best suited to English conditions, taking into account the quantity of imported hard wheats in the millers' grist.

An integral part of this method of dough processing is an operation known as 'knocking-back', 'cutting-back' or 'punching-down'. This can be carried out manually or in the mixer and involves the partial degassing of the dough in order to control its overall volume and prevent overflow from the dough bowl. It is also thought to bring about a redistribution of yeast and its substrate, thereby re-intensifying the rate of gas production, as well as improving the gas-retaining ability of the dough as a result of work hardening. In a 3 h bulk fermented dough it is usual to carry out the knock-back operation twice, first at 1 h and again at 2·5 h, thus allowing sufficient time for dough recovery prior to the dough-scaling operations.

Although short-time doughs considerably reduced processing times and also offered economic advantages, overall bread quality was markedly diminished. To maintain the quality normally associated with lengthy dough procedures, experience has shown that it is essential to include so-called enriching agents such as malt and lard.

Delayed salt method: This method is a variation of the conventional bulk fermentation procedure and was popular in England in the 1950s. All ingredients except the salt are mixed together to form a dough and, after completing two-thirds of its 'floor time' (total fermentation in bulk), knock-back is carried out while salt is added. Salt delays gluten development and also inhibits yeast action, thus, in the absence of salt, dough maturity is achieved more rapidly.

Delayed fat method: In a manner similar to the delayed salt method, fat is incorporated only at the knock-back stage of dough development. Doughs processed in this manner display superior machinability and tolerance to mechanical abuse and are, therefore, more suited to automated production units.

Sponge and dough process: This two-stage process accounts for a large proportion of the bread produced in the USA (Chamberlain, 1975) and can be successfully employed in the manufacture of a variety of breads and other baked goods.

The first stage of the process involves mixing part of the flour, usually 50–80 % of the total, yeast, yeast nutrients, shortening and some sugar, together with part of the water, into a fairly stiff dough called the sponge. The amount of water used depends on the absorption capacity of the flour and the degree of stiffness required. The aim in this mixing operation is to achieve homogeneity of the ingredients rather than to encourage development of the dough, although some 'mellowing' of the gluten proteins takes place during the fermentation period.

Fermentation is allowed to proceed for about 3–5 h, at 26–27 °C and 75–80 % RH. The sponge develops a weblike structure due to gas evolution and may increase in volume about 4–5 times before it reaches its limit of extensibility and collapses, 'drops' or 'falls'. This collapse normally occurs after 65–70 % of the total fermentation time has elapsed.

In the second stage of the process, the sponge is mixed with the balance of the dough ingredients ('doughing up'), although salt addition may be delayed. The high speed mixing employed encourages development of the gluten, after which the dough is allowed to ferment for a further 15–30 min, in order to recover and expand prior to dough make-up.

The above description refers to a typical US system. In the UK, although this type of process is much less popular, the tendency is to use a lower proportion of flour, about one-third, in the sponge, and a longer fermentation period of approximately 12 h. Two modifications to this system are known as (a) 'flying sponges', where fermentation at the sponge stage is markedly accelerated by the addition of greater quantities of yeast, which reduces overall process time, and (b) 'half-sponges' which refers to the quantity of flour used at the sponge stage, resulting in a stiffer sponge.

Although the sponge and dough process is more demanding than the straight-dough method with respect to time, labour, energy and equipment, the nature of the process allows a greater degree of control flexibility, which makes it amenable for mass production methods. Owing to the mellowing effect in the fermentation stage, the sponge and dough process is preferred for bread made from strong flours, whereas the straight-dough process is more suited to weaker flours.

Liquid ferment process: Not to be confused with barm-making, liquid ferment systems, as well as the sponge and dough method of breadmaking, are adaptations of the straight-dough process. A ferment is a watery medium containing all the essential nutrients for yeast growth and, after the period of fermentation, constitutes all or part of the total dough liquor.

Significant interest in liquid ferment processes has been generated in the USA only since the mid-1950s. The two main factors responsible for this were the introduction of the Stable Ferment Process by the American Dry Milk Institute (McLaren, 1954) and the tremendous effort put into development of equipment for continuous breadmaking.

The original Stable Ferment Process was introduced to simplify the sponge and dough method of breadmaking by replacing the sponge stage with a pumpable, liquid ferment. This method employed a high milk (6 % non-fat dry milk on a flour basis) and sugar (3 %) level in the ferment to stabilise the fermentation. Water, yeast, yeast food, malt, sugar, non-fat dry milk and salt were gently stirred in fermentation tanks for about 6 h at 35–38 °C. The mature ferment could then be used directly for dough mixing or cooled to 10 °C and stored for 48 h or more. The milk had two main functions, that of buffering the medium within the optimum pH range for yeast activity and of providing yeast nutrients, including phosphates and soluble nitrogenous material. Many benefits were claimed for the process, but it did not

become popular for several reasons, which included the high cost of the milk and yeast, problems of product uniformity and the organoleptic attributes of the bread.

Several other formulations were subsequently introduced, the principal change being the substitution of milk by buffering salts and yeast food. Generally, concentrated brews were used, which contained all the solid ingredients, except salt and flour, but only half the dough water, which improved the practical capacity of the fermentation tanks and permitted manipulation of the final dough temperature by adjusting the temperature of the remaining dough water (Pyler, 1970).

Several liquid ferment systems are described in detail by Euverard (1967). For the production of the 'liquid sponge' it is usual to employ components designed for continuous breadmaking, the dough being further processed in conventional equipment.

The advantages claimed for such systems include savings of labour, plant space and processing time, waste reduction, elimination of sponge-making equipment and increased production flexibility.

Processes employing mechanical dough development

Processes employing mechanical dough development have now largely replaced bulk fermentation as the principal method of breadmaking in developed countries and have eliminated the need for biological development by yeast (Williams, 1975). In South Africa almost 50% of the bread output is made using such a system, while in Britain between 75 and 80% of all bread is produced by mechanical development. In the USA almost all the bread is made by either the sponge and dough method or by continuous mechanical development processes using brews.

Historical aspects: The possibility of using mechanical dough development was first reported by Swanson & Working (1926). They showed that traditional bulk fermentation could be largely replaced by an intense mechanical energy input to a dough by a mixer with an action described as 'pack–squeeze–pull–tear'. It was almost 30 years later, however, that this principle was used commercially.

Baker & Mize (1937) found that after intense mixing of a dough, the addition of bromate had a far greater effect than expected and concluded that the dough had become activated in some way. In 1941, Baker developed an experimental 'no-time' system of breadmaking using a rapid-acting oxidising agent. On completion of mixing, the dough was extruded directly into a baking pan. He concluded that the type of mixing applied to a dough is of importance to gluten structure and the type of gas-cell and its distribution in the final product. Devices which rub, cut or tear appeared to be detrimental to gluten structure, whilst a kneading or compression (stretching action) tended to improve it. This work culminated in the commercial application of a new method of dough development, the 'John C Baker Do-Maker', described by Baker (1954).

Continuous breadmaking systems: The potential economic advantages of an automated bread production system provided a great incentive for research in this area. The Do-Maker process was introduced in the USA in 1953 and in England

three years later. A competitor, the 'Amflow' system (American Machine and Foundry Co., 1959) was soon added and these two systems are responsible for producing a major portion of bread in the USA today.

The main features of these continuous systems are similar. Basically, they rely on sugar-containing pre-ferments to provide flavour and the desired leavening action. Initially, the Do-Maker system was designed to operate using a 'liquid brew' or 'broth' containing yeast, sugar, milk, salt, yeast food, enrichment and mould inhibitor, whereas the Amflow process employed a 'liquid sponge' containing flour. However, due to a desire by the bakers to use flour in the pre-ferment (mainly for economic reasons since flour could be used to replace part of the more expensive sugar) the Do-Maker system has now been modified to allow flour inclusions.

After a fermentation period of about 2–4 h, the pre-ferment is mixed with the balance of the dough ingredients. It is then metered via a dough pump to a developer working under pressure of the continuous ingredient flow, where it is subjected to intense mechanical action for less than 1 min. From the developer it is extruded, cut and deposited directly into the baking pan for proving and then transferred to the oven.

The heart of these continuous systems is the developer, which transforms the mass of ingredients into a dough possessing the desired attributes of elasticity and extensibility. The developer chamber is oval in cross-section and contains two high-speed, counter-rotating impellers, operating under a pressure of 20–60 psi (140–410 kPa). The dough is rapidly kneaded, folded, stretched and compressed, power consumption being in the range of 0·3–0·4 hp min/lb dough (30–40 kJ/kg). The operating conditions of the developer are critical in determining the physical properties of the final loaf.

Thus the continuous process eliminates the dough make-up stages of rounding, intermediate proof and moulding, with consequent savings in time and cost. Space savings of up to 75 % are also possible. In addition, process absorption is increased and loss of solids in bulk fermentation is eliminated, with the result that yield figures are higher. Other benefits include the elimination of dough-to-dough variation, resulting in improved scale-weight accuracy and greater product uniformity.

