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Separation and Characterisation of Chymotryptic Peptides from α- and β-Purothionins of Wheat[a]

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Both α- and β-purothionins of wheat were readily hydrolysed by chymotrypsin into peptides which were separable by ion exchange chromatography. Hydrolysis occurred rapidly at the single tyrosine residue but there was no apparent cleavage at the phenylalanine residue. Leucine residues were hydrolysed at a slower rate. Reduction and alkylation of the purothionins increased the rate at which they were hydrolysed by chymotrypsin. Seventeen peptides were isolated from α-purothionin digests and β-purothionin yielded 15. From amino acid analyses of the chymotryptic peptides, there were at least four differences in the primary structures of the purothionins with β-purothionin containing 2 Asx, 1 Lys and 0.5 Leu residues which had replaced 2 Gly, 1 Arg and 0.5 Ileu residues of α-purothionin. Tyrosine is probably the thirteenth residue from the amino terminus of both α- and β-purothionins.

1. Introduction

Purothionins are low molecular weight, basic proteins extractable from wheat flour by non-polar solvents. They were obtained in crystalline form by Balls and Hale in 1942.[1] They are extracted as lipid complexes which may be artifacts. Crude purothionin extracts can be fractionated by gel electrophoresis or ion exchange chromatography into two similar components, α- and β-purothionins.[2] They have amino acid compositions quite distinct from other wheat proteins, containing large proportions of cystine (~18%), lysine (~10%) and arginine (~10%) and small amounts of proline and glutamic acid. The amino acids histidine, tryptophan and methionine, as well as free sulphhydril groups, are absent.[2-4]

Some physical properties of wheat purothionins have recently been reported.[4] Their molecular weights appear to be around 5000 Daltons, about half the 10 000–12 000 value reported by several earlier workers.[1,3] It has been estimated that they possess about 40% α-helical structure and probably assume a compactly folded, globular shape in solution.[4]

The two purothionins are very similar in amino acid compositions,[2,3] terminal amino acid residues[2] and physical parameters.[4]

This article deals with the dissimilarities of α- and β-purothionins from one wheat variety which are revealed by comparing the peptides obtained by chymotryptic digestion.

2. Experimental

2.1. Preparation of α- and β-purothionins

A crude mixture of α- and β-purothionins was extracted from flour milled from Canadian hard red spring wheat (cv. Manitou). The method described by Redman and Fisher[3] was used except that the petroleum ether was percolated through the flour in a glass column of 11.5 cm diameter and the final preparation was desalted with a Sephadex G-25 column instead of by dialysis.

The α- and β-purothionins were separated on a carboxymethyl cellulose (CM-cellulose, Whatman

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CM 52) column (2 × 25 cm). The protein sample was eluted with a linear concentration gradient of ammonium acetate (pH 5.2) from 0.4 to 0.7 M, 300 ml of each. The flow rate was 30 ml/h and the effluent was monitored simultaneously at 254 nm and 280 nm. The protein-containing fractions were freeze-dried twice to remove the volatile ammonium acetate. A yield of about 200 mg α- and 100 mg β-purothionin was normally obtained from 4 kg of flour.

2.2. Reduction and pyridylethylation of proteins and peptides

Proteins or chymotryptic peptide mixtures (15 mg) were dissolved in 2 ml of 0.05 M Tris buffer, pH 7.5, containing 3 M urea. After flushing the solution with nitrogen for 2 min, 80 μl of 2-mercaptoethanol was added and reduction proceeded at room temperature for 2 h.[5] After reduction, 80 μl of freshly distilled 4-vinylpyridine was added and the solution was again flushed with nitrogen (2 min) at room temperature. The reaction was stopped after 1.75 h by adjusting the pH to 3.0 with glacial acetic acid.[6] The reduced and pyridylethylated material was desalted on a Sephadex G-25 column (1.8 × 180 cm) eluted with 0.1 N acetic acid.

The use of the alkylating agent 4-vinylpyridine has been very helpful in this study. Not only is alkylation with this chemical easy and fast, but it also has the following advantages:

1. As long as one uses freshly distilled 4-vinylpyridine, the reaction appears to be quantitative and the pyridylethylcysteine formed is stable to 6 N HCl hydrolysis and is easily determined by standard amino acid analysis procedures.[6,7]

2. Each pyridylethylcysteine residue formed adds a positive charge to the peptide it occurs in. This means that, in the present study, every peptide except one had at least one positive charge at pH 5.2 and very good separations were obtained by a single ion exchange step.

3. Pyridylethylcysteine has a strong absorption maximum at 254 nm.[8] This allows one to determine where all the peptides except chαl and chβl elute from ion exchange columns by simply monitoring the effluent at 254 nm. Since native purothionin contains only 1 Tyr and 1 Phe, most of the peptides formed on hydrolysis would not absorb at 254 nm if they were not derivatised before being applied to the ion exchange column.

4. We have found (unpublished results) that it is possible to detect the phenylthiohydantoin (PTH) derivative of pyridylethylcysteine by gas chromatography. This is an advantage for sequencing the purothionins since the PTH derivatives of the compounds obtained when cysteine residues are reacted with other common alkylating reagents cannot be determined by gas chromatography.

2.3. Hydrolysis with α-chymotrypsin

Each purothionin was hydrolysed with α-chymotrypsin either before or after reduction and pyridylethylation. Protein sample (15 mg), either native or reduced and pyridylethylated, was dissolved (or suspended) in 1 ml of 0.5% ammonium bicarbonate, pH 7.7. Chymotrypsin solution (0.5 ml of 0.6 mg/ml enzyme in the same buffer) was added to the protein solution and the mixture was maintained at 37°C. Native purothionins were hydrolysed for 15 and 30 min, while reduced and pyridylethylated ones were hydrolysed for 30 min and 18 h. Hydrolyses were terminated by adjusting the reaction mixtures to pH 3.0 with glacial acetic acid. The hydrolysates were frozen in liquid nitrogen and freeze-dried. The dried peptide mixtures from the hydrolysis of native proteins were reduced and pyridylethylated before separation on a CM-cellulose column.

2.4. Fractionation of chymotryptic peptides

Chymotryptic peptides were separated by ion exchange chromatography. The CM-cellulose was equilibrated with 0.07 M ammonium acetate buffer, pH 5.2, and packed in a 0.9 × 25 cm column. The freeze-dried peptide mixture, normally 15 mg and pyridylethylated in all cases, was dissolved in 1 ml of the 0.07 M ammonium acetate buffer and applied to the column.

Peptides were eluted with a 0.07 to 1.0 M linear concentration gradient of ammonium acetate buffer, pH 5.2. The eluant was monitored simultaneously at 254 nm and 280 nm. The collected fractions were freeze-dried twice to remove ammonium acetate.

2.5. Amino acid analysis

Each peptide or protein sample, 0.5–2.0 mg, was dissolved in an equal weight of 3× distilled 6 N HCl in a vial fitted with a teflon-lined screw cap. The solution was flushed with nitrogen for 5 min, capped and placed in an oven at 110°C for 24 h. After hydrolysis, excess HCl was removed by placing the opened tubes in an evacuated desiccator over NaOH pellets. The amino acid compositions were determined on a Beckman 121 amino acid analyser. Pyridylethylated cysteine appears as a single peak between ammonia and arginine from the short column when the pH of the sodium citrate is changed to 5.5.

2.6. Determination of C-terminal peptide residues with carboxypeptidase A or B

An aqueous suspension of crystalline carboxypeptidase A (carboxypeptidase A-CAO, Mann Research Laboratories) was washed free of contaminating amino acids and dissolved in 0.02 M sodium phosphate buffer, pH 8.0, at a concentration of 0.6 mg/ml by the method of Ambler[9] as adapted from Harris.[10] Carboxypeptidase B (Worthington, DFP treated) was used without further washing.

The peptide sample (0.5 μmole) was dissolved in 200 μl of 0.02 M sodium phosphate buffer (pH 8.0). Carboxypeptidase A solution (300 μl, see above) and/or 30 μl carboxypeptidase B (6 mg/ml) was added. Aliquots (one-third of reaction mixture) were removed after 15, 60 and 120 min of incubation at 37°C. Each aliquot was immediately acidified with 50 μl of glacial acetic acid. One ml of sodium citrate buffer, pH 2.2, was added and the sample was applied to the column of an amino acid analyser for determination of the free amino acids present.

3. Results

3.1. Amino acid composition

Table 1 compares the amino acid compositions of native and of reduced and pyridylethylated α- and β-purothionins. The reduction and alkylation did not significantly affect the amount of any of the amino acids.

The compositions reported here were in good agreement with the data reported by Nimmo *et al.*[4] and by Redman and Fisher.[2]

Table 1. Amino acid composition of α- and β-purothionins. The results are expressed as mole ratios with respect to tyrosine

Amino acid	Pyridylethylated α	Native α	Pyridylethylated β	Native β
Lysine	5.4	5.6	6.0	6.5
Histidine	—	—	—	—
Arginine	5.3	5.6	4.0	4.6
Aspartic acid	2.1	2.2	4.4	4.3
Threonine	2.2	2.4	2.0	2.1
Serine	4.8	4.9	4.0	4.0
Glutamic acid	1.1	1.1	1.0	1.1
Proline	2.0	2.2	2.2	2.2
Glycine	4.8	5.0	3.4	3.4
Alanine	2.3	2.3	3.0	3.2
Half Cystine	8.3[a]	8.0	8.4[a]	8.1
Valine	1.0	1.0	1.0	1.0
Methionine	—	—	—	—
Isoleucine	0.5	0.6	—	—
Leucine	4.6	4.9	5.4	5.5
Tyrosine	1.0	1.0	1.0	1.0
Phenylalanine	1.0	1.1	1.0	1.1

[a] Values determined as pyridylethylated cysteine (4-PEC).

Table 2. Amino acid composition of chymotryptic peptides from α-purothionin. The results are expressed as mole ratios with respect to the amino acid marked [a] in each column

Amino acid	chα1	chα2	chα3	chα4	chα5	chα6	chα7	chα8	chα9	chα10	chα11	chα12	chα13	chα14	chα15	chα16	chα17
Lysine	0.4	1.2	0.4	2.2		1.8	0.8	1.0	1.0	1.0	1.8	2.0	3.3	1.0	0.8	2.0	4.5
Arginine			0.4		1.0		1.2	1.3	1.0	2.0	2.4	1.0	1.2	2.0	1.2	3.2	3.4
4-PEC	0.5	1.5	2.9	1.2	1.1	1.1	1.0	1.4	1.0			1.9	4.6	2.8		4.6	4.6
Aspartic acid	0.5	0.3			1.0			0.2	0.2	0.3	0.5			1.0		1.1	1.0
Threonine	1.0	0.9	0.8	1.0				0.2	1.0	0.6	1.0			1.0	0.3	1.0	1.0
Serine	1.2	1.4	1.0	2.0		0.9	1.3	0.5	1.0	1.0	2.0	2.0	3.1	1.8	1.0	1.6	3.5
Glutamic acid	0.3	0.3						0.1	0.2	0.6	1.2					1.1	0.8
Proline		0.8		2.0		1.9						1.4	2.0				2.1
Glycine	1.0[a]	1.2	0.8	2.2	1.0	1.1	1.0	0.4	0.2	0.6	2.0	1.4	3.3	1.0	1.0	1.5	4.2
Alanine	0.2	0.6	0.8				0.3	0.3	0.2	1.0	2.0		1.1		1.0	1.3	3.0
Valine	0.2	1.0[a]	1.0[a]							1.0[a]	1.0[a]		1.0			1.0[a]	1.0[a]
Isoleucine							0.6						0.7				0.8
Leucine	1.6	0.9	0.5	1.3			1.0[a]	1.0[a]	1.0[a]	1.6	2.0	1.0[a]	1.5	1.0[a]	1.0[a]	3.1	3.3
Tyrosine					1.0[a]									0.8			
Phenylalanine	0.1	0.5		1.0[a]		1.0[a]							1.0[a]				1.0

3.2. Chymotryptic peptides

For automated sequence studies on proteins, relatively large peptides are needed owing to solubility problems often encountered with smaller peptides.[11] Since both purothionins contain over 20% arginine + lysine residues, hydrolysis with trypsin would yield a large number of small peptides. To obtain large peptides (required for the sequence analysis that is part of this study), timed hydrolyses with α-chymotrypsin were performed.