However, despite the many economic and practical advantages of the above systems, their acceptance outside the USA has been limited, primarily due to the textural properties of the finished product. Such bread is typified by its fine and uniform grain, possibly due to a combination of carbon dioxide dissolution under pressure and the intense shearing action to which the dough is subjected (Ponte, 1971). The crumb lacks resilience and bears little resemblance to bread made by the conventional British bulk fermentation systems. Criticism lodged by the British public was that the bread tended to be 'chewy', 'doughy' or 'cake-like', and lacked flavour. As a result, only a few Do-Makers are currently operating successfully in Britain, while in South Africa such plants have been extensively modified to produce a product acceptable to the public.

The Chorleywood bread process: The Chorleywood Bread Process (CBP) is a no-time batch process of mechanical dough development characterised by intense mechanical working of the dough for a short time (5 min or less) in conjunction with the addition of ascorbic acid as an improver.

The historical background of the CBP is described in detail by Williams (1975). Work leading to the development of the CBP began in 1958 at the British Baking Industries Research Association (BBIRA), Chorleywood. At that time, attention was being devoted to those factors which appeared to be important in producing bread by a mechanical development method, particularly with a view to adapting the 'Do-Maker' to local conditions. The main difficulties which had to be overcome were:

(a) the process had to suit local lean formulations containing less shortening, sugar and other enriching agents than American-type doughs.
(b) it was necessary to incorporate large quantities of softer, home-grown wheats in the millers' grist.
(c) oxidants such as potassium iodate, used in the USA and which played an important role in dough modification, were not permitted in the UK.
(d) the fine and uniform crumb structure of bread produced by current continuous methods was not acceptable to the British consumer.

It became apparent that the amount of work expended on a dough during its development was critical. This important finding was to be employed as a means of measuring the extent of dough development. Further investigations revealed that the presence, in the dough, of a fast-acting oxidising agent, was essential to this system.

In 1961 the 'Chorleywood Bread Process' was introduced as a batch method for the production of good quality small goods with a substantial reduction in time and cost. The application of the process was described by Collins (1963). Compared with conventional bulk fermentation procedures the CBP is characterised by:

(a) intense mechanical working of the dough, preferably for 2–4 min, with a work input of 0·4 hp min/lb dough (40 kJ/kg). This amount of energy is about six times that used in mixing a dough intended for bulk fermentation and is carried out in a high speed mixer operating under a vacuum of 380–510 mm Hg ($50–67 \times 10^3$ N/m^2).
(b) the presence of high levels of oxidising agents in the dough. The original process employed 75 ppm ascorbic acid but better results are obtained by using a mixture of oxidising agents. A combination of potassium bromate and potassium iodate is particularly effective but iodate is not permitted in the UK. Commonly a mixture of 30 ppm bromate and 30 ppm ascorbic acid is added to flours already treated at the mill with 15 ppm bromate and 15 ppm chlorine dioxide.

(c) the addition of fat to the dough. A level of about 0·7 %, based on flour mass, is normally required. Since the improving effect of fat is related to the solid fraction, it is essential that the slip point be higher than the final dough temperature. Fat with a slip point of 35 °C will give good results at a dough temperature of 30 °C.
(d) the addition of extra yeast. The higher dough density and absence of a starter brew or long fermentation period necessitates a higher level of compressed yeast to maintain normal proof times. Additions of 1·8–2·2 % yeast are usual, compared with about 1·6 % for bread produced using bulk fermentation.
(e) the addition of extra water, up to 3·5 % based on flour mass. To achieve normal dough consistency, process absorption must be increased. The marked increase in dough density is due to the exclusion of air at the mixing stage and the absence of a long leavening period.
(f) improved bread quality. It is generally accepted that the CBP results in better quality bread than the conventional straight-dough process. Alternatively, it is possible to maintain quality using a lower proportion of strong flour, which, in the UK, allows a better utilisation of the local, softer wheats.

Although this process was favourably received by the British baking industry, there remained a major obstacle to its successful application. No commercial dough mixer would adequately supply the required rate of energy input to the dough. However, testing revealed that the 'Tweedy' mixer, with slight alterations, proved well-suited for this purpose. Today, the numbers of batch machines available for mechanical dough development run into double figures.

The CBP has had a tremendous impact on the baking industry in Britain and in many other countries. About 70 % of the bread now produced in the UK is being made by means of the CBP.

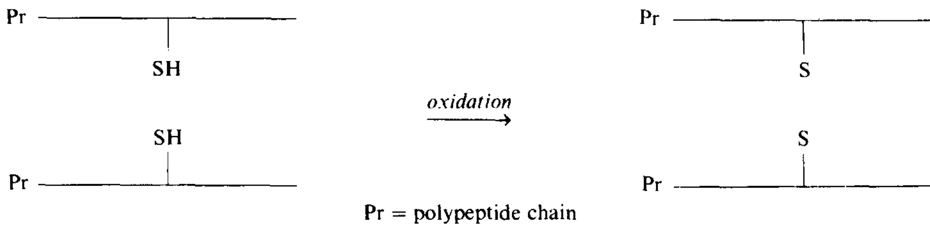
Theory of mechanical dough development: Before discussing the mode of action of mechanical dough development, it is necessary to outline the changes occurring when dough is developed conventionally. Since our knowledge in this area is far from complete, it is not possible to give a precise account of these changes.

At maturity, as mentioned earlier, bread dough has characteristic rheological properties, exhibiting the ability to deform elastically and by viscous flow. In general, viscoelastic polymers consist of long-chain molecules with occasional intermolecular cross links and developed dough is believed to consist of a network of protein molecules, with an inert starch filler, whose rheological properties are dependent on the quantity and strength of the cross links between the proteins.

Although bonds such as salt links, Van der Waals forces, and particularly hydrogen bonds, may play a significant role in determining dough properties, our understanding is restricted mainly to the disulphide bonds, which are important for dough cohesion (De Keken & Mortier, 1955; Wren & Nutt, 1967), forming cross

links between polypeptide chains and which, by undergoing exchange reactions with sulphydryl groups, confer mobility to the dough.

In glutenin, the disulphide bonds are intermolecular, involving extended protein chains, whereas in gliadin they are mainly intramolecular, resulting in a more compact molecule (Walls & Beckwith, 1969). Disulphide linkages are dependent on the amino acid cysteine, which has the ability to form a covalent link between two portions of protein chain, corresponding to the formation of cystine, thus:



Pr = polypeptide chain

Bloksma (1971) has pointed out that not only the disulphide bonds, but also the sulphydryl (thiol) groups are important, for two main reasons; first, certain thiol reagents, which are unable to form disulphide cross links, have similar effects on dough rheological properties as oxidising agents; secondly, although mature dough exhibits some elasticity, its deformation is primarily viscous, which is only possible if the cross links are not permanent.

The above phenomena can be best explained by the process of *disulphide/sulphydryl interchange*. It was suggested by Goldstein (1957), and later demonstrated by McDermott & Pace (1961), that an interchange can occur in dough between free sulphydryl groups and disulphide bonds. The interchange participants must be brought into contact and the disulphide bond should preferably be under some strain. During dough mixing only about 1–2% of the gluten disulphide bonds undergo exchange reactions (Mauritzen, 1967) which suggests that these few reactive sites have a profound effect on dough properties. Rupture of disulphide bonds may occur as a result of mechanical action, the action of added enzymes or those which are naturally present, or the effect of dough constituents which contain active sulphydryl groups (Jackel, 1977).

During mixing, as protein chains are rapidly moved relative to each other, there are many possibilities for exchange reactions to occur. Such reactions are not restricted solely to gluten protein. It is possible for water-soluble proteins to undergo exchange with strained disulphide bonds between glutenin molecules which, although not directly contributing to the building of the network, reduces strain on the glutenin and results in the formation of a free sulphydryl group in the glutenin which may take part in further reactions. If the sulphydryl group in a glutenin chain reacts with a strained disulphide bond in another glutenin chain, then this reduces the strain in the network and, at the same time, contributes to increasing the elastic nature of the dough structure. In summary, therefore, permanent cross links

between gluten polypeptide chains impart elasticity to the dough, while the reactive disulphide bonds which are able to undergo exchange reactions impart mobility to the dough.

Although dough development appears to be dependent on the above exchange reactions, optimum development cannot usually be achieved unless a small amount of oxidising improver is added. The effect of oxidants is normally recognised by the stiffening or strengthening of the dough, and their properties have recently been reviewed by Jackel (1977). Their mode of action is generally considered as that of oxidising sulphydryl groups (Bloksma, 1964; Mecham, 1968), removing a portion of the non-gluten, protein sulphydryls from the system, and increasing the amount of glutenin cross-linkage. There is, however, evidence to suggest that oxidising agents do not improve dough rheological properties solely by reactions involving disulphide bonds and sulphydryl groups (Kovats & Lasztity, 1965), although a precise explanation for their action is still not available.

There are several oxidants available, differing mainly in their reaction rates. Potassium bromate is slow-acting and is more active in the oven whereas azodicarbonamide and potassium iodate, being rapid-acting, function during mixing and proofing. Ascorbic acid is intermediate in rate and, although itself a reducing agent, is converted to dehydroascorbic acid, an oxidising agent, by ascorbic acid oxidase present in the flour. In practice, a combination of oxidants is commonly employed. Care has to be taken that a dough is not over or under-oxidised, the latter condition giving rise to a soft, sticky dough, while over-oxidation may cause excessive cross-linkage to occur, giving a stiff, dry dough.

During mechanical development of dough, the objective is to produce dough with characteristics similar to those developed during bulk fermentation. In order to accomplish this in a short time, a combination of intense mechanical work and a high level of oxidant is essential. The high energy input results in more advanced gluten development than in conventional mixing, so there is less need for further development by fermentation and punching. After the initial rapid flour hydration, fairly drastic modification of the gluten proteins, particularly glutenin, must occur. It would seem logical to assume that the intense shearing action causes cleavage of disulphide bonds with a reorientation of protein chains, bringing new reactive sites together very quickly. It seems likely, however (Williams, 1975), that a more important mechanism is the exposure of 'masked' or 'unreactive' sulphydryl groups, present in the glutenin, which are not immediately available for reaction with an oxidant in a conventional dough (Lee & Lai, 1969). Without the presence of a high level of oxidant, however, the optimum dough structure is not formed, since the most mobile sulphydryl groups are contained in the water-soluble non-gluten protein fraction and which will preferably react with any strained disulphide bonds. According to Williams (1975) the main effect of the oxidants used in the CBP is to oxidise the non-gluten sulphydryl groups and, effectively, remove them from the

system. The result would be a shift of the reaction balance towards the formation of inter-glutenin disulphide bonds, producing a more coherent and elastic structure.

In the CBP, iodate and bromate can give particularly undesirable results if used slightly in excess of optimum, resulting in a large volume loaf with a weak crumb, coarse texture and a tendency to form holes at the moulding line. The use of excess ascorbic acid is much less critical. It is thought that excess bromate or iodate may cause direct oxidation of glutenin sulphydryl groups, which can then take no further part in exchange reactions, whereas the more bulky dehydroascorbic acid molecule is not able to gain access to many of the sulphydryl groups in the glutenin chain (Williams, 1975).

Other dough development processes

There are several other methods of dough development available, some of which are still in the experimental stage, and others which have not found widespread commercial application. Brief descriptions of a selection of these are given below.

One process of bringing about rapid dough development, without the need for intense mechanical work, is called *activated dough development* (ADD) or *chemical development*. An essential feature of this process is the addition of a reducing agent, usually L-cysteine hydrochloride (at a level of about 35–40 ppm on a flour mass basis), although sodium metabisulphite is also effective (Collins, 1972). Dough maturity is completed by a relatively high level of oxidising improver.

Presumably, the cysteine ruptures disulphide bonds, encouraging unfolding of the gluten protein during mixing, and promotes disulphide/sulphydryl interchange. As a result, the dough can be developed with a relatively low input of mechanical energy. However, cysteine only activates the sequence of interchange reactions, addition of an oxidant being necessary to oxidise excess sulphydryl groups after development is complete. In order for the cysteine to carry out its function before either itself or the dough is oxidised, the oxidant employed must be relatively slow-acting. Potassium bromate alone or in a blend with ascorbic acid is generally satisfactory, at levels of 40 ppm and 50 ppm on a flour mass basis, respectively (Collins, 1972). Since the presence of cysteine greatly reduces the energy requirements for mechanical development, it is possible to produce good quality bread using conventional mixers without the need for bulk fermentation.

Bulk fermentation is also eliminated in the *Blanchard Batter Process*, developed in 1963 (Blanchard, 1965), although the principle is rather different. Gluten development is achieved through a two-stage mixing process, based on an initial batter stage. The batter is composed of three-quarters of the total flour, all the water, yeast and oxidant, which is mixed to hydrate and develop the gluten. The remaining ingredients are subsequently blended into the developed batter. In this method, gluten development can be achieved with a relatively low energy input, requiring only minor modification of conventional mixing equipment (Blanchard, 1966).

Since less frictional heat is developed during mixing, lower dough temperatures can be used than in the CBP. Oxidant requirements are also much lower. This process is used to a limited extent in the UK.

Research in Australia has led to the introduction of a no-time system designed for small bakeries (Marston & Bond, 1966) and a mechanical development process, similar to the CBP, called the *Brimec process* (Marston, 1967). The Brimec process is essentially a batch method, designed to produce two distinctly different types of bread, a 'texturised' product, having a fine, even crumb structure, similar to bread made on a continuous basis, and an 'aerated' product, with a more open crumb structure, resembling the conventional loaf. The main features of the process are the requirements of fat and a moderate oxidant addition, and that the dough is developed in a specially designed 'Brimec' mixer to an optimum energy input as indicated by a recording Watt meter.

In the UK, several attempts at designing continuous breadmaking plants, including a Baker Perkins continuous sponge and dough plant and the Simon–Strahman plant, were unsuccessful, although the Oakes continuous mixer/modifier achieved some commercial popularity (Williams, 1975). The sponge and dough plant produced a good loaf, without the poor textural characteristics of bread produced by other continuous methods, although the high cost of plant, labour and maintenance and the low yield offset this advantage. The Oakes machine extrudes dough in a continuous ribbon which is then handled on conventional equipment. The above processes, apart from other considerations, mainly owe their failure to the success of the Chorleywood Bread Process.

Other types of continuous breadmaking plants operate elsewhere in Europe (Pomeranz & Shellenberger, 1971), including a Russian process for rye breads, the Iverson process in Sweden for traditional baked goods and, in Germany, the Strahman process for coarse bread types.

Bread possessing satisfactory physical attributes has been produced on an experimental scale with the omission of yeast from the formula (Fowler & Priestley, 1977). A CBP process was used in conjunction with glucono delta-lactone as a leavening agent. Thus, the function of yeast in promoting gluten development was replaced by mechanical development, and its biological leavening action was substituted by a chemical system. As would be expected, however, the bread had a rather bland flavour.

FUTURE TRENDS IN BREADMAKING

It is not possible to accurately predict the changes which will occur in breadmaking technology over the next 10, 20 or 50 years, although the fields of current intensive research efforts and present economic and social conditions can provide some indication of the areas in which changes are likely to take place.

Future developments in baking technology will probably involve refinements of existing breadmaking procedures. Traditionally the consumer, the ultimate judge of bread quality, has come to recognise good bread by certain physical attributes. Any change in manufacturing procedure, raw material specification or formula balance must therefore not alter the final product significantly. The Do-Maker process serves well as an illustration. Its introduction was acknowledged as a significant achievement both in cereal chemistry and baking technology. However, its success was limited to few countries on account of the lack of acceptance of the end-product by the consumer. Thus, to a large extent bread type dictates manufacturing practice.

In the past, considerable interest has been devoted to the development of bread made from composite flours containing cheaper starchy materials, particularly those indigenous to non-wheat producing areas. In spite of the volume of research carried out there has been a notable lack of success, which can be traced mainly to the unwillingness of the consumer to accept bread possessing unfamiliar characteristics.

Despite the resistance of the majority of consumers to changes in bread type, there appears to be a steady growing interest in speciality items such as reduced calorie, high fibre and whole-wheat bread. However, this is probably more a reflection of changing dietary attitudes rather than changes of taste.

Breadmaking, originally a craft, at present utilises a number of scientific disciplines which contribute towards maintaining consistent bread quality. The baking industry recognises the importance of technology and is aware that a sound scientific basis is essential for future progress. Past successes in baking technology have mainly been achieved through raw material manipulation and formulation, the use of optional ingredients, particularly bread improvers, and process alterations aimed at reducing process time and increasing production throughput. Future major developments in raw material processing and baking techniques are expected to be limited, however. The main concentration of effort is likely to be directed towards attaining reductions in process time or improving production capacity. The use of microwaves shows some promise in this area but has yet to prove its value in commercial applications. In view of the high energy input in dough development, processing and baking, it is certain that efficient use of energy will become much more critical as fuel availability becomes limited while its cost escalates considerably.

The introduction of highly automated, large-volume bread plants has emphasised the need for greater process and raw material control. One of the major obstacles to achieving good control is the use of yeast as a leavening agent and source of flavour. Together with mechanical dough modification systems, there may be a future for alternative chemical leavening systems, although public acceptance is likely to be a major problem.

Bread consumption has often been used as a yardstick to indicate affluence. In developing countries bread is a luxury item and prosperity leads to an increase in

consumption. In the West, however, increasing affluence tends to lead to a drop in bread consumption. South Africa has a rather unique position in that the baking industry enjoys an expanding market of around 5 % per annum, largely attributed to a change in eating habits of the black population. Whatever the consumption trend, however, continued price rises will lead to greater quality awareness on the part of the consumer, which will create the need for even greater control of quality.

Accompanying a decline in bread consumption and a desire by the consumer to reduce wastage, will be the demand for bread which will maintain its 'fresh' condition for a longer period. A breakthrough in overcoming the bread staling problem would have an enormous impact in such a situation. Although progress is being made, there is little hope that a solution will be found in the immediate future.

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EFFECT OF FERMENTATION ON THE NUTRIENT STATUS OF LOCUST BEANS

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ABSTRACT

Both fermented and unfermented locust beans were analysed for proximate composition (carbohydrate, moisture, lipid, crude protein and ash) as well as for content of amino acids and mineral elements. There were changes in the nutrient contents. The amount of protein was increased by fermentation as was the amount of fat. The amount of carbohydrate was greatly decreased by fermentation. The content of essential amino acids was also influenced by fermentation. The importance of fermented food in the traditional diets of people in Africa is discussed.