3.2.1. Fifteen minute hydrolysis

When α-purothionin was hydrolysed with chymotrypsin for short periods (15 min) and the resulting peptides were separated on CM-cellulose (Figure 1A) two major and several minor peptides were found. The β-purothionin was also cleaved into two major peptides under these conditions (Figure 2A). The two main peptides from each hydrolysis (chα14 and chα17 from α-purothionin and chβ14 and chβ15 from β-purothionin) were collected, hydrolysed and their amino acid compositions were determined (Tables 2 and 3). Peptides chα14 and chβ14 each contained one tyrosine residue and no phenylalanine, while peptides chα17 and chβ15 contained phenylalanine but no tyrosine.

Table 3. Amino acid composition of chymotryptic peptides from β-purothionin. The results are expressed as mole ratios with respect to the amino acid marked [a] in each column

Amino acid	chβ1	chβ2	chβ3	chβ4	chβ5	chβ6	chβ7	chβ8	chβ9	chβ10	chβ11	chβ12	chβ13	chβ14	chβ15
Lysine	0.2	2.0	2.2	0.4			1.0	1.0	1.0	1.0	1.6	1.0	4.8	1.8	4.2
Arginine					1.2	2.0	1.0	2.0	1.3	0.8	1.0	1.5	1.4	1.0	3.5
4-PEC		0.9	1.0	1.0	1.3		2.4	2.4	1.2	1.2	3.4	0.6	6.4	2.8	5.2
Aspartic acid	0.3	1.1	1.3	1.3	1.2	1.0	1.0	1.0		0.3	0.3	0.4	2.4	1.0	3.0
Threonine	1.0	0.8		1.0[a]		1.0	0.3	1.0		0.3	0.3		1.2	0.9	1.0
Serine	1.0	1.9	1.0	0.9		0.4	0.9	1.4		0.8	1.2		3.5	1.8	3.1
Glutamic acid	0.1			0.4		1.5	0.3			0.3	0.3	0.4			1.2
Proline		1.9	2.0				0.3						2.1		2.2
Glycine	1.0[a]	1.1			1.0		0.5	0.8		0.3	0.3	0.5	1.2	1.0	2.0
Alanine	0.1					1.0	1.0	1.0		0.5	0.6	1.2	1.3		3.3
Valine	0.1						0.3				0.2	0.3	1.0[a]		1.0[a]
Isoleucine															
Leucine	1.2	1.1				1.0[a]	1.0[a]	2.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	2.3	1.1	4.1
Tyrosine					1.0[a]									1.0[a]	
Phenylalanine		1.0[a]	1.0[a]										0.9		0.9

Since no third peptide was formed in large amount, cleavage at one of the two theoretically susceptible residues must be much slower than at the other. This could be due either to one of the two residues being shielded from the chymotrypsin by the folding of the purothionin or to a proline residue being located next to the non-susceptible residue, in which case chymotryptic activity would be inhibited.[12]

To elucidate where chymotryptic cleavage had occurred, the C-terminal residues of each of the 15-min peptides were determined with carboxypeptidase A and B (Table 4). Both peptides chα14 and chβ14 had C-terminal tyrosine residues, while peptides chα17 and chβ15 had lysine as terminal residues. It has been reported previously[2] and we have confirmed that the C-terminal residues of both α- and β-purothionins are lysines, so it is probable that the tyrosine peptides are located at the N-terminal ends of both α- and β-purothionins, that the phenylalanine peptides are at the C-terminal ends, and that the peptide bond involving the phenylalanine residue is resistant to chymotryptic hydrolysis. This is supported by the finding that the phenylalanine-containing peptides had lysine as the C-terminal residue (Table 4).

3.2.2. Thirty-minute hydrolysis

When chymotrypsin digestion was extended to 30 min, the pattern of peptides eluted from CM-cellulose changed considerably (Figures 1B and 2B). The amount of material in the two original

Table 4. Comparison of amino acid compositions of similar chymotryptic peptides from α- and β-purothionins

Peptide pairs	No. of residues	C-terminal residue	Residue differences[a]
chα1, chβ1	4	Leu	similar
chα4, chβ2	12	Lys	−1 Asx, +1 Gly
chα5, chβ5	5	Tyr	nil
chα6, chβ3	8	Lys	−1 Asx, +1 Gly
chα9, chβ10	mixture	Leu	similar
chα14, chβ14	13	Tyr	−1 Lys, +1 Arg
chα17, chβ15	33	Lys	−2 Asx, +2 Gly, −8 Leu, +0.8 Ileu

[a] Composition of α-purothionin peptide relative to β-purothionin peptide.

peptide peaks (chα14 and 17, chβ14 and 15) decreased and several new peptides appeared. These generally eluted at a lower salt concentration indicating that they contained fewer basic residues. Amino acid analyses of these new peaks (Tables 2 and 3) also indicated they were smaller peptides. Chymotrypsin has been reported to catalyse the hydrolysis of peptides at leucine residues, as well as at aromatic amino acids.[12] Since each of the new peptides formed contained at least one leucine residue (or Tyr or Phe) it was probable that they were arising from hydrolysis of the original two peptides via hydrolysis at leucine residues. This was confirmed by determining the C-terminal residues of those peptides present in relatively large amounts (Table 4). All peptides had C-terminal lysine, tyrosine or leucine.

3.2.3. Hydrolysis of reduced and alkylated purothionins

When either α- or β-purothionin was first reduced and pyridylethylated and then subjected to chymotryptic hydrolysis for 30 min, they were both hydrolysed at faster rates than the native proteins (Figures 1C and 2C). In both cases, the amounts of the two large peptides which

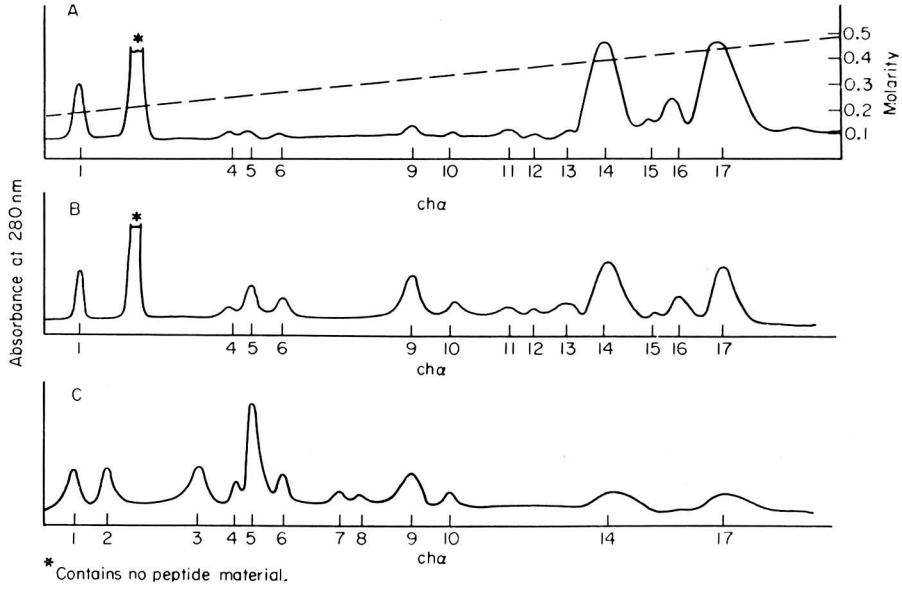

Figure 1. Elution patterns of chymotryptic peptides of α-purothionin from the CM-cellulose column. A, 15-min hydrolysis, native protein, B, 30-min hydrolysis, native protein, C, 30-min hydrolysis, reduced and pyridylethylated protein, ———, absorbance at 280 nm; – – – – –, molarity of ammonium acetate in gradient.

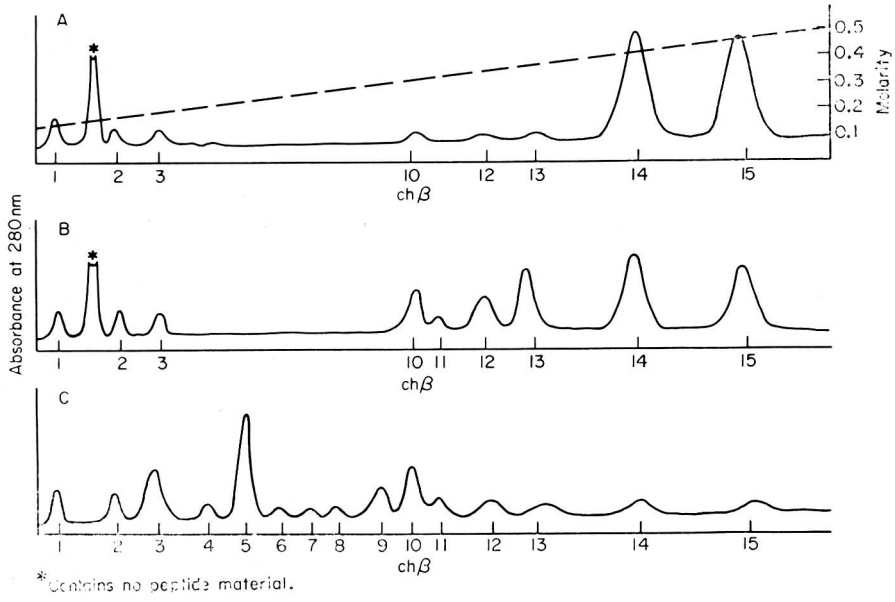

Figure 2. Elution patterns of chymotryptic peptides of β-purothionin from the CM-cellulose column. A, 15-min hydrolysis, native protein; B, 30-min hydrolysis, native protein; C, 30-min hydrolysis, reduced and pyridylethylated protein; ———, absorbance at 280 nm; – – – – –, molarity of ammonium acetate in gradient.

predominated in the 15-min native protein hydrolysates were greatly reduced and the smaller peptides predominated. The 30-min treatment of the reduced and alkylated proteins gave almost complete hydrolysis since the CM-cellulose elution patterns of (Figures 1C and 2C) were identical to those obtained when reduced and pyridylethylated purothionins were hydrolysed for 18 h. It is to be expected that reducing and alkylating the purothionins would have a large effect on the hydrolysis rate since the native protein, with almost one fifth of the amino acids being involved in disulphide bonding, is probably a compactly folded protein whose potential sites of hydrolysis would presumably be at least partially sheltered from chymotryptic attack.