INTRODUCTION

The African locust bean plant (*Parkia filicoidea*. Welw.) is widely distributed in the northern parts of Nigeria. It bears fruits in the form of bunches of pods containing yellow powdery pulp with dark brown to black seeds. The sweet pulp is usually made into a traditional carbohydrate food known as *dorowa* by the Hausas. This food is often supplemented with cereal grains in meat stew or soup. The seeds are usually boiled and fermented and then pounded to give a popular traditional food known by the Hausas as *daddawa*.

Oyenuga (1968) discussed the value of the African locust beans as stock feed. All parts of the fruit, including the pod, the mealy pulpy material and seeds are claimed to constitute valuable cattle food.

Several Nigerian foods and also West African foods are fermented at some stage in their preparation. The African locust bean seeds are normally fermented before they are used either as food or as flavouring agents. The main aim of fermentation is to enhance the flavour and the keeping quality. During fermentation, changes both

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desirable and undesirable may occur in the food material. There are only a few scanty reports on the effect of fermentation on nutritive value of traditional foods in Nigeria (Oke, 1965; Akinrele, 1970; Eka & Edijala, 1972; Okoh & Eka, 1978; Eka, 1978).

The present series of investigations were carried out to observe the changes in the nutrients as a result of fermentation of African locust beans.

MATERIALS AND METHODS

The locust beans used were purchased from the local markets at Zaria. Fermentation of the beans to produce fermented meal (*daddawa*) was carried out using the traditional methods described by Simons (1976).

In essence, the procedure involves washing the locust bean seeds thrice with water and cooking with added potash until slightly soft. The cooked seeds are then left overnight in a basket. On the following day, ashes are added to the cooked seeds and the mixture pounded until the husks are removed. The edible portion is again emptied into a basket and the yellow portion of the fruit pulp sprinkled over it; leaves are then used to cover the basket. The preparation is left for 48 h at room temperature to ferment. After the fermentation, the fermented bean is dried in the sun for 24 h. The semi-dry material is pounded in a mortar into a pulpy mass and flattened into cakes known as *daddawa*. The cakes are dried again in the sun since they tend to keep for a long time when dry. The *daddawa* is usually hawked around and also sold at the local markets.

ANALYSIS OF THE SAMPLES

The methods of treatment of samples and analyses were similar to those recommended by the Association of Official Analytical Chemists (AOAC, 1970) and by Joslyn (1970). The cooked edible portion of the locust bean seed and the fermented beans were analysed for proximate composition, mineral elements and amino acids. The chemical analyses, except for moisture determination, were carried out on oven-dried samples. In all cases the material was dried at 60–70 °C to constant weight using a vacuum oven and then ground into powder until it passed through a 40 mesh sieve (AOAC, 1970). The dry material was preserved in the refrigerator for subsequent analysis.

The methods of analysis followed were those described by the AOAC (1970). The ash was determined by incineration of known weights of the samples in a muffle furnace at 550 °C until ash was obtained. The lipid composition was determined by exhaustively extracting a known weight of the sample with petroleum ether (boiling point 40–60 °C) using a Soxhlet apparatus. Protein ($N \times 6{\cdot}25$) was determined by the macro-Kjeldahl method. The carbohydrate content was obtained by the

difference method, that is, by subtracting the total crude protein and crude lipid from the organic matter. Crude fibre was determined by acid and alkaline digestion methods described by Joslyn (1970) and the AOAC (1970).

The elemental composition was also determined using the methods of the AOAC (1970). Sodium and potassium were determined by flame photometric methods: calcium, magnesium, zinc, iron, and copper were determined using absorption spectrophotometer; phosphorus was determined by colorimetric methods using ammonium molybdenate. The amino acid contents of the samples were determined using the automatic amino acid analyser (Beckman 120C).

The vitamins were determined using the methods of the Association of Official Vitamin Chemists (AOVC) (1966). Thiamine was estimated as thiochrome by the fluorimetric method, riboflavine was also estimated fluorimetrically, using a Locarte fluorimeter. Vitamin C (ascorbic acid) was estimated by the N-bromo-succinimide method described by Evered (1960). Oxalate was determined by the method of Dye (1956) and as modified by Oke (1965). Hydrocyanic acid content was determined by the alkaline titration method (AOAC, 1970). Phytic acid was estimated by a photometric method adapted from the methods of McCance & Widdowson (1935), and Briggs (1922).

RESULTS AND DISCUSSION

The results are shown in Tables 1–4. Table 1 shows the proximate composition of the fermented and unfermented samples of locust bean expressed as an average of six

TABLE 1
PROXIMATE COMPOSITION OF FERMENTED AND UNFERMENTED SAMPLES OF LOCUST BEAN IN g/100 g (±SE)

	g/100 g wet weight	*g/100 g dry weight of material*				
Sample	*Moisture*	*Ash*	*Crude protein*	*Petroleum ether extract*	*Total carbohydrate*	*Crude fibre*
Unfermented locust beans	12·66 ± 1·12	5·14 ± 0·75	30·57 ± 1·55	15·20 ± 1·20	49·09 ± 2·25	7·83 ± 0·86
Fermented locust bean cakes	13·75 ± 1·11	6·75 ± 0·95	38·5 ± 1·16	31·20 ± 1·35	23·55 ± 2·13	6·23 ± 0·55

determinations. The moisture contents of both samples were close in value and also low since sun-dried samples were used. There were high values of ash content in both samples and the fermented samples showed slightly higher total ash content. The crude protein was high in both, being comparable to the amount in soya beans. The fermented samples had a higher protein content than the unfermented samples. The

petroleum ether extract of the fermented samples was double that of the unfermented samples. The total carbohydrate was greatly reduced in the fermented samples and was almost 50 % that of the unfermented samples. The crude fibre was slightly higher in the unfermented samples than in the fermented samples. On the whole, the locust bean seed appears to be a good source of nutrients and fermentation seems to improve the nutrient status of the locust bean food. The high fibre content may, however, influence the availability of the nutrients to man and animals.

Table 2 shows the mineral element content of the fermented and unfermented samples of locust bean. The samples were rich in all the elements determined and there was a slight increase in element content in fermented samples. The elements were adequate to meet the recommended daily allowance. For example, the recommended daily allowance for iron is 12 mg/100 g dry matter (Oke, 1972; Lutham, 1969). The actual amount of the mineral elements available to the consumer may depend on various factors such as the limitation of absorption by phytic acid or oxalic acid present in the food.

TABLE 2
MINERAL ELEMENT CONTENT OF FERMENTED AND UNFERMENTED SAMPLES OF LOCUST BEANS IN mg/100 g DRY MATTER (±SE)

Sample	*K*	*Na*	*Zn*	*Mg*	*Ca*	*Cu*	*Fe*	*P*
Unfermented locust beans	250±5·5	240±3·8	15·0±1·2	80·5±3·5	330±4·3	1·5±0·1	22·5±1·4	280±3·5
Fermented locust bean cakes	550±4·8	250±2·0	18·0±1·1	83·5±2·7	360±4·1	2·0±0·1	28·0±1·2	320±2·5

Table 3 shows the amino acid composition of the fermented and unfermented locust bean samples. The samples were rather low in the sulphur-containing amino acids such as cystine and methionine. They were also low in the essential amino acids leucine, isoleucine, phenylalanine and tryptophan. Tryrosine was low and this was also true of histidine. Fermentation resulted in increased yield of most of the amino acids except arginine, leucine and phenylalanine. The results indicate that even though the locust bean seeds are high in crude protein content, the quality of the protein is not very high. The deficiency in some of the essential amino acids and the sulphur-containing amino acids tends to mitigate against classifying the locust bean, fermented and unfermented, as a source of high quality protein.

Table 4 shows the content of vitamins B_1 (thiamine), B_2 (riboflavine) and C (ascorbic acid) in both the fermented and unfermented locust bean samples. The vitamins B_1 and B_2 and C were low in both samples but there were higher amounts of thiamine and riboflavine in the fermented samples. The level of vitamin C (ascorbic acid) was, however, adversely affected by fermentation (Oke, 1967*a*).

TABLE 3
AMINO ACID COMPOSITION OF FERMENTED AND UNFERMENTED SAMPLES OF LOCUST BEAN SEEDS IN mg/100 g SAMPLES (±SE)

Amino acids	*Unfermented locust beans*	*Fermented locust bean cake*
Lysine	4·24±0·55	6·72±0·65
Histidine	1·60±0·02	1·68±0·02
(Ammonia)	(3·20±0·05)	(4·96±0·07)
Arginine	2·84±0·05	2·88±0·03
Tryptophan	0·12±0·01	0·24±0·02
Aspartic acid	8·40±1·10	12·00±1·10
Threonine	3·80±0·65	4·48±0·55
Serine	3·22±0·55	5·62±0·55
Glutamic acid	16·00±2·11	19·60±2·24
Proline	4·24±1·12	6·11±1·15
Glycine	6·84±0·95	9·20±1·17
Alanine	5·60±0·85	7·84±1·15
Cystine (half)	1·20±0·01	2·10±0·45
Methionine	0·12±0·01	0·24±0·01
Isoleucine	0·88±0·03	0·40±0·01
Leucine	1·44±0·04	0·80±0·03
Tyrosine	1·12±0·04	0·80±0·04
Phenylalanine	0·32±0·02	0·16±0·01

TABLE 4
VITAMINS AND SOME TOXIC SUBSTANCES IN FERMENTED AND UNFERMENTED SAMPLES OF LOCUST BEAN SEEDS IN mg/100 g DRY SAMPLES (±SE)

Samples	*Vitamin B_1*	*Vitamin B_2*	*Vitamin C*	*Oxalate as soluble oxalic acid*	*HCN*	*Total phytic acid P*	*Phytic acid P as % of total P*
Unfermented locust beans	0·65±0·05	0·45±0·03	7·50±1·1	0·21±0·01	26×10^{-4}	51·0±1·85	15·0±1·22
Fermented locust bean cakes	1·35±0·08	1·30±0·07	5·20±0·90	0·12±0·12	12×10^{-4}	31·0±2·10	7·5±0·83

Table 4 also shows the levels of oxalic acid, phytic acid and hydrocyanic acid in the two locust bean samples. All these toxic substances were found in reduced amounts in the fermented samples. The hydrocyanic acid levels were below the dangerous levels (Oke, 1967*b*, 1969*a*). The percentage of total phosphorus present as phytic acid phosphorus was 15·0 in the unfermented samples and 7·5 in the fermented samples.

The percentage of soluble oxalate present was 0·21 in the unfermented samples and 0·12 in the fermented samples. The soluble oxalate is known to be the fraction of oxalate that can be toxic to animals but the levels in both samples were rather low (Oke, 1969*b*). Phytic acid and oxalic acid are also known to form insoluble salts with

mineral elements and therefore prevent their utilisation. In this aspect the levels of phytic acid and oxalic acid may call for some consideration.

It appears from the results that the seed of locust bean is high in nutrients, particularly in protein. The amino acid content of the proteins tends to indicate that there are deficiencies of some essential amino acids. Even though these results agree with those of previous workers in certain respects (Oyenuga, 1968) the method of assay of these amino acids might have influenced the results, for example, in the case of the low content of tryptophan and cystine. There is a need for biological studies involving feeding experiments to be able to confirm or disprove the evidence presented by chemical scores.

It is also clear from the results of the experiments that fermentation in most cases resulted in better quality food material both in terms of contents of nutrients and toxic substances. The traditional practice of fermenting the locust beans before consumption should be encouraged and efforts should be made to understand what goes on during the fermentation processes. Studies in all these directions are now in progress.

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PROTEIN SUPPLEMENTATION OF BROAD BEANS WITH SESAME

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ABSTRACT

Feeding experiments with weanling rats indicated that the protein of broad beans (Vicia faba *var. major) and sesame* (Sesamum indicum) *are mutually supplementary. A standard diet in which 75 % of the protein was provided by broad beans and 25 % by sesame had a protein efficiency ratio (PER) of 2·36 compared to a casein PER adjusted to 2·50. A similar diet in which broad beans and sesame contributed equal amounts of protein had a PER of 2·41. The PER of broad beans was 0·99 and that of sesame 1·55.*

INTRODUCTION

Broad beans (*Vicia faba*), also known as fava beans, are very popular in many parts of the world, especially in the Middle East and Mediterranean areas. The protein content of broad beans is high, but this protein is deficient in the sulphur-containing amino acids, methionine and cysteine (Orr & Watt, 1957). On the other hand, sesame (*Sesamum indicum*) is one of the very few plant sources of protein rich in sulphur-containing amino acids (FAO, 1970). Furthermore, sesame protein contains very little lysine, but broad bean protein contains more than twice as much lysine as sesame. A nutritional supplementary effect of these two proteins would be, therefore, expected. The main objective of the present study was to demonstrate the mutually supplementary nature of the proteins of broad beans and sesame by means of a biological experiment.

Previously, Boloorforooshan & Markakis (1979) showed that the proteins of navy beans (*Phaseolus vulgaris*) and sesame supplement each other, and Antunes &

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Markakis (1977) demonstrated the high supplementary value of Brazil nut (*Bartholetia excelsa*) protein for the navy bean protein. Similar protein–protein supplementation experiments involving foods of plant origin have been conducted by a number of other workers (Evans & Bandemer, 1967; Krishnamurthy *et al.*, 1960).

MATERIALS AND METHODS

Preparation of flours

Broad beans (*V. faba* var. major), grown in Iraq, were cooked (a) as it is usually done in the home, and (b) by a quick-cooking method. The home process consisted in soaking the beans for about 12 h and then cooking them in 2·5 % NaCl solution until ready to eat. This cooking lasted approximately 2 h. In the quick-cooking process the beans were first blanched in boiling water for 7 min, then soaked in a solution containing 0·2 % $NaHCO_3$, 0·1 % Na_2CO_3 and 0·1 % Na_3PO_4 for 12 h and then cooked in 2·5 % NaCl solution to organoleptic acceptability. This cooking lasted about 20 min.

The cooked beans were drained, dehulled by hand, dried in a forced-air cabinet maintained at 65 °C and then ground to 60-mesh size.

Decorticated sesame seeds obtained from a local market were first cracked and then extracted three times with cold hexane. Each time, 3 vol. of hexane were mixed with 1 vol. of sesame for 1 h. The defatted meal was placed in a forced-air cabinet at 65 °C for complete removal of the solvent and ground to 60-mesh size.

Chemical analysis

Crude protein (N × 6·25 for broad beans, N × 5·30 for sesame), crude fibre, crude fat, ash and moisture contents of the broad bean and sesame flours were determined according to AOAC (1975) methods. Amino acid analysis was carried out using a Beckman Model 120C Amino Acid Analyser. Cysteine and methionine were determined after prior oxidation with performic acid (Lewis, 1966). Tryptophan was determined colorimetrically after hydrolysis with pronase as described in Procedure W by Spies (1967). Duplicate samples were analysed.

Biological assay of protein quality

Protein efficiency ratios (PER) were determined for diets containing as a sole source of protein (a) flour from home-cooked broad beans; (b) flour from quick-cooked broad beans (B); (c) sesame flour (S); (d) B and S flours in the ratio of 87·5 B protein to 12·5 S protein; (e) B and S flours in the ratio of 75 B protein and 25 S protein; (f) B and S flours in 50:50 ratio of B and S proteins; and (g) casein as a reference. The official AOAC (1975) method for the biological evaluation of protein quality was employed using 10 Sprague–Dawley rats per diet.

Apparent nitrogen digestibility was determined by collecting the faeces of 5 rats from each diet during the last 7 days of the PER experiment and using the formula

$$\% \text{ apparent N digestibility} = \frac{\text{N in food} - \text{N in faeces}}{\text{N in food}} \times 100$$

On the first and the last day of the digestibility test period carmine was added to the diet for marking the faeces.

RESULTS AND DISCUSSION

The proximate composition of the flours tested in this experiment is shown in Table 1. The two broad bean flours did not differ substantially from each other; the higher

TABLE 1
COMPOSITION OF THE SESAME, QUICK-COOKED AND HOME-COOKED BROAD BEAN FLOURS USED IN THIS STUDY

%	*Sesame flour*	*Quick-cooked broad bean flour*	*Home-cooked broad bean flour*
Moisture	4·0	8·5	8·7
Crude protein	31·4	27·0	26·4
Crude fat	14·6	2·0	2·1
Crude fibre	3·4	2·9	2·8
Ash	3·7	3·8	4·7
Carbohydrates (by difference)	42·9	55·8	55·3

ash content in the home-cooked beans is probably due to uptake of salts during soaking. A considerable amount of oil was left in the sesame flour (originally the seeds contained 54% crude fat), but this did not interfere with the balancing of the diets.

The amino acid composition of the sesame flour and uncooked broad bean flour is shown in Table 2. A comparison of these data with those of the FAO (1973) pattern shows that broad beans are deficient in sulphur-containing amino acids, but they contain a high level of lysine. On the contrary, sesame is rather rich in sulphur-containing amino acids but deficient in lysine. A mutual protein supplementation effect between these two commodities would be expected. Indeed, Table 3 shows that while broad beans and sesame separately are sources of poor quality protein, mixtures of these two commodities contain protein of very high PER. In fact, a diet in which these commodities contributed equal amounts of protein, and a diet in which $\frac{3}{4}$ of the protein originated from broad beans and $\frac{1}{4}$ from sesame, had PER values statistically equal to the PER of casein. Mixing of these two commodities also improved the N digestibility of the diets.