3.2.4. Amino acid composition of chymotryptic peptides

The elution patterns of the α- and β-purothionin chymotryptic peptides from CM-cellulose are similar (Figures 1 and 2). The u.v.-absorbing fractions were collected, freeze-dried, hydrolysed and analysed for amino acid composition (Tables 2 and 3). Several pairs of peptides from α- and β-purothionin have identical or similar compositions. These peptide pairs and the amino acid differences between comparable peptides from α-purothionin and β-purothionin are listed in Table 4.

Chα peaks 2, 3, 10 and 16, and chβ4 and 9 peaks did not have any readily apparent counterparts in the other chromatogram. Yields of the peptides chα7, 8, 11, 12, 13 and 15, and chβ6, 7, 8, 11 and 13 were very small. From this data, it is probable that α- and β-purothionins have very similar primary structures with 2 Asx, 1 Lys and 0.5 Leu of β-purothionin being replaced by 2 Gly, 1 Arg and 0.5 Ileu in α-purothionin.

4. Discussion

There has been considerable disagreement about the molecular weights of the purothionins with the older literature reporting molecular weights of 10 000–12 000, while recent physical measurements

suggest a value about half this large. It is generally agreed that there is one tyrosine and one phenylalanine residue for every 6000 mol.wt. Chymotryptic hydrolysis should then yield three (mol.wt 6000) or five (mol.wt 12 000) peptides depending on which of these size estimates is correct. We find that only two major peptides are formed when either α- or β-purothionin is cleaved by chymotrypsin for short periods of time. This is indicative that both proteins have molecular weights of 6000. This conclusion is further strengthened by the finding that after prolonged chymotryptic hydrolysis of α-purothionin only two peptides, chα5 and chα14, were found to contain tyrosine. From the amino acid compositions, it seems likely that one of these peptides (chα5) arises from the other (chα14) via hydrolysis at the single leucine residue present in chα14. If there were two tyrosine residues per molecule (i.e. if mol.wt = 12 000) they would probably occur in peptides which could be differentiated by their amino acid content.

A similar situation is seen in β-purothionin where the tyrosine peptide chβ5 probably arises from chβ14 by a single cleavage at a leucine residue.

The situation is similar for phenylalanine, where all the phenylalanine-containing peptides probably arise from further hydrolysis of the large peptides chα17 and chβ15. The data thus indicate that the purothionins probably have molecular weights of 5000–6000.

Part of the confusion about the molecular weights of the purothionins has come from the fact that α-purothionin contains approximately one residue of isoleucine per 10 000 mol.wt. This has been ascribed to heterogeneity of the α-purothionin preparation.[3,13,14] Our data support this idea, since peptides chα7, chα13, and chα17 differ from their β-purothionin peptide counterparts by having gained about 0.6–0.8 residue of isoleucine and having lost a corresponding amount of leucine. It appears that there are probably two forms of α-purothionin present, one containing leucine at some site, while the other has isoleucine at that position.

The finding of 0.6–0.8 isoleucine residues in the isoleucine-containing peptides instead of the expected 0.5 residues arises from the fact that the chymotrypsin hydrolyses bonds adjacent to isoleucine at a much slower rate than those adjacent to leucine.[15] If then, one has a peptide containing equal amounts of isoleucine and leucine sharing a single position, part of the leucine-containing peptide will be further hydrolysed by chymotrypsin so that the remaining larger peptide pool will appear to be enriched in isoleucine. There was no evidence for the presence of any isoleucine in the β-purothionin examined in the present study.

In addition to the isoleucine–leucine replacement between α- and β-purothionins, the α-purothionin contains two aspartic acid or asparagine residues and one lysine residue in place of two glycine and one arginine residues of β-purothionin (Table 4). All these substitutions could arise from single base changes in the codons for the various amino acids except for the Gly → Asn, which, using codons determined for bacteria, would need two base changes. One of the peptides containing an Asx (chβ3 from β-purothionin) elutes much sooner than the corresponding α-purothionin peptide containing Gly (chα6). This may reflect the introduction of a negatively charged Asp group in place of the neutral Gly. Introduction of a neutral Asn would not be expected to have much effect on the elution point of the peptide.

The amino acid composition data from the chymotryptic peptides (Tables 2 and 3), as well as the C-terminal residue analyses, allow certain deductions to be made relative to the primary structures of the two wheat purothionins. The two large peptides which first appear during hydrolysis (chα14 and chα17 from α-purothionin and chβ14 and chβ15 from β-purothionin) together contain the full complement of amino acids of the parent proteins and probably result from a single hydrolytic scission. Since tyrosine is the C-terminal residue of one peptide and lysine, the C-terminal residue of whole α- and β-purothionins, is C-terminal on the other peptide, which contains phenylalanine, it appears that the cleavage occurs at the tyrosine residue while the proteins are not hydrolysed at phenylalanine. It also indicates that the tyrosine peptide is from the amino terminal end of the purothionins, so that tyrosine should be the thirteenth residue from the amino terminus of both α- and β-purothionin. From the different amino acid compositions of chα14 and chβ14, there is at least one difference in the primary structures of the purothionins prior to residue 13 since the α-purothionin peptide contains 1 Lys and 2 Arg, while the one from β-purothionin has 2 Lys and 1 Arg. The other structural changes must be in the region C-terminal to residue 13.

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Triglyceride Composition of *Papaver somniferum* seed oil

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Poppy seed oil (*P. Somniferum*) contains palmitic (12%), stearic (3%), oleic (20%) and linoleic acid (65%). Lipolysis with pancreatic lipase indicates the following glyceride composition: S_3 (tr), S_2U (5%), SU_2 (34%) and U_3 (61%) or saturated dilinolein (19%), oleo-dilinolein (25%), and trilinolein (27%).

1. Introduction

Papaveraceae is a family of 100 species, including the poppy (*Papaver somniferum*). Poppies are cultivated extensively in India: the oil content of the seed usually varies between 44–50%. The principal use of poppy seed oil is as an edible oil.[1]

Earlier reports[2–6] on the fatty acid composition of *P. somniferum* seed oil of different origins, indicate that it is a linoleic-rich (62–73%) oil. The demonstration[7–9] of the dietary effect of linoleic-rich oils like corn or safflower oils in lowering the serum cholesterol content has added an extra medicinal importance to the use of poppy seed oil as an edible oil.

Seed fats rich in linoleic acid seem to be particularly susceptible to the environmental conditions in which the seeds mature. An outstanding example is the sunflower seed oil, whose linoleic acid content has been reported to vary between 70–30% depending upon the conditions of its growth.[10] But the fatty acid composition of poppy seed oil of Indian origin was found to be very similar to that of oil from seeds growing in the United Kingdom.[5] Since most of the published analyses of poppy seed oil[2–6] preceded the development of the g.l.c. method of analysis, the reported fatty acid composition of this oil needs further confirmation.

The triglyceride composition of *P. somniferum* seed oil has been studied previously by Bridge *et al.*[5] who used the low temperature crystallisation procedure which has proved to be inadequate for arriving at the correct triglyceride composition of linoleic-rich seed oils.[10] The computed figures for the triglyceride composition of the poppy seed oil[10] from its fatty acid composition, on the basis of mode-I of Gunstone's theory[11] also showed considerable variations from that reported by Bridge *et al.*[5]

Gunstone and Qureshi[12] estimated the triglyceride compositions of safflower, tobacco and sunflower seed oils, each with more than 60% linoleic acid content, by lipolysis and also by chromatographic separation on thin layers of silica gel impregnated with silver nitrate. Good agreement between the results obtained by these two methods provides support for the assumptions adopted in handling lipolysis and also for the correctness of the postulates of Gunstone's theory.[11]

The present communication reports the determination of the triglyceride composition of poppy seed oil of Indian origin by the technique of selective enzymatic hydrolysis[13] preceded by the determination of the fatty acid composition using combined techniques of urea complexation, spectrophotometric analysis and gas–liquid chromatography.

2. Experimental and results

P. somniferum seeds were procured from the local market. On extraction with petroleum ether (b.p. 40–60°C), the seeds yielded a light yellow coloured oil which on analysis by standard pro-

cedures showed the following characteristics: % free fatty acid, 13.7; saponification equivalent, 294; iodine value, 133.

One hundred grams of the mixed fatty acids, free from the non-saponifiable matter (saponification equivalent, 277) were next segregated into four fractions (A–D) by urea complexation techniques,[14] increasing stepwise the proportion of urea to fatty acids (0.5–1.5). The mixed fatty acids were always dissolved in five times the volume of methanol. Mixed fatty acids of *P. somniferum* seed oil and also its fractions (A–D) were examined spectrophotometrically after alkali isomerisation, for their contents of polyethenoid acids.[15, 16] Results are given in Table 1.

Table 1. Spectrophotometric analysis of the MFA and of the fractions obtained from MFA by urea complexation

Sample	% yield	Iodine value	$E_{1\,cm}^{1\%}$ at 234 nm isomerised at 180°C for 1 h	Fatty acid composition (wt %)		
				Linoleic	Monoethenoid as oleic	Saturated by difference
MFA		137.3	580.3	64.1	24.3	11.6
A	23.7	69.5	221.3	24.4	28.1	47.5
B	19.3	136.6	497.1	54.5	42.2	3.3
C	29.9	160.9	724.7	80.0	17.9	2.1
D	27.1	176.0	856.1	94.5	5.5	—
Computed values from (A–D)		138.0		65.8	21.7	12.5

Lipolysis of the *P. somniferum* seed oil was carried out as suggested by Coleman[17] at pH 8.5 and 37.5°C using a purified pancreatic lipase preparation with the addition of Ca^{2+} ions and bile salts. The partial glycerides were separated on a thin layer (0.3–0.4 mm) of silica by developing with a solvent system of n-hexane, diethyl ether and acetic acid (80:20:0.25). The 2-monoglyceride fraction, detected with 2′,7′-dichlorofluorescein, was extracted with hot alcohol. The isolated 2-monoglycerides were converted into methyl esters by Luddy's method.[18] Methyl esters of the mixed fatty acids of the seed oil were prepared by acid catalysed esterification.

All the methyl esters were analysed by g.l.c. for their fatty acid composition; g.l.c. was carried out with an F and M analytical Gas-chromatograph (Model 700 R 12) equipped with a flame ionisation detector. The column (6 ft × ¼ in) packed with 10% polyester of diethyl glycol succinate on 60–80 mesh gas chrom-Z was operated at 155°C with a carrier gas flow of 40 ml/min. Peak areas were determined as the product of peak height and the width at half height. The weight percentages thus obtained were converted to mole percentages. Results are given in Table 2.

Table 2. Fatty acid composition (mole %) of the triglycerides and of the 2-monoglycerides of *P. somniferum* seed oil

Sample	16:0	18:0	18:1	18:2
Triglycerides	11.9	2.9	20.4	64.8
2-Monoglycerides	2.3	—	26.8	70.9

The triglyceride composition of *P. somniferum* seed oil was then calculated from the fatty acid composition of the original triglyceride and of the 2-monoglycerides formed, using the assumptions

of Vanderwal[19] and Coleman.[17] In the calculations fatty acids have been grouped as S, 16:0 and 18:0; O, 18:1 and L, 18:2. The results are given in Table 3.