TABLE 2
AMINO ACID COMPOSITION OF SESAME AND BROAD BEANS, ALONG WITH THE FAO PROVISIONAL AMINO ACID SCORING PATTERN (g/16 g N)

Amino acid	*Broad bean*	*Sesame**	*FAO (1973) pattern*
Isoleucine	4·2	3·0	4·0
Leucine	7·7	5·4	7·0
Lysine	7·3	2·3	5·5
Methionine	0·7	3·1	3·5 (Methionine + Cysteine)
Cysteine	1·3	1·6	
Phenylalanine	4·5	3·9	6·0 (Phenylalanine + Tyrosine)
Tyrosine	3·6	2·9	
Threonine	3·9	3·0	4·0
Tryptophan	1·0	1·5	1·0
Valine	5·8	3·1	5·0
Histidine	2·8	2·2	—
Arginine	12·9	11·6	—
Aspartic acid	11·6	6·7	—
Serine	4·9	3·6	—
Glutamic acid	15·7	17·1	—
Proline	4·0	3·0	—
Glycine	3·9	3·6	—
Alanine	4·3	3·5	—

* The sesame data are taken from Boloorforooshan & Markakis (1979). The same shipment of sesame was used in both studies.

TABLE 3
PROTEIN EFFICIENCY RATIOS (ADJUSTED TO CASEIN PER = 2·50), AND %N DIGESTIBILITY OF DIETS INVOLVING SESAME, BROAD BEANS AND MIXTURES OF BOTH

Diet	*PER ($\bar{X} \pm SEM$)*	*%N Digestibility*
Casein	$2·50 \pm 0·09^a$	$91·4 \pm 0·3^a$
Quick-cooked broad beans (B)	$0·94 \pm 0·03^b$	$82·1 \pm 0·9^b$
Home-cooked broad beans	$0·99 \pm 0·06^b$	$82·4 \pm 1·0^b$
Sesame (S)	$1·55 \pm 0·02^c$	$87·4 \pm 0·7^c$
B + S (50:50)	$2·41 \pm 0·05^a$	$86·7 \pm 0·4^c$
B + S (75:25)	$2·36 \pm 0·07^a$	$86·6 \pm 0·3^c$
B + S (87·5:12·5)	$1·86 \pm 0·04^d$	$84·5 \pm 0·3^c$

Identical letter superscripts indicate differences among means which are not significant at $p = 0·05$.

When the amino acid scores of these diets were calculated according to the FAO (1973) method and using the amino acid analysis of the raw broad beans rather than the cooked ones, the values were: 57 for broad beans (B); 42 for sesame (S); 87 for B:S at 50:50 protein ratio; 76 for B:S at 75:25 protein ratio; and 67 for B:S at 87·5:12·5 protein ratio. The limiting amino acid in the sesame and the 50:50 sesame:broad bean diet was lysine. In the other diets, methionine plus cysteine were the limiting amino acids.

While both the chemical scores and the PER values clearly reflect the mutual nutritional improvement of the protein of broad beans and sesame, the chemical scores of these two commodities, taken separately, are in reverse order to the PER

values. If the sulphur-containing amino acids of broad beans are as poorly utilised by the rat as those of white beans (*Phaseolus vulgaris*), then the low PER values for broad beans are understandable. Evans *et al.* (1974) found that approximately 50 % of the methionine and 75 % of the cystine of white beans are available to the growing rat.

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THE NUTRITIONAL VALUE OF TWO CEREAL-BASED FOODS IN NIGERIA

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ABSTRACT

Two cereal-based foods fortified with soybean flour, vitamins and minerals were evaluated for their nutrient composition and protein quality. Results presented suggest that both 'Soy-ogi' and 'Belona' contain no trypsin inhibitors or other growth-inhibiting substances. Both have adequate amounts of the essential amino acids, high protein content and in vitro *digestibility values of 78 % and 80 %, respectively. Soy-ogi and Belona have protein efficiency ratio (PER) values of 1·35 and 2·54, respectively, assuming respective digestibility values of 78 % and 80·8 % for the samples and 93 % for casein.*

INTRODUCTION

Cereal foods provide a major portion of the protein and calories in human diets in many developing countries. In these countries, animal sources contribute about 10 % and cereals 70 % of proteins consumed. Maize contains, on average, 7–12 % protein while soybean contains about 40 % protein.

Traditional foods are nutritionally inferior since they are made from maize. It is now known that poor quality protein is not the principal cause of malnutrition. It is possible to correct the imbalance in a particular essential amino acid, vitamin or mineral, thereby upgrading traditional diets to a level at which they provide suitable means for meeting protein/calorie needs (HMSO, 1975).

The traditional weaning food in some parts of West Africa consists mainly of a maize preparation called 'Ogi' in Nigeria, and 'Akasa' in Ghana. Akinrele *et al.*

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(1970) have described the traditional method of Ogi preparation. The biological quality of Ogi is so poor that it does not support growth in rats. However, when Ogi is supplemented with heated full-fat soyflour, the protein efficiency of rats increases threefold (Akinrele, 1966).

Recently, with the aim of improving the nutritional quality of traditional weaning foods, two commercial cereal-based products have been marketed in Nigeria. The first, 'Soy-ogi' is a corn–soyflour mixture developed by the Federal Institute of Industrial Research in Nigeria. Akinrele & Edwards (1971) reported that Soy-ogi compares favourably with some commercial infant foods. Luse & Okwuraiwe (1975) have shown that Soy-ogi contains three times the protein, sixteen times the lysine and about three times the sulphur amino acids of normal Ogi.

Soy-ogi is made up of 70 parts heated full-fat soyflour and 30 parts Ogi. The composition and preparation of Soy-ogi have been previously described by Akinrele & Edwards (1971).

The second cereal-based product is 'Belona', developed by the ARS–USDA, a special committee for world food needs set up by the Millers' National Federation and Protein Cereal Products International. It is specially processed for, and packaged in, Nigeria by Industrial and Commercial Resources Limited. The promoters of this product in Nigeria claim that Belona is made up of 73·35 % wheat-based products and wheat protein concentrate, 20 % defatted soyflour, 4 % refined soybean oil and 2·65 % vitamins, minerals and iodised salt and has a PER value of 2·4.

The purpose of this study was to evaluate the nutritional characteristics of these commercial products and their effectiveness as weaning foods in Nigeria.

MATERIALS AND METHODS

Samples of Soy-ogi were obtained from the Federal Institute of Industrial Research, Oshodi, Nigeria. Samples of Belona were supplied by Industrial and Commercial Resources Limited, Nigeria. Casein was purchased from the Nutritional Biochemicals Corporation.

Chemical analysis

The two cereal-based foods, Soy-ogi and Belona, were analysed for their proximate chemical constituents following the methods of the Association of Official Agricultural Chemists (AOAC, 1970).

Air-dried samples for lipid extraction were ground in a Waring blender and placed in Whatman cellulose extraction thimbles (80 mm × 20 mm). The meals were extracted in a Goldsfisch apparatus with refluxing petroleum ether for 4 h. The ether was evaporated and the residues dried under the hood overnight.

Wet digested samples were used for determining individual mineral contents using

the Perkin-Elmer atomic absorption spectrophotometer. Phosphorus was determined from aliquots of the mineral solution by the method of Fiske & Subbarow (1925) on a Beckman model DU spectrophotometer. Tryptophan was determined using an alkaline solution of $Ba(OH)_2$ following the method of Pataki (1968). Cystine and methionine were determined as cysteic acid and methionone sulphone by a performic acid oxidation followed by an acid hydrolysis (Moore, 1963).

All hydrolysates, after a 6N HCl, 24-h vacuum hydrolysis at 110 °C for all amino acids except tryptophan, were analysed on a Beckman-120C amino acid analyser (Beckman Instruments, 1973). The data were computed automatically (Cavins & Friedman, 1968).

Determination of Trypsin Inhibitor Activity (TIA)

The trypsin inhibitor activity of soybean, Soy-ogi and Belona was assayed using the method of Kakade *et al.* (1974). One trypsin unit (TU) is defined as an increase of 0·01 absorbance units at 410 nm/10 ml of reaction mixture under experimental conditions. Trypsin inhibitor activity is expressed in terms of trypsin units inhibited (TIU).

In vitro *protein digestibility*

A fast but reliable multi-enzyme automatic recording technique for *in vitro* protein digestibility developed by Hsu *et al.* (1977) was used.

The multi-enzyme solution was made up of trypsin (Type IX) with 14,190 BAEE units per milligramme of protein; bovine pancreatic chymotrypsin (Type II) with 60 units per milligramme of powder and porcine intestinal peptidase (Grade III) with 40 units per milligramme of powder. The enzymes were purchased from the Sigma Chemical Company, St Louis, Missouri.

A protein suspension of the samples was made with glass distilled water. Five millilitres of the multi-enzyme solution were added to the protein suspension previously adjusted to pH 8 and maintained at a constant temperature of 37 °C in a water bath for 30 min. The drop in pH on addition of the multi-enzyme solution was monitored every 2 min over a 10-min period using a recording pH meter.

Computed–PER (C–PER)

The computed PER (C–PER) was determined using the model of Kendrick *et al.* (1976). This model provides estimates of protein quality based on the amino acid profiles and *in vitro* digestibilities of different protein sources.

RESULTS AND DISCUSSION

The results presented in Table 1 show the percentage composition of Ogi from normal corn, supplemented ogi (Soy-ogi and corn–soya) and infant foods marketed

TABLE 1
PERCENTAGE COMPOSITION OF CORN-OGI, SOY-OGI AND BELONA COMPARED WITH SOME COMMERCIAL PROTEIN FOODS

	Corn-ogi	*Soy-ogi*	*Belona*	*Corn-soya*[a]	*Farlene*	*Superamine*[b]
Moisture	49·12	8·77	16·59	9·50	4·00	7·3
Crude protein	3·44	22·12	22·00	20·90	25·00	20·2
Fat	1·19	8·42	5·36	1·20	5·50	3·5
Crude fibre	0·43	2·14	1·07	—	—	1·2
Ash	0·14	3·19	4·48	2·10	4·00	2·9
Carbohydrates (by difference)	45·68	55·36	50·50	61·00	60·50	51·2
Calcium		0·48	0·58	0·40	0·78	0·35
Phosphorus		0·45	0·56	0·44	—	0·40
Calories	107·19	385·70	338·24	358·00	390·00	317·00

Sources: [a] Akinrele & Edwards (1971).
[b] Kapsiotis (1969).

in a number of developing countries. Farlene and Superamine are commercially produced cereal-based infant foods. When maize is processed into Ogi, there is a significant loss in protein, fat, crude fibre, ash and carbohydrates. In Table 1 carbohydrate is calculated by difference. The value obtained for carbohydrates is much lower than that obtained by Banigo & Muller (1972). The proximate composition reported by these authors and their method of calculating carbohydrate by difference does not appear to be in line with approved AOAC methods (AOAC, 1970), hence the difference in results.

While the protein contents of Soy-ogi and Belona are shown to be almost identical (except for Farlene), there is a marked difference in the fat and fibre levels and in carbohydrate content. The Soy-ogi formula provides more calories than Belona and Soy-ogi in this context is similar to Farlene.

When maize is supplemented with soybean, there is an increase in calorific value. Corn-ogi supplemented with legumes, as in Soy-ogi or corn–soya, shows an improvement in the major nutrients as well as the calorific value. In this study, the comparison between Soy-ogi and Belona was carried out on the products as marketed and not as consumed. Unpublished results by the author have shown that when corn-based weaning foods are processed into consumable foods there is some loss in nutrients. This confirms earlier results of other authors (Oke, 1967; Banigo & Muller, 1972). Legumes, generally, are very low in the sulphur-containing amino acids while their content in lysine is about twice that of cereals. Most proteins respond well to amino acid supplementation when fed at low levels. It is well accepted that maize proteins are deficient in lysine and tryptophan. Soybean proteins are adequate in lysine but deficient in lysine and tryptophan. Soybean proteins are adequate in lysine but deficient in methionine which is adequate in maize proteins. The complementary effect of combining two or more protein sources —for example, maize and soybean or wheat flour and soybean—results in an amino

acid pattern with a higher protein level and a higher net protein utilisation (NPU).

Table 2 shows the amino acid composition of Soy-ogi and Belona compared with some other infant foods and protein sources.

The iysine level of Soy-ogi used in this study is well below that obtained and reported on earlier by Akinrele & Edwards (1971). This difference could be attributed to type of sample, age of sample, processing conditions and other undetermined factors. Belona, on the other hand, has a lysine content similar to that

TABLE 2
AMINO ACID COMPOSITION OF SOY-OGI AND BELONA COMPARED WITH OTHER PROTEIN SOURCES (g/100 g PROTEIN)

Amino acid	*Soy-ogi*	*Belona*	*Lactogen*[a]	*Whole egg*[b]	*FAO pattern*
Aspartic acid	9.11	6·84	—	1·52	—
Threonine	3·62	3·28	7·15	1·60	2·8
Serine	4·41	4·17	9·14	2·07	—
Glutamic acid	19·27	23·36	—	3·70	—
Porline	5·92	7·09	—	7·96	—
Glycine	4·19	4·16	—	6·01	—
Alanine	5·15	4·01	—	3·81	—
Valine	5·09	4·69	10·64	2·60	4·2
Cystine + methionine	1·60	2·80	5·18	3·62	4·2
Isoleucine	1·47	4·11	9·89	3·93	4·2
Leucine	4·65	7·10	15·21	5·51	4·8
Tyrosine + phenylalanine	14·83	7·27	7·52	7·20	2·8
Lysine	3·92	4·40	12·05	4·36	4·2
Histidine	2·24	2·41	4·08	—	—
Arginine	6·16	5·99	—	3·20	—
Tryptophan	1·51	1·14	2·18	6·18	1·4

Sources: [a] Akinrele & Edwards (1971).
[b] FAO/WHO/UNICEF (1970).

of whole egg (4·36) and the FAO suggested amino acid pattern (4·20). The sulphur amino acid level is higher in Belona than in Soy-ogi. This higher methionine level could be due not only to the complementary effects of combining both protein sources (cereals and legumes) but also to the faster rate of liberation of methionine during heat treatment occurring in processing. This effect has been adequately demonstrated by Melnick *et al.* (1946) who showed that heat treatment increased the rate of liberation of methionine to a relatively greater degree than either leucine or lysine.

Compared with whole egg and the FAO amino acid pattern, both Soy-ogi and Belona have adequate amounts of threonine, serine, leucine, the aromatic amino acids and lysine. It is concluded from this that both Soy-ogi and Belona have nutrient profiles which are similar to that of whole egg and which agree well with the FAO suggested amino acid pattern.

Table 3 shows the essential amino acid (EAA) pattern of Soy-ogi, Belona and

TABLE 3

THE ESSENTIAL AMINO ACID CONTENT OF SOY-OGI, BELONA AND CASEIN AS A PERCENTAGE OF THE FAO PATTERN

Protein source	*Essential amino acid (g/100 g of protein)*							
	Lysine	*Methionine + cystine*	*Threonine*	*Isoleucine*	*Leucine*	*Valine*	*Tyrosine + phenylalanine*	*Tryptophan*
Soy-ogi	3·92(56)[a]	1·61(35)	3·62(70)	1·47(28)	4·65(51)	5·09(79)	14·83(188)	1·51(121)
Belona	4·40(65)	2·80(64)	3·28(66)	4·11(83)	7·10(82)	4·69(76)	7·27(97)	1·14(96)
Casein	5·72(95)	4·01(102)	3·84(86)	6·59(148)	10·07(127)	5·62(102)	14·61(216)	1·43(134)

[a] Values in parentheses represent percentage of the FAO pattern adjusted for digestibility.

casein as a percentage of the FAO pattern. The values shown in parentheses in this table represent the percentage of FAO values adjusted for the *in vitro* digestibility.

The closest to casein in terms of these percentages is Belona. The deficient amino acids in maize and legumes, when taken singly, are lysine, methionine and tryptophan. A combination of these protein sources as occurs in Soy-ogi and Belona shows that Belona is a better protein source than Soy-ogi.

To further compare the protein quality of these two cereal-based infant foods and relate them to casein, Table 4 presents comparative values obtained for all these protein sources. Except for the total essential amino acids (EAA), Belona appears to

TABLE 4
COMPARATIVE PROTEIN QUALITY OF SOY-OGI, BELONA, SOYBEAN AND CASEIN

Protein quality parameters	*Soy-ogi*	*Belona*	*Raw soybean*	*Heated soybean*	*Casein*
% Protein	22·12	22·00	39·00	38·10	90·60
% *in vitro* digestibility	77·22	80·84	70·52	81·65	90·54
% lysine	3·92	4·40	4·09	3·83	5·72
% *S*-amino acids	1·60	2·80	4·20	5·12	4·00
% Total EAA	36·69	34·79	37·49	36·77	51·88
P-PER[a]	1·35	2·54	2·29	—	2·66
C-PER[b]	0·51	2·11	1·86	—	2·20
Trypsin activity*	10·40	9·40	88·40	11·5	—

[a] Predicted PER obtained from the computer assuming a digestibility of 77·22%.
[b] Corrected PER obtained from the computer assuming a digestibility of 80·84%.
* Trypsin activity calculated as TIU per milligramme of sample where TIU = trypsin inhibitor units.

be superior to Soy-ogi in terms of the protein quality parameters studied. Belona is more easily digested than Soy-ogi and has higher PER values which are similar to those of casein. Both products have low trypsin activity. This is important since sufficient heat applied during the processing of Belona and Soy-ogi not only destroys the trypsin inhibitor in the legume but also modifies the protein in such a way that it is exposed to attack by proteolytic enzymes. Raw soybean meal does not support optimum growth. This is due to the presence of trypsin inhibitors. The data in Table 4 show the trypsin activity in the raw soybean to be 88·4 trypsin inhibitor units per milligramme of sample (TIU/mg sample) as compared with 11·5 TIU/mg for heated soybean and 10·4 and 9·4 TIU/mg for Soy-ogi and Belona, respectively. Although there is this problem caused by the presence of trypsin inhibitors, the effect of adequate heat treatment is an elimination of these anti-growth factors, leading to an improved biological value.

Feeding tests carried out elsewhere (Akinrele & Edwards, 1971) indicated that rats fed Soy-ogi had an optimum growth rate, normal litter sizes and normal livers. The results presented in Table 4 show that the trypsin activity was low for both Soy-ogi

and Belona, although not completely absent. Although nutrient losses occur in the preparation of Corn-ogi (Oke, 1967; Banigo & Muller, 1972), such losses would be minimal in the case of Soy-ogi and Belona. The limiting amino acid in Ogi has been reported by Makinde & Lachance (1976) to be tryptophan. Since both cereals and legumes have complementary amino acid effects, the supplementation of one with the other would result in an improved amino acid profile and higher protein content. Based on results presented here, Belona seems to be a better formula for infant feeding.