Table 3. Triglyceride composition[a] (mole %) of *P. somniferum* seed oil

	Glycerides	Mole %		Glycerides	Mole %
SSS	SSS	0.1	SUU	SOO	2.0
				SOL	6.9
SSU	SSO	0.2		SLO	5.2
	SSL	0.6		SLL	18.4
Total		0.8	Total		32.5
SUS	SOS	1.2	UUU	OOO	0.8
	SLS	3.1		OOL	5.7
Total		4.3		LOL	10.2
				OLO	2.1
USU	OSO	0.1		OLL	15.0
	OSL	0.4		LLL	27.1
	LSL	0.9	Total		60.9
Total		1.4			

[a] The fatty acids have been grouped as, S, 16:0 and 18:0; O, 18:1 and L, 18:2.

3. Discussion

The fatty acid composition of the *P. somniferum* seed oil, as determined in the present investigation, is given in Table 4 along with the findings of previous workers.

As the spectrophotometric method for unsaturated acids can provide a relatively quick check on the results obtained by g.l.c.,[10] the sample was analysed by both methods. The fatty acid composition of *P. somniferum* seed oil as computed from its different fractions (A–D) obtained by urea complexation, agrees well with the results obtained by g.l.c.

Table 4. Fatty acid composition (wt %) of the seed oil of *P. somniferum* of different origin

Habitat	% weight					
	14:0	16:0	18:0	20:0	18:1	18:2
Temperate and subtropics[2]	—	4.8	2.9	—	30.1	62.2
Russia[3]	—	10.0		—	25.0	65.0
Europe[4]	—	10.0		—	25.0	65.0
England[5]	0.7	9.5	1.4	0.3	16.5	71.6
England[5]	—	8.3	2.7	—	16.4	72.6
India[5]	—	11.0	4.2	0.4	11.4	73.0
Argentina[6, a]	0.3	7.4	1.2	0.1	20.5	69.7
Argentina[6, b]	0.1	10.6	1.3	0.1	16.0	70.3
—[20, c]	—	10.0	2.0	—	11.0	72.0
India (present work)	—	11.1	3.0	—	20.6	65.3

Also reported, the presence of minor acids as follows: [a] 0.8% of 16:1; [b] 1.6% of 16:1; [c] 5% of 18:3.

From Table 4, it is evident that the fatty acid composition of Indian *P. somniferum* seed oil as determined in the present investigation shows considerable deviations from that reported by earlier workers,[5] particularly in the case of linoleic and oleic acids, the percentage contents in the two samples being respectively 65.3 and 73.0 for linoleic acid and 20.6 and 11.4 for oleic acid. If the fatty acid composition of the poppy seed oil grown in the United Kingdom as reported by Bridge *et al.*[5] is taken into consideration, the present findings on the composition of Indian poppy seed oil seem to agree better with the hypothesis that the species yields oil with relatively lower linoleic acid content if grown in hotter climatic conditions.

The only available data based on g.l.c. analysis for the fatty acid composition of poppy seed oil[20] of unknown origin indicate that the seed oil is composed of 72% linoleic, 11.0% oleic and 5.0% linolenic acids. Linolenic acid, which has not been reported by any of the previous groups of workers, could not be detected in the present sample by either spectrophotometric or g.l.c. analysis. The discrepancy between the present study and other work based on g.l.c.[20] regarding the content of linoleic and oleic acids, might be due to differences in the environmental conditions under which the seeds developed. Using urea complexation the mixed fatty acids could be segregated to yield 27% of the product as a fraction with linoleic acid content as high as 94.5%. Thus the method may easily be followed for preparation of linoleic acid concentrate from the poppy seed oil.

The triglyceride composition of the seed oil of *P. somniferum* as determined in the present investigation is given in Table 5 along with the findings of Bridge *et al.*[5] and the figures computed from the previously reported fatty acid composition based on mode-I of Gunstone's hypothesis.[10]

Table 5. Component glycerides[a] (mole %) of *P. somniferum* seed oil

Type	Poppy seed (English[5])	Poppy seed (Indian[5])	Computed figures from Indian species[10]	Poppy seed (present work)	Sunflower seed[21]
S_3	—	—	—	0.1	—
S_2O	—	—	1	1.4	0.5
S_2L	1	1	5	3.7	2.5
SO_2	—	—	1	2.1	2.4
SOL	6	9	10	12.5	9.9
SL_2	35	37	25	19.3	14.5
O_3	—	—	—	0.8	1.3
O_2L	—	—	4	7.8	11.2
OL_2	31	32	20	25.2	28.8
L_3	27	21	34	27.1	28.1

[a] The fatty acids have been grouped as, S, 16:0 and 18:0; O, 18:1 and L, 18:2.

For comparison, the triglyceride composition of linoleic rich sunflower oil (fatty acid composition: 16:0, 6.5; 18:0, 3.3; 18:1, 23.0; 18:2, 66.5 and minor acids, 0.7%)[21] as determined by enzymatic hydrolysis has also been included in the same Table.

The triglyceride composition of *P. somniferum* seed oil as determined in the present investigation differs considerably from the composition[5] determined by the method of low temperature crystallisation. The variations between the present findings and the computed figures are not of much significance as the latter were based on a different fatty acid composition determined by the same group of workers.[5] The triglyceride composition of *P. somniferum* seed oil determined in the present investigation agrees well with the triglyceride composition of sunflower oil of similar fatty acid composition as determined by Jurrien[21] using lipolysis.

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Sterol Composition of Butters and Margarines

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Analysis of the sterol and triterpene alcohol fractions of polyunsaturated margarine, normal butter and soft butter by gas chromatography–mass spectrometry revealed that the major components in margarines were β-sitosterol, stigmasterol, Δ 7-stigmasterol and campesterol, while cholesterol was the major sterol in butter. The hydrogenation process used in the manufacture of margarines had no effect on the composition of the unsaponifiable components of the parent oils. Crystallisation as used in the manufacture of soft butter did not affect the quantity of cholesterol present in this product.

1. Introduction

In recent years there has been much controversy regarding the role of cholesterol[1] in arteriosclerosis and a reduction in dietary content of saturated fatty acids and cholesterol has been advocated as a means of reducing blood cholesterol. It has been suggested that plant sterols in polyunsaturated oil could well be responsible for lowering of serum cholesterol; however, during the process of manufacture of margarines these sterols may be hydrogenated.

Recently polyunsaturated margarines manufactured from hydrogenated soyabean oil, sunflower or safflower oil[2] have become available in New Zealand.

Though few reports are available on the sterols and triterpene alcohols present in margarine, the unsaponifiable fractions of vegetable oils have been widely studied. Gas chromatography has shown a very complex distribution of sterols and triterpene alcohols and only recently has much progress been made in identifying the major constituents. Work on the lipid composition of vegetable oils and on phytosterols has been reviewed.[3,4] However, much of this earlier information has been greatly augmented and corrected in the recent investigations of Itoh and co-workers.[5–7] They have very thoroughly analysed the sterol and triterpene alcohol fractions of the oils from over 40 varieties of seeds. As well as the well known major components, campesterol, stigmasterol and β-sitosterol, they have isolated and identified nine other sterols and seven triterpene alcohols that are present in amounts varying from traces (less than 0.1 %) up to several per cent of the unsaponifiable fraction depending on variety.

In response to a demand for more spreadable products, soft butter, a fractional crystallate of normal butter, has been developed. While it is known that the major sterol in butter is cholesterol,[8] the effect of crystallisation on the sterols present was not known. In this paper detailed analyses of the sterols and triterpene alcohols in margarines are presented and compared with the levels associated with alternative dairy products. Also of interest is the effect of the margarine manufacturing process on the composition of the vegetable oils used. The methodology of Itoh *et al.*[5,6] was followed quite closely except that analytical rather than preparative techniques were in general used.

2. Methods

2.1. Materials

Samples of the following margarines and butters were purchased from retail sources: Butter (normal)—Anchor, Auckland Co-op, NZ; soft butter—Anchor, NZ Dairy Co-op, Hamilton;

Dixibell polyunsaturated margarine—Kaipara Foods, Helensville, NZ; Meadow-Lea—ASPAK Manufacturers and Distributors, Auckland; Meadow-Lea—Meadow-Lea Margarine Co. Pty Ltd, Sydney, NSW, Australia; Miracle—Abels Ltd, Auckland, NZ.

2.2. Saponification

Duplicate 5 g samples were refluxed with 50 ml methanolic potassium hydroxide for 30 min, 30 ml of distilled water was added and the unsaponifiables extracted with three 50 ml portions of diethyl ether. The combined ether extracts were washed with distilled water until neutral, dried with anhydrous sodium sulphate, filtered, and taken down to dryness on a rotary evaporator. The unsaponifiables were redissolved in 2 ml diethyl ether and 1 mg progesterone (Sigma, USA) added as an internal standard in determining the total sterols and triterpene alcohols by gas chromatography.

2.3. Moisture determination

Duplicate 30 g samples of the spreads were dried at 105°C for 18 h.

2.4. Thin-layer chromatography (t.l.c.)

Approximately 10% of the unsaponifiable material was applied to a 20 × 5 cm plate coated with 1 mm of Silica Gel G (Merck, Germany). Development was carried out twice in hexane ether (8:2). A tracer lane was cut down one outer edge of the plate and visualised with a spray of 1:1 ethanol/saturated ethanolic dodecaphosphomolybdic acid (BDH, England).

Bands corresponding to sterols, 4-methyl sterols and triterpene alcohols (R_F 0.24, 0.35 and 0.42 respectively) were scraped off and eluted with ether. To each extract, 0.1 mg progesterone was added as an internal standard for gas chromatography. It was assumed that the percentage recovery was constant for all bands.

2.5. Gas chromatography (g.c.)

The total sterol and triterpene alcohol content of the extracts was determined using a Hewlett Packard model 402 gas chromatograph with flame ionisation detectors. Glass U columns (4 mm i.d. × 2 m) were packed with 1% OV17 on 100/120 mesh Gas-Chrom Q (Applied Science Lab., USA). Injector, column and detector temperatures were kept at 250°C and the nitrogen carrier gas flow was 25 ml/min (retention time for β-sitosterol 45 min). Individual t.l.c. fractions were analysed on a Varian 2740 gas chromatograph with a coiled glass column (3 mm i.d. × 2 m) and a flame ionisation detector. The column packing and operating conditions were as above except that the column temperature was 265°C and helium was used as the carrier gas (retention time β-sitosterol 25 min).

Components were determined by comparing the area of the peak with that of the internal standard, progesterone. Sterols in the margarine were determined using the calibration factor of β-sitosterol in relation to progesterone. For the butters all components were calculated using the calibration factor of cholesterol in relation to progesterone.

Identification of the individual components relied in general on a comparison of the relative retention time (RRT, β-sitosterol 1.00) and the mass spectrum with those of standards or with values quoted in the literature, as mentioned in the results.

2.6. Gas chromatography–mass spectrometry (g.c.–m.s.)

Analyses were carried out on a Varian-MAT model CH5 single focusing mass spectrometer coupled by a two-stage glass frit separator to a Varian 2740 g.c. (column and conditions as above). The interface temperature was 250°C and the ion source was operated at 220°C, 100 μA trap current and 24 eV electron energy.

3. Results

The levels of total sterols plus triterpene alcohol in the butters and margarines on a wet weight basis together with their corresponding moisture content are presented in Table 1. These were

Table 1. Total sterols plus triterpene alcohols of various butters and margarines

Sample	mg % (wet wt)	% moisture
Butter (normal)	206	15.9
Soft butter	212	13.4
Dixibell	498	14.4
Meadow-Lea (NZ)	378	14.5
Meadow-Lea (Aust.)	515	14.3
Miracle	402	14.9

calculated from the total area of the peaks in the retention time region (relative to β-sitosterol) of 0.6–1.6, g.c.–m.s. having failed to reveal any non-steroidal contaminants in this region. The percentage composition of these components calculated from the t.l.c. fractions is given in Table 2 along with the observed relative retention times. Cholesterol, campesterol and β-sitosterol were matched in retention time and mass spectra against standard samples. These components and stigmasterol also gave retention behaviour and mass spectra as their trimethyl silyl derivatives corresponding to the literature data.[9,10] A small peak on the leading edge of β-sitosterol in the margarine samples was identified as 24-methyl-cholest-7-en-3β-ol from its RRT of 0.94 and mass spectrum.[11] The molecular ion was at *m/e* 400 with a prominent M-CH_3 ion, and strong peaks at 273 and 255 correspond to loss of (side chain) and (side chain plus water). This component was less than 1% of the β-sitosterol and was included with it in the determination of the sterol levels. The mass spectral and retention data for Δ^5-avenasterol (mw 412, strong 314, base peak 296/weak 271 and 253), Δ^7-stigmasterol (mw 412, strong 273, base peak 255), and Δ^7-avenasterol (mw 412, strong 314, base peak 271) follow that of Itoh and co-workers.[5,9,11]

For the 4-methyl sterol t.l.c. fractions the components of RRT 0.93 and 1.50 gave mass spectra that agreed very well with those for obtusifoliol and cistrostadienol respectively.[6] Obtusifoliol gave mw 426, strong 411, 245 and 233, weak 293, 327 and 313. Cistrostadienol gave a weak molecular ion 426, M-CH_3 and M-CH_3-H_2O peaks, strong 328, base peak 285 and a 267 peak, the latter two ions corresponding to loss of (side chain 2H) and (side chain $+2H+H_2O$). A minor component with RRT 0.89 and mw 412 was observed on the leading edge of the obtusifoliol peak. A peak at RRT 1.10 in the margarine fractions gave mixed spectra with three components mw 426, 412 and 414 being indicated. The first two made up the major proportion of the g.c. peak in all margarines and from the spectral and relative retention data are tentatively assigned to cycloeucalenol and gramisterol respectively.[6] Cycloeucalenol was not reported as a component of soyabean, sunflower or safflower oil.[6] A component, A, at RRT 1.35 gave a mass spectrum with mw 426, M-15, *m*-15-18, 328, 313, base peak 285. The latter ion corresponds to the (side chain + 2H) loss observed in citrostadienol and indicates that one of the double bonds is in the side chain. It is possibly the Δ^7 isomer of citrostadienol, although the retention time ratio for these two components (1.11) is not identical to that for the two pairs of Δ^5/Δ^7 isomers, stigmasterol/α-spinasterol (ratio 1.17) and Δ^5-avenasterol/Δ^7-avenasterol (1.18). This RRT 1.35 component has been observed but not identified in the 4-methyl sterol fraction of various vegetable oils.[6,7] No 4-methyl sterols were detected in the butter samples.

The triterpene alcohol t.l.c. fractions from the margarines gave poorly resolved g.c. peaks and therefore the assignments and relative amounts have a greater margin of uncertainty than in the other fractions. The component at RRT 1.08 was well resolved and gave a good mass spectrum (mw 426, *m*-15, 393, 331, base peak 218, strong 203). These data correspond to that for β-amyrin.

Table 2. Per cent composition of the sterol and triterpene alcohol fraction of butters and margarines

	RRT[a]	Butter	Soft butter	Dixibell	Meadow-Lea (NZ)	Meadow-Lea (Aust.)	Miracle	30/70 Soy/sunflower oil
Sterols[b]								
Cholesterol	0.60	98.8	96.4	—	—	—	—	—
Campesterol	0.79	—	—	7.4	6.0	8.1	6.2	8.2
Stigmasterol	0.86	—	—	9.4	4.6	5.9	6.1	8.2
β-Sitosterol	1.00	—	—	47.7	52.4	55.8	51.6	41.2
Δ^5-Avenasterol	1.10	—	—	1.2	1.9	1.9	1.8	2.6
Δ^7-Stigmastenol	1.17	—	—	9.5	8.6	7.7	7.8	8.0
Δ^7-Avenasterol	1.30	—	—	2.9	2.5	1.8	2.1	2.2
4-Methyl sterols								
Obtusifoliol	0.93	—	—	4.7	4.3	6.1	3.3	4.6
Cycloeucalenol + gramisterol	1.10	—	—	2.3	2.5	3.5	2.1	4.4
A	1.35	—	—	0.8	0.8	0.4	3.3	1.8
Cistrostadienol	1.50	—	—	6.0	7.0	3.5	6.2	6.7
Triterpene alcohols								
Lanosterol	1.06	1.2	3.6	—	—	—	—	—
β-Amyrin	1.08	—	—	1.1	1.5	0.5	1.2	0.9
Cycloatenol + α-amyrin	1.25	—	—	3.4	4.2	2.5	4.5	4.3
B	1.30	—	—	1.3	1.6	0.8	0.6	2.4
24-Methylene-cycloartenol	1.36	—	—	2.3	2.1	1.5	3.2	2.4
Cyclobranol	1.65	—	—	Trace	Trace	Trace	Trace	0.2

[a] Relative to β-sitosterol.

[b] Systematic names:
Cholesterol: 5β-Cholestene-3β-ol.
Campesterol: [24R]-24-methyl-Δ^5-cholestene-3β-ol.
Stigmasterol: [24R]-24-ethyl-$\Delta^{5,22}$-cholestadiene-3β-ol.
β-Sitosterol: [24R]-24-ethyl-Δ^5-cholesten-3β-ol.
Δ^5-Avenasterol: [24Z]-24-ethylidene-Δ^5-cholesten-3β-ol.
Δ^7-Stigmastenol: [24R]-24-ethyl-Δ^7-cholesten-3β-ol.
Δ^7-Avenasterol: [24Z]-24-ethylidene-Δ^7-cholesten-3β-ol.
Obtusifoliol: 4α,14α-dimethyl-24-methylene-Δ^8-cholesten-3β-ol.
Cycloeucalenol: 4α,14α-dimethyl-9,19-cyclopropane-24-methylene-cholesten-3β-ol.
Gramisterol: 4α-methyl-24-methylene-Δ^7-cholesten-3β-ol.
Cistrostadienol: 4α-methyl-[24Z]-24-ethylidene-Δ^7-cholesten-3β-ol.
Lanosterol: lanosta-$\Delta^{8,24}$-dien-3β-ol.

A major component at RRT 1.21 overlapped another at about RRT 1.27. Mass-spectral scans across these fused peaks showed them to be cycloartenol (mw 426, *m*-15, strong 302 and 287) and α-amyrin (mw 426, *m*-15, *m*-18, *m*-18-15, 365, 286, base peak 218) respectively. They have been combined in Table 2. An unidentified component, B, at RRT 1.30 gave a mass spectrum almost identical to the preceding α-amyrin. It is approximately estimated in Table 2. A major component at RRT 1.36 gave mass spectral data (mw 440, *m*-15, *m*-18, *m*-15-18, strong 379, 327, strong 300, base peak 216) that matches that for 24-methylene-cycloartenol RRT 1.38.[5]

Traces of a component RRT 1.65, were detected in the margarines which may be cyclobranol, as found in soyabean, sunflower and safflower oils at RRT 1.68.[5–7] The only triterpene alcohol found in the butters had RRT 1.06. Its mass spectrum and retention time corresponded exactly with that of a sample of lanosterol extracted from wool grease. The molecular ion is at *m/e* 426 and there are strong *m*-15 and *m*-15-18 peaks. The other major ions are 341, 311, 273, 271, 259, 255, 241, 229, 109 (base peak) and 71.

Lanosterol, dihydrolanosterol and β-sitosterol have been identified in the unsaponifiable fraction of milk fat.[8] Quantitative data were not presented but the latter two components were apparently present in quantities less than 10% of the lanosterol. These trace amounts were not detectable in our butter samples.

4. Discussion

The cholesterol levels in butter and soft butter are comparable and in broad agreement with literature values for milk fat.[12,13]

The higher figure for lanosterol in soft butter may be a result of regional variation in milkfat composition or of the fractional crystallisation manufacturing process. No significant fractionation of cholesterol is apparent. The total sterol plus triterpene alcohol contents of the margarines are fairly similar and exceed the levels in butter by about a factor of two. American margarine (not necessarily polyunsaturated) is reported to contain 165–279 mg %, corn oil 861–1443 mg %, safflower oil 343 mg %, and soyabean oil 990 mg % wet weight sterols as determined by the Liebermann–Burchard colour reaction.[13,14]

When converted to a dry weight basis our results range from 440 to 600 mg % which is the range reported for soyabean, sunflower and safflower oils.[5] The composition of Dixibell margarine is 70% sunflower oil and 30% partially hydrogenated soyabean oil. The expected sterol and triterpene alcohol composition of the mixture of oils was calculated from their published individual composition[5] and the result is listed in Table 2 as sunflower/soy 70:30. It can be seen that there is a very close correspondence between this composition and that measured for the Dixibell sample in this study. This result indicates that the manufacturing process, which includes heat deodorisation and partial hydrogenation has not appreciably changed the sterol and triterpene alcohol composition. There is no evidence for significant hydrogenation of the margarine sterols. For example hydrogenation of the side chain of stigmasterol would lead to β-sitosterol but as shown in Table 2 there is no significant drop in stigmasterol relative to sterols with a saturated side chain (campesterol, β-sitosterol, Δ^7-stigmasterol) when compared with the 70:30 sunflower/soyabean oil mixture. The slightly higher β-sitosterol value and lower triterpene alcohol values in the margarine are probably not significant. The oil composition of the other margarines is not precisely known although a similar mix of partially hydrogenated soyabean oil and sunflower or safflower oils is involved. The sterol composition of Meadow-Lea (NZ and Australia) and Miracle margarines are remarkably similar to that of Dixibell indicating their similar origin. Safflower and sunflower oils have very similar sterol and triterpene alcohol compositions, the only significant difference being a lower citrostadienol content for safflower oil.

Differentiation between these sources of oil is therefore difficult although the lower value for citrostadienol in the Australian Meadow-Lea margarine compared with the New Zealand Meadow-Lea may indicate the use of safflower oil in the Australian product.

Australian polyunsaturated margarine is reported by Parodi to contain 440–500 mg % of sterols on a dry weight basis not including 4-methyl sterols and triterpene alcohols. Parodi also measured the relative amounts of campesterol, stigmasterols, β-sitosterol and Δ^7-stigmastenol. While our results are in general agreement he found rather more Δ^7-stigmastenol and did not measure the avenasterols.[15]

Adulteration of cottonseed oil by other oils can be detected by detailed sterol composition analysis[16] and the contrary adulteration of butter by oils containing β-sitosterol is also readily determined.[17] The detailed composition analysis of butters and margarines also shows that adulteration of the vegetable product by animal product and vice versa would be readily apparent.