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BOOK REVIEWS

Food Processing and Nutrition. Edited by Arnold E. Bender, Academic Press Inc., London. 1979. Price: £7·80 (US$15·25).

In the opening paragraph the author dispels the widely held belief that mother's cooking is always best and points out, quite rightly, that mass-produced food can be made with a minimum loss of nutritional value. The difficulties encountered in measuring losses of nutrients by processing are discussed and the seemingly forgotten (by many people) benefits of food processing are described.

Section B details the effects of processing on the nutrients in foods, dealing with each nutrient (fat, protein, carbohydrate, etc.) separately and in conjunction with other nutrients in foods.

The third section deals with the effects of individual processes on the nutritional values of foods and individual nutrients found in foods. Conventional processing methods are described—e.g. canning and drying—and also the less well known methods such as microwave heating and ionising radiations.

Effects of processing on selected commodities are dealt with in Section D and the effects on many common foods are documented. Finally, food enrichment is discussed both from a manufacturing and a legal viewpoint. At the end of the main text are almost 50 pages of references from the main text which are given according to the subject or commodity to which they refer. For example, all references throughout the book concerning vitamin C are brought together under that heading. This gives rise to such anomalies as the first reference in the book being number 677.

The book is well written and invaluable to anyone requiring a knowledge of food chemistry, either as a student or in industry. At the reasonable price of £7·80 it should be within the means of any prospective buyer.

MALCOLM W. KEARSLEY

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Dietary Fibers: Chemistry and Nutrition. Edited by George E. Inglett and S. Ingemar Falkenhag. Academic Press, New York, San Francisco, London. 1979. xiii + 285 pp. Price: £11·20.

This new book in the Academic Press Rapid Reproduction Series is the proceedings of a symposium on Dietary Fibres: Chemistry and Nutrition, held at the American Chemical Society meeting, Miami Beach, Florida, from 11th to 15th September, 1978. The symposium has some international character because, although the majority of the contributors are from the USA or Canada some are from Great Britain, Germany and Sweden. Chapters include 'Interaction of Small Molecules with Hydrogenated Polymer Networks' (W. Brown), 'Lignin Distribution by Energy Dispersive X-Ray Analysis' (S. Saka, R. J. Thomas and J. S. Gratzl), 'Interaction of Pectinaceous Dietary Fibre with Some Metals and Lipids' (I. Furda), 'Dietary Fibre–Bile Acid Interactions' (J. A. Story, D. Kritchevsky and M. A. Eastwood), 'Differential Absorption of Bile Acids by Lignins' (R. M. Kay, S. M. Strasberg, C. N. Petrunka and M. Wayman), 'Food Fibre Analysis: Advances in Methodology' (D. Baker, K. H. Norris and B. W. Li), 'Dietary Fibre Evaluation of Wheat Products by *In Vitro* and *In Vivo* Methods' (R. M. Saunders and E. Hautala), 'Chemical and Physical Characteristics of Dietary Cereal Fibre' (V. F. Rasper), 'The Nutritional Significance of Chemically Defined Dietary Fibres' (G. C. Fahey, Jr.), 'Effects of Dietary Fibre and Protein Level on Mineral Element Metabolism' (H. H. Sandstead, L. M. Klevay, R. A. Jacob, J. M. Munox, G. M. Logan, Jr., S. J. Reck, F. R. Dintzis, G. E. Inglett and W. C. Shuey), 'Some Effects of Baking and Human Gastro-intestinal Action upon a Hard Red Wheat Bran' (F. R. Dintzis, J. B. McBrien, F. L. Baker, G. E. Inglett, R. A. Jacob, J. M. Munoz, L. M. Klevay, H. H. Sandstead and W. C. Shuey), 'Effect of Dietary Fibre on Parameters of Glucose Tolerance in Humans' (S. Reiser), 'Digestion of Plant Cell Wall Polysaccharides by Bacteria from the Human Colon' (A. Salyer, J. K. Palmer and J. Balascio), '*In Vitro* Binding of Bile Acids by Rice Hemicelluloses' (F. L. Normand, R. L. Ory and R. R. Mod), 'The Chemistry, Morphology and Analysis of Dietary Fibre Components' (O. Theander and P. Aman), 'European Efforts in Dietary Fibre Characterisation' (O. Theander and P. James), 'The Interactions of Carcinogens and Co-Carcinogens with Lignin and Other Components of Dietary Fibre' (M. A. Rubio, S. Ingemar Falkehag, B. A. Pethica and P. Zuman), 'The Influence of Lignin on Lipid Metabolism of the Rat' (C. Leitzmann, A. Meier-Ploeger and K. Huth).

This book contains much up-to-date research data on fibre and the volumes' attractive price should ensure a wide readership among food scientists engaged in this important field.

G. G. Birch

Nutritional and Safety Aspects of Food Processing. Edited by S. R. Tannenbaum. Marcel Dekker Inc., New York and Basel. 1979. x + 448 pp. Price: £29·00.

This important new book in a food science series of monographs contains chapters on: 'The Consumer', 'The Product and The Promise' (Goldblith), 'Human Nutrient Requirements and Dietary Allowance' (Young and Scrimshaw), 'Vitamins' (Archer and Tannenbaum), 'Lipids' (Witling, Perkins and Kummerow), 'Minerals' (Tannenbaum and Young), 'Proteins and Amino Acids' (Cheftel), 'Technology of Fortification' (Borenstein), 'Prediction of Nutrient Losses and Optimisation of Processing Conditions' (Karel), 'Antinutritional and Toxic Substances: Naturally Occurring and Accidental Contaminants' (Wogan), 'Agricultural Chemicals' (Sissons and Telling), 'Preservatives Added to Foods' (Sinskey) and 'Immunological Aspects of Foods and Food Safety' (Catsimpoolas).

With such an authorative line-up of food scientists the twelve chapters promise a rewarding account of the frontiers of knowledge in these important areas and detailed reading brings the promise to fruition. The two chapters in which the editor is co-author set a poignant style for the book and the helpful structural depictions of the vitamins are of great value to a student of food science concerned with their stability and reaction mechanisms. Loss or gain of minerals during processing is now of topical interest and the less usual chapter on fortification is welcome in a time of general concern about the nutritional value of processed foods. The references cited for this subject reflect its applied nature with selected relevant patents. Cheftel's chapter on amino acids and proteins (232 references) gives a profound coverage of important reaction mechanisms affecting food value and the notable chapter on lipids (363 references) deals with their technology in relation to particular physiological effects. Karel's chapter on current lines of research aimed at predicting nutrient losses on processing presents a masterly approach based on mathematical models.

Although all the chapters in this book are of a high scientific standard certain of them are of more immediate interest to a food chemist concerned with processing. The chapters on preservatives (covered in complete and appropriate depth) and agricultural chemicals come into this category. Both of these chapters are very enlightening and the latter one fully explains how the processing of oils and fats decreases their pesticide content, an advantage not enjoyed by certain other food products, e.g. chocolate.

It is difficult to find fault with this book which is written in a condensed style by authors anxious to impart much of their extensive knowledge. The editor deserves every credit for unifying the diverse subject area so successfully and all food chemists must use this volume.

G. G. Birch

Liquid Chromatographic Analysis of Food and Beverages. Volume 1. Edited by George Charalambous, Academic Press, New York, San Francisco and London, 1979. Price: £12·40.

This book represents part of the proceedings of a symposium on the Analysis of Foods and Beverages by HPLC, organised by the Agricultural and Food Chemistry Division's Flavour Subdivision, of the American Chemical Society. The stated purpose of the symposium was to review the most recent developments in the analysis of foods by HPLC.

The thirteen papers that go to make up this book fall roughly into two sections, one covering the developments of technology and instrumentation while the other reports analytical results and the developments in analytical methods. The chapters that could be considered to make up the technology and instrumentation section include a paper on stopflow spectroscopic scanning as an aid to identification, two reporting the use of microprocessor based systems, one for optimising solvent systems, and the other describing a time programmable variable wavelength detector. Others describe the use of a dual (UV and fluorescence) detector system, the use of a fluorescence detector and the use of liquid chromatography in conjunction with ^{13}C NMR spectroscopy.

The papers concerned with reporting analytical results and the developments in analytical methods include two papers describing methods for determining aflatoxins in foods, one using reversed phase HPLC, and the other a radical chromatographic method. Other topics covered include the analysis of naturally occurring capsaicins, anthocyanins in fruits and beverages, vitamins in citrus juices, monosaccharides in avocado, amadori compounds in model systems and coumestrol in germinated and ungerminated soyabean.

The style and approach adopted varies considerably from chapter to chapter; some review a specific topic, while other papers are research reports. This has resulted in a most uneven book. Although printed on reasonable quality paper the author's original typescripts are directly reproduced for speed of publication. The typescripts of all the papers are clearly legible; this however, cannot be said of a number of the chromatograms which are too small to be clearly read.

This volume by itself does not fulfil the original object of the symposium.

R. D. KING

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