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Chemical Methods for the Reduction of the Purine Content of Baker's Yeast, a Form of Single-cell Protein

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Chemical procedures, suggested by methods formerly used to prepare yeast RNA, or in the analysis of nucleic acids, were applied to the preparation of nucleic acid-reduced baker's yeast. Water at 80–100°C extracted nucleotides but no nucleic acid from the cell, with a loss of 20% total solids and 16% total nitrogen. Water at 120°C removed part of the nucleic acid, and nearly all was extracted by 5% NaCl solution at 120°C. At room temperature, 0.5 N-HCl removed RNA from a suspension of heat-killed yeast. NaOH at a pH of about 12.5 extracted RNA from suspensions of fresh or of heat-killed yeast. In both cases the cells lost 25–35% solids and a similar proportion of crude protein (Kjeldahl N). The RNA-reduced yeast preparations may require further treatment, such as extraction with organic solvents, to improve appearance or palatability.

1. Introduction

As discussed previously,[1] single-cell protein (SCP, the dried cells of yeast, filamentous fungi, bacteria, or algae) commonly has a high content of nucleic acid, the purines from which are metabolised in the human body to uric acid. Unless steps are taken to reduce the nucleic acid content, only limited amounts of SCP (10–30 g per day) may be safely incorporated in the diet. The difficulty may be circumvented by restricting SCP to use as animal feed, but this is a relatively inefficient process in terms of the protein finally made available for human consumption.

PAG Statement No. 4 on Single Cell Protein[2] states that the nucleic acid content of SCP can be reduced "by any of several methods available". Whatever the process, some drawbacks may be anticipated, providing one reason for the continued interest in the preparation of pure protein isolates rather than SCP, e.g. yeast protein, by the Anheuser-Busch[3] and other[4] processes. SCP has to be a low-cost product; extra processing steps tend to erode any competitive advantage over other forms of protein. If cells are made sufficiently permeable for nucleic acid or its breakdown products to diffuse out, other constituents will be lost, e.g. nutritionally valuable amino acids and vitamins, though these may be returned to the fermenter in which SCP is propagated and partially re-assimilated.[5] The effluent disposal problem, already severe in the fermentation industries,[6] may be aggravated. A related problem is smell, an important consideration in yeast-processing plants.[7] Recovery and drying processes may be complicated, while the taste, colour and functional properties of the product may be altered, in undesirable as well as in desirable ways. With autolytic procedures for the reduction of nucleic acid content, there is also the risk of microbiological contamination.

An appreciable number of processes for the reduction of the nucleic acid content of SCP preparations have been published, often in the form of patent specifications. They may be divided into chemical and enzymic methods, the latter including autolytic procedures which rely on the RNase of the SCP microorganisms themselves. The variety is an indication of current interest, but also

suggests that many procedures are applicable only to a particular type of microorganism, and perhaps one grown on a particular medium and harvested at a certain stage of the growth cycle. Disadvantages, such as those mentioned above, are rarely discussed.

We therefore decided to investigate some general methods for removing nucleic acid from SCP, using, because of its ready availability, baker's yeast (*Saccharomyces cerevisiae*). This is probably more resistant to extraction procedures of all types than SCP harvested from continuous fermenters in an actively growing condition. It was thought that the findings might assist in the assessment of the future potential of SCP for food use, especially in developing countries.

2. Experimental

2.1. Baker's yeast

This was obtained from the United Yeast Co. Ltd, Bushey Road, Raynes Park SW20. The 450 g blocks were stored at 4°C until use. The dry matter content was 28–29%, total (Kjeldahl) N 8.8–9.2%, and total purine content 6.5–7.2%, expressed as RNA.[1] Most experiments were carried out with suspensions containing 20% w/v pressed yeast.

2.2. Analytical methods

Measured amounts of yeast suspension were centrifuged and the cell residue washed twice with water. *Dry matter content* of the cells was determined by weighing the residue left after drying in a fan-ventilated oven for 20 h at 105°C. *Total nitrogen* estimation was by a Kjeldahl method, with copper sulphate and selenium dioxide as catalysts. *Nucleic acid* was computed from the results of the determination of total adenine and guanine by a slight modification of the procedure described previously.[1] Solutions of the bases in 1 N-perchloric acid were mixed with an equal volume of 1 N-K_2HPO_4 and the $KClO_4$ precipitate filtered off. The filtrate was applied by means of a 0.5 ml sample loop to a 6.5 × 160 mm column of Bio-Rad AG 50W X4 cation exchange resin, and the purines eluted with 0.5 M-KH_2PO_4. The absorbance at 254 nm of the eluate was recorded on an ISCO (Instrument Specialities Company, Lincoln, Nebraska, USA) UA-5 Absorbance Monitor and Type 6 Optical Unit. Peak heights, which were reproducible to 1.5% (s.d.) were compared with those from a standard solution of adenine + guanine.

Occasionally citrate buffers were also used. Citrate buffer pH 4 + 0.5 M-NaCl, and the appropriate alkaline citrate solution for neutralisation[1] were used, in conjunction with a larger column (10 × 200 mm) to test for *cytosine*. A pH 3 buffer (0.125 M-citric acid + 0.025 M-sodium citrate) + 0.5 M-NaCl gave well-separated peaks for *xanthine* and *hypoxanthine*. The composition of this eluant was designed to give the same base-line absorbance as the pH 4 + NaCl system with which guanine and adenine were eluted.

2.3. Recovery of product

Most experiments were on a small scale but occasionally 400 g lots of yeast were processed. The treated cells were collected on a 300 ml capacity basket-head centrifuge (solid bowl) operated at 100 ml/min. The cells were washed twice with 2 litres of water and, after aspirating excess liquid from the bowl, the final centrifuge cake was freeze-dried *in situ*.

3. Results

3.1. Effect of heat on yeast suspensions

Most constituents of pressed baker's yeast are retained within the cell by a lipoprotein cell membrane which is permeable to water, but exhibits high selectivity towards other materials. Washing with cold water removed 3.8% of total solids, none of which contained guanine or adenine.

On heating aqueous suspensions of yeast to 80–100°C for 2–20 min the membrane was ruptured, allowing coenzymes and other cell constituents of low molecular weight to diffuse into the medium.

In one case the extracellular fluid contained 16.4 μmol adenine/g initial yeast solids (24% of total) and only 1.6 μmol/g of guanine (3% of total).

The washed cell residue was considerably shrunken, and somewhat darker than the original yeast both when hydrated, and after drying. The colour of dried yeast has complex determinants, depending on surface roughness, air-spaces within the particles, and the opacity of the cell wall; preparations of isolated cell walls dry to a transparent glass (unpublished work). The ratio of adenine to guanine + adenine was 48.2% (s.d. 0.3, 6 samples) as against 55% for the pressed yeast: this agrees with the percentage found in a preparation of yeast RNA (47%).[8] Thus nucleotides, but not macromolecular RNA, were extracted by water at 80–100°C. The method of heating—by adding yeast to water at 80–100°C, by immersing a flask of suspension in a bath of water, by pumping yeast suspension through a stainless steel coil surrounded by water at 80–90°C—was not critical. The cells lost about 20% of total solids and 15% of total N. On centrifuging, the cell residue was often covered by a loose, greyish, fluffy layer of unknown origin.

When the extraction temperature was raised to 120°C both nucleotides, and about one-half of the macromolecular RNA, were removed from the cells (Table 1). The additional loss of solids was about 6%, or 3 g per g RNA extracted.

Table 1. Reduction of RNA content of yeast by autoclaving for 15 min at 120°C

Sample	Suspension medium	Total solids recovery (%)	Guanine (μmol/g yeast taken)	Adenine (μmol/g) yeast taken)	Total purines	
					As RNA (%)	Recovery (%)
Pressed yeast	—	100.0	46.5	58.4	6.75	100.0
Washed cells after autoclaving	Water	73.8	29.0	30.0	5.14	56.2
	1.0 M-NaCl	69.0	5.9	6.6	1.14	12.0
	Water	69.4	24.4	25.4	4.62	47.5
	0.4 M-NaCl	66.3	12.1	12.6	2.39	23.5
	0.8 M-NaCl	66.1	4.5	4.8	0.91	8.9
	1.2 M-NaCl	66.8	3.2	4.2	0.71	7.2
	1.6 M-NaCl	67.2	2.6	3.3	0.57	5.6
	2.0 M-NaCl	69.1	3.9	3.4	0.70	7.0

A sour, unpleasant smell was produced when yeast suspensions were heated.

3.2. Extraction of RNA by heating yeast with NaCl solution

Clarke and Schryver[9] described a method for the preparation of RNA from yeast whereby alcohol-extracted, air-dried yeast was stirred with 10% NaCl solution at 60–80°C for 4–5 days. In the last 20 years a number of variations of the method have been described in Japanese patent specifications; it is said to be used in that country to prepare RNA for use in the manufacture of 5′-nucleotides[10] (flavour-enhancing agents).[11]

Baker's yeast lost 53.1% of its purines, 26.8% of its total solids, and 26.2% of its total N when a suspension in 0.5 M-NaCl was heated for 0.5 h at 75°C. At 120°C the loss of RNA was increased, and exceeded 90% provided the strength of the NaCl solution was greater than 0.8 M (5% w/v; Table 1).

Heating with salt solutions appeared to be less effective when yeast was first heated at 80°C to remove nucleotides.

3.3. Extraction of RNA with NaOH solutions at room temperature

This is the classical method of preparing yeast RNA, and has been widely used for that purpose

commercially.[12] A number of patent specifications describe variations of the process, often being concerned with methods of recovery, e.g. as the calcium[13] or iron[12] salts.

With excess alkali the extraction is rapid. Thus, analysis of the washed cell residue obtained after a 20% suspension of pressed yeast had been treated with an equal volume of 1 N-NaOH for 15 min showed that 94% of purines had been extracted, together with 30% of the cell solids and 35% of Kjeldahl N. As prolonged exposure caused loss of protein, the effect of adjusting the pH before harvesting the cells was investigated (Table 2). The pH could be lowered to 7 without appreciable effect, but at pH 5 the reduction of yeast RNA content was impaired.

Table 2. Effect of final pH on RNA content of alkali-treated[a] yeast suspension

Sample	pH after adjustment	Dry matter recovery (%)	Guanine (μmol/g yeast taken)	Adenine (μmol/g yeast taken)	Total purines	
					As RNA (%)	Recovery (%)
Yeast before treatment	—	100.0	43.5	53.2	6.2	100.0
Washed cells	>12	66.8	2.5	3.3	0.6	6.0
	9.0	73.3	3.8	5.4	0.8	9.5
	7.0	75.6	4.3	5.9	0.9	10.5
	5.0	77.0	15.8	16.9	2.7	33.8

[a] 10 g pressed yeast in 50 ml water agitated with 10 ml 1 N-NaOH for 30 min at 24°C, then pH adjusted with HCl. Cells collected by centrifuging, washed twice.

When a suspension of yeast (100 g = 28.3 g dry matter + 200 ml water) was titrated with 1 N-NaOH, the pH rose steeply until after 12 ml had been added it reached 11.7. The pH then remained steady when more NaOH was added, due probably to the membrane of one cell after another breaking down and allowing contact between the titrant and intracellular protein. pH values drifted downwards with time. If the yeast suspension was first heated (10 min at 100°C) to destroy the permeability barrier, addition of NaOH initially caused a relatively slow increase in pH, but, after 45 ml the pH was the same as with yeast which had not been heat-treated. This is a measure of the amount of NaOH required just to destroy the membrane in all the yeast cells (under the conditions of the experiment). With heat-treated yeast, pH values were stable.

Washed, heat-treated cells required half as much alkali to establish a pH in the range 9–12 as compared with the original heat-treated suspension. In one case 32.5 ml of 1 N-NaOH was added to a suspension of washed, heat-treated cells from 100 g pressed yeast to give a pH of about 12.5. After 30 min the pH was brought to 9 and the cells centrifuged and washed. They contained only 0.5% RNA, with a solids recovery of 68%. From analyses on washed, heat-treated yeast cells, it was computed that the treatment at pH 12.5 had extracted 1.83 g of RNA. By acidifying the alkali extract to pH 2, a crude (61% pure) RNA preparation was recovered, accounting for 1.41 g of RNA.

3.4. Extraction of RNA from yeast with HCl solutions

Nucleic acid can be extracted from single-cell protein by perchloric acid or trichloroacetic acid (0.5–1.0 M), both of which are protein precipitants, over a wide range of temperatures, a property which is applied for analytical purposes.[1] A procedure for reducing the nucleic acid content of (bacterial) SCP has been patented,[14] although its commercial feasibility must be doubted.

Commercially, mineral acids such as hydrochloric acid are the only acids likely to be used. Refluxing yeast with 1–2 N-HCl was the original extraction method used in the analysis of purine content. A less drastic treatment suffices for a considerable reduction in RNA content. Thus, a 20% suspension of baker's yeast heated for 0.5 h at 75°C with an equal volume of 1 N-HCl lost 98%

of its total purines, together with 38.9% solids and 35.3% Kjeldahl nitrogen. Loss of protein when yeast is heated with HCl is said to be reduced by the addition of alcohol.[15]

The cell membrane of yeast was resistant to the action of HCl in the cold. When 10 ml of 1 N-HCl was added to a suspension of 100 g pressed yeast in 200 ml water, the pH was 2.1; if the membrane had been destroyed by heat, the pH was 4.7. Unlike the case with alkali, this pH difference still existed when as much as 60 ml acid had been added. For this reason, experiments on the extraction of RNA by HCl at room temperature were confined to heat-treated yeast.

As shown in Table 3, a concentration of about 0.5 N-HCl was required to reduce the purine

Table 3. Effect of acid concentration on the extraction[a] of RNA from a heat-treated suspension of baker's yeast

Concentration of HCl, mol/ litre suspension	pH	Dry matter recovery[b] (%)	Guanine (μmol/g yeast taken)	Adenine (μmol/g yeast taken)	Total purines	
					As RNA (%)	Recovery[b] (%)
0	6.1	80.3	47.6	43.1	7.27	81.1
0.25	1.1	79.8	40.3	36.2	6.17	68.4
0.50	0.7	74.7	4.8	3.3	0.69	7.2
1.00	0.4	72.2	0.9	0.4	0.11	1.1

[a] Yeast suspension (20% w/v pressed yeast) heated 2 min at 80°C (stainless steel coil) and cooled, then mixed with 25 ml acid and agitated 20 h at room temperature.

[b] Compared with pressed yeast containing 50.4 μmol/g guanine and 61.4 μmol/g adenine, and 28.4% dry matter.

content of yeast by more than 90%. The loss in solids was surprisingly low at 25%, which raises some questions as to the form in which the purines were extracted.

Treatment of yeast with acid produced a sharp, unpleasant odour.

4. Discussion

The results presented here show that it is feasible to prepare, from commercial baker's yeast, SCP which has a content of nucleic acid of less than 2% together with a crude protein content which is not less than that of the starting material. The effects of the treatment on the protein itself are not known, though Lindblom[16] has discussed the influence of alkali and heat treatment on yeast protein. It is probably the fear of such effects which underlies the present trend, in patent specifications and other publications, towards autolytic methods for the reduction of nucleic acid content. Chemical methods, however, are likely to be more generally applicable than autolytic procedures.

The SCP preparations were sometimes dark in colour or possessed an unpleasant sharp smell or taste, features referable, it is thought, to lipid components. Extraction of these with an organic solvent may be necessary for a really attractive product.

In all cases about 30% of cell solids were lost, two-thirds of which were low-molecular weight constituents which diffused from the cell when the membrane was destroyed by heat or chemical action. These would include vitamins and free amino acids, both nutritionally valuable constituents of yeast. These substances, which account for some 15% of total yeast nitrogen, can be removed in advance of the RNA reduction step by heating a yeast suspension briefly to 80–100°C, and used in the preparation of yeast extract, or returned to the propagation fermenter as a contribution to the growth medium. Some protein is lost together with RNA, but the amount is quite small, as the overall loss in cell N is about 30%, and a 90% reduction in nucleic acid content would account for 10%, or a third of this. Extraction with alkali or with salt solutions has the advantage that RNA can be recovered as a by-product.

Reduction of RNA content with acid is not thought to be commercially attractive because of the expense of acid-resistant equipment, and of the effluent disposal problem. Alkali treatment may require careful timing, but because of its rapidity could probably be readily put on a continuous basis. The alkaline extraction of yeast which has been treated with isopropyl alcohol has been patented[17] as has been a variant of the procedure in which ammonia is the alkali.[18] Autoclaving yeast with water or with salt solutions appears to be fairly straightforward; Russian[19] and Japanese[20] patents for the preparation of RNA from yeast by such a procedure have been taken out.

It is clear, however, that all procedures must add considerably to the cost of SCP both directly and indirectly (through loss of material), and are likely to be technologically fairly sophisticated.

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Efficiency of Bacterial Protein Synthesis in the Rumen of Sheep Receiving a Diet of Sugar Beet Pulp and Barley

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Eight sheep fitted with both rumen and duodenal re-entrant cannulae were used to investigate the digestion of a diet consisting of 60% molassed sugar beet pulp and 40% ground barley. Molar proportions of acetic acid in the rumen varied from 55 to 66%, of propionic acid from 15 to 26% and of butyric acid from 12 to 19%. On average, 69.1 ± 1.2% of the dietary organic matter was digested in the rumen and 89.9 ± 0.4% in the whole digestive tract. Corresponding figures for gross energy were 64.4 ± 1.4% and 87.4 ± 0.4%, for cellulose 82.8 ± 1.4% and 86.3 ± 0.8% and for α-linked glucose polymers were 91.6 ± 0.9% and 100 ± 0%. There were only small differences in the extent of digestion of these constituents in the rumen between animals and the mean production of total short-chain fatty acids was 55.5 ± 1.6% of the digestible energy.

The mean daily amount of nitrogen entering the duodenum was 11.1 ± 5.1% greater than the dietary intake and the apparent digestibility of nitrogen was 76.7 ± 1.0%. Calculation of bacterial protein using α-ε-diaminopimelic acid as a marker, indicated that bacterial protein constituted about 80% of the total protein entering the duodenum and that the average efficiency of rumen protein synthesis was 14.31 ± 0.75 g crude protein/100 g OM "disappearing" in the rumen. With the exception of one animal in which the rumen ammonia concentration was especially high, the efficiency of protein synthesis varied between animals over only a narrow range and there was no evidence that efficiency was influenced by variation in the pattern of fermentation in the rumen.

1. Introduction

Ishaque *et al.*[1] found that in sheep given a high-concentrate diet of hay, barley and flaked maize there were marked variations between animals in the efficiency of synthesis of bacterial protein in the rumen. High rates of protein synthesis were correlated with a high molar proportion of propionic acid[1] and a low molar proportion of acetic acid[2] in the mixture of rumen acids, and with a low ruminal digestion of cellulose and gross energy. The object of the experiment reported here was to determine the efficiency of ruminal protein synthesis in animals receiving a diet of a different type to that used by Ishaque *et al.*, but one with which there were substantial between animal differences in the composition of the mixture of short-chain fatty acids in the rumen. Preliminary studies indicated that a diet of molassed sugar beet pulp and ground barley met these requirements.

2. Experimental

2.1. Animals and their management

Eleven cross-bred wether sheep 12–18 months old were used. All the animals were surgically prepared with rumen cannulae and eight of the animals were also fitted with duodenal re-entrant cannulae. Four of the doubly cannulated animals weighed about 26 kg each, the remaining animals weighed about 45 kg. Food was provided each day in four equal meals at 06.00, 10.00, 16.00 and

22.00 hours at a level calculated to provide 1.3 times the metabolisable energy requirement for maintenance.[3] There was free access to mineral licks and to drinking water but to minimise fluctuations in fluid input into the rumen due to intermittent drinking all animals were given a continuous intraruminal infusion of water at a rate calculated from their *ad libitum* intake (1.5–2.00 litres/day). Under these conditions they drank little.

2.2. Experimental diet

The experimental diet was a mixture of 60 parts molassed sugar beet pulp and 40 parts coarse ground barley, both constituents being taken from single uniform batches of material. Chromic oxide powder which was used as an indigestible marker was thoroughly mixed with the barley at a rate sufficient to provide each animal with a chromic oxide intake of 2 g/day. The concentrations of some chemical constituents in the barley, the sugar beet pulp and the complete diet are given in Table 1.

Table 1. Concentration of some chemical constituents in the experimental foods and in the complete diet (g/100 g)

	Dry matter (DM)[a]	Ash (in the DM)	Cellulose (in the DM)	α-linked glucose polymers (in the DM)	Nitrogen (in the DM)	Gross[b] energy (in the DM)
Sugar beet pulp	86.9	7.79	17.31	6.81	1.79	17.25
Barley	83.6	3.13	5.79	63.4	1.73	18.54
Complete diet	85.6	5.93	12.70	31.2	1.77	17.75

[a] In the fresh material.
[b] kjoules/100 g.

2.3. Experimental plan and procedures

The main part of the experiment consisted of eight determinations of the digestion of dietary constituents in the rumen and intestines, each conducted with one sheep. The determinations were made in two sets, each with four animals, carried out about 4 months apart. In each determination the procedure was the same. Each sheep was established on the experimental diet and then maintained at a constant level of feeding for a period of at least 14 days. Faecal collections were made over a further period of 5 days. Following this, samples of duodenal digesta were taken at intervals of 4 h throughout a 48 h period. On each occasion the cannula was opened, the fluid in the cannula allowed to drain, and a fresh sample of 60 g of digesta collected from the proximal duodenum. Following the completion of the collections of duodenal digesta, small samples of rumen fluid were taken on two consecutive days at 10.00, 12.00, 14.00 and 16.00 hours.

In the subsidiary part of the experiment the three animals with rumen fistulae only were used to provide samples of about one litre of rumen fluid for the preparation of isolated bacteria. Following their establishment on the experimental diet for a period of at least 14 days the animals were sampled over a period of 1 day. Sampling was repeated after an interval of 7 days and for 1 sheep again after a further 7 days.

2.4. Preparation and analysis of samples

Samples of duodenal digesta taken over a 48 h period were bulked and homogenised. An aliquot was taken for analysis for ammonia and non-protein nitrogen and the remainder stored at −20°C. Samples of faeces obtained daily were subsampled, an aliquot preserved in hydrochloric acid for analysis for nitrogen and the remainder bulked to provide a sample for the determination of other chemical constituents. In the main part of the experiment, the pH of rumen fluid was taken immediately following its withdrawal from the rumen. The digesta was filtered through muslin and an aliquot taken for the determination of ammonia. The remainder was centrifuged at 3000 rev/min

and the supernatant stored at −20°C. In the subsidiary part of the experiment rumen fluid was filtered through muslin and centrifuged at 1500 *g* to remove food particles and protozoa and then centrifuged at 22 000 *g* to isolate the bacteria. The bacteria were washed with saline, re-centrifuged and dried.

Samples of feed, digesta, faeces and bacteria were analysed as appropriate for dry matter by drying to constant weight in a forced draught oven at 60°C, for ash by ignition at 550°C, for calorific value using an adiabatic bomb calorimeter and for nitrogen by a Kjeldhal method. Non-protein nitrogen and ammonia were determined in tungstic acid filtrates by Kjeldhal analysis and by the method of Sweetsur[4] respectively. Determinations were also made of cellulose,[5] α-linked glucose polymers,[6] soluble sugars,[7] chromic oxide[8] and total and individual short-chain fatty acids.[9] Samples for amino acid analysis were oxidised with performic acid and hydrolysed by refluxing with 6 N hydrochloric acid for 24 h under an atmosphere of nitrogen. Separation and determination of the individual acids was carried out using an automatic amino acid analyser (LKB—Biocal BC 100) following the procedure described by Moore *et al.*[10]

2.5. Calculation of results

The passage of constituents to the duodenum was calculated by reference to the duodenal flow of chromic oxide.[11]

Production rates for the rumen short-chain fatty acids were calculated from the disappearance of gross energy in the stomach and the composition of the mixture of rumen acids using the stoichiometric equations suggested by Hungate.[12] The amount of bacterial crude protein in the duodenal digesta was calculated from the α-ε-diaminopimelic acid concentration.[13]

3. Results

There were difficulties in obtaining a free flow of digesta from the duodenal cannula in one of the first group of sheep. Subsequently the results for this animal were found to be anomalous and were excluded. The values for the remaining 7 animals are summarised below.

3.1. Digestion of organic matter, gross energy and carbohydrate constituents

The results for the digestion of organic matter and energy are given in Table 2. For both constituents, differences in faecal loss between animals were small and there were only minor variations in the extent of digestion of the constituents in the rumen. For α-linked glucose polymers, digestion in the whole tract was invariably complete and in each sheep about 90% of the polymers consumed were fermented in the rumen (Table 3). For cellulose, rumen digestion was less extensive (Table 3) and more variable. In some animals the quantity of cellulose passing to the duodenum was similar to that lost in the faeces but in others there was evidence of digestion of cellulose in the intestines.

3.2. Fermentation in the rumen

The composition of the mixture of short-chain fatty acids in the rumen was fairly constant in each sheep from time to time but there was considerable variation in the composition of the mixture of acids between animals (Table 4). In animal 6, rumen pH was especially low but this appeared to have little significance in relation to the composition of the mixture of fatty acids formed. In contrast to other studies,[1,15] there was no relationship between the rumen ammonia concentration and the mixture of rumen acids, although in one animal (515) rumen ammonia levels were relatively high.

As indicated by the molar proportions of fatty acids present, there were marked differences in the relative production of individual acids between sheep but except for animal 515 the production of total fatty acids was relatively constant between animals at about 54% of the digested energy (Table 4).

3.3. Digestion of nitrogenous constituents

With the exception of animal 515, the nitrogen entering the duodenum in each animal was greater

Table 2. The amounts of organic matter (g/day) and gross energy (Mjoules/day) consumed, entering the duodenum and excreted in the faeces in each animal and the proportions (%) of the intake digested in the stomach and in the whole digestive tract

Animal no.	Intake	Duodenum	Faeces	Proportion digested	
				In the stomach	In the whole tract
Organic matter:					
512	332	114	37	65.7	88.9
515	322	79	29	75.5	91.0
28	419	136	40	67.5	90.4
37	555	178	56	67.9	89.9
6	634	196	75	69.1	88.2
15	634	203	57	70.0	91.0
47	655	211	68	67.8	89.6
Mean with standard error				69.1 ±1.2	89.9 ±0.4
Gross energy:					
512	6.28	2.46	0.83	60.8	86.8
515	6.08	1.70	0.78	72.0	87.2
28	7.92	2.87	1.00	63.8	87.4
37	10.48	3.76	1.29	64.1	87.7
6	11.98	4.14	1.68	65.4	86.0
15	11.98	4.38	1.30	63.4	89.1
47	12.38	4.80	1.55	61.2	87.5
Mean with standard error				64.4 ±1.4	87.4 ±0.4

Table 3. The amounts of cellulose and α-linked glucose polymers (g/day) consumed entering the duodenum and excreted in the faeces in each animal and the proportions (%) of the intake digested in the stomach and in the whole digestive tract

Animal no.	Intake	Duodenum	Faeces	Proportion digested	
				In the stomach	In the whole tract
Cellulose:					
512	45	7	8	84.4	82.2
515	44	6	6	86.4	86.4
28	57	7	7	87.7	87.7
37	76	15	10	80.3	86.9
6	85	19	13	77.6	84.8
15	85	17	10	80.0	88.4
47	88	15	11	82.9	87.8
Mean with standard error				82.8 ±1.4	86.3 ±0.8
α-Linked glucose polymers:					
512	111	14	0	87.4	100
515	107	8	0	92.5	100
28	139	11	0	92.1	100
37	184	10	0	94.6	100
6	211	21	0	90.0	100
15	211	13	0	93.8	100
47	218	20	0	90.8	100
Mean with standard error				91.6 ±0.9	100 ±0.0

Table 4. The mean pH, ammonia nitrogen concentration, molar percentage of short-chain fatty acids and production rates of short-chain fatty acids in the rumen in each sheep

			Molar percentage of						Short-chain fatty acid production	
Animal no.	pH	NH_3-N (mg/100 ml)	Acetic acid	Propionic acid	Iso-butyric acid	Butyric acid	Iso-valeric acid	Valeric acid	Moles/day	% of digested energy
512	6.43	13.6	58.5	21.2	0	19.1	0	1.2	2.27	53.0
515	6.48	17.3	55.5	25.5	0	16.9	1.0	1.1	2.20	63.7
28	6.58	11.6	60.8	25.6	0.2	12.4	0.1	1.0	3.19	57.6
37	6.24	10.0	61.9	18.4	0.6	17.6	0.5	1.1	4.04	54.4
6	5.51	12.0	57.7	23.5	0.4	16.0	1.1	1.3	4.78	56.1
15	6.39	14.8	63.5	18.6	0.9	14.7	1.3	1.0	4.70	53.0
47	6.63	10.4	66.1	15.3	0.9	16.0	1.0	0.8	4.62	51.0
Mean with standard error	6.32 ±0.14	12.8 ±1.0	60.6 ±1.3	21.2 ±1.5	0.4 ±0.15	16.1 ±0.8	0.7 ±0.2	1.1 ±0.06	— —	55.5 ±1.6

than the nitrogen intake, the increase varying from 4 to 28% (Table 5). Faecal losses also differed between sheep but variations were smaller than in duodenal flow.

In Table 6 results are given for the constituent fractions of the duodenal nitrogen and for the amino acid composition of the dietary ingredients, the complete diet and the samples of duodenal digesta. Values for the amino acid composition of rumen bacteria isolated in the subsidiary part of the experiment are also included.

There were substantial differences between animals in the concentration of ammonia and non-protein nitrogen in the duodenal digesta but there was no clear pattern of association between the two fractions. The amino acid composition of the digesta was similar in the 7 animals but there were differences in the concentration of some amino acids which could be of nutritional significance. The average concentration of methionine for example was 2.25 g/16 g N but values as low as 1.61 g/16 g N and as high as 2.74 g/16 g N were recorded. For most amino acids the concentration in the digesta was intermediate between that in the food and that in the rumen bacteria, the values being biased towards the latter. However, the concentration of glycine tended to be higher than in either the bacteria or the food. A high bacterial protein content in the digesta was indicated by the

Table 5. Amount of nitrogen (g/day) consumed, entering the duodenum and lost in the faeces in each animal and the proportions of the intake digested in the stomach and in the whole digestive tract

				Proportion digested	
Animal no.	Intake	Duodenum	Faeces	In the stomach	In the whole tract
512	6.24	8.00	1.62	−28.2	74.0
515	6.05	5.29	1.34	12.6	77.8
28	7.88	9.39	1.84	−19.2	76.6
37	10.43	11.80	2.63	−13.1	74.8
6	11.92	12.38	3.16	−3.9	73.5
15	11.92	12.62	2.46	−5.9	79.4
47	12.31	14.80	2.38	−20.2	80.7
Mean with standard error				−11.1 ±5.1	76.7 ±1.0

Table 6. The amino acid composition (g/16 g N) of the sugar beet pulp, barley and total diet consumed, of rumen bacteria and of the duodenal digesta[a] in each animal, and the ammonia and non-protein nitrogen content (% of total N) of the duodenal digesta

Constituent	Sugar beet pulp	Barley	Complete diet	Rumen bacteria[b]	Duodenal digesta							
					Animal no.							Mean with standard error
					512	515	28	37	6	15	47	
Ammonia	—	—	—	—	7.7	8.4	5.0	7.8	3.1	5.6	5.0	6.1 ±2.4
Non-protein nitrogen	—	—	—	—	22.3	30.6	29.3	22.4	18.7	20.6	18.7	23.2 ±1.8
Amino acids:												
Aspartic acid	7.08	5.95	6.65	12.43	9.81	11.18	8.08	8.80	10.29	10.59	8.94	9.67±0.42
Threonine	7.82	4.65	6.62	4.48	4.69	5.81	4.22	3.41	4.92	5.32	4.59	4.71±0.29
Serine	5.49	4.04	4.94	3.81	4.00	4.56	4.17	3.38	4.32	4.62	4.01	4.15±0.16
Glutamic acid	9.94	18.22	13.08	10.79	10.97	11.03	9.23	9.94	12.46	10.96	9.41	10.57±0.43
Proline	5.89	6.70	6.20	2.29	3.67	2.50	1.82	2.74	3.83	3.41	3.22	3.03±0.27
Glycine	2.81	4.22	3.34	4.74	4.81	4.65	4.86	6.22	5.40	5.71	4.36	5.14±0.25
Alanine	4.37	4.44	4.40	5.85	5.66	6.18	5.03	5.67	6.30	6.82	5.65	5.90±0.21
Cystine	1.68	3.82	2.50	1.64	1.59	1.50	2.10	2.03	1.94	2.10	1.95	1.89±0.09
Valine	4.94	4.90	4.92	5.07	4.85	4.67	4.77	4.96	5.17	5.38	4.97	4.97±0.09
Methionine	2.12	3.28	2.56	2.79	2.74	2.07	1.61	1.68	2.63	2.59	2.45	2.25±0.18
α-ε-Diamino-pimelic acid	—	—	—	0.77	0.55	0.62	0.59	0.65	0.64	0.64	0.63	0.62±0.01
Iso-leucine	3.25	3.81	3.47	5.48	4.30	4.71	4.58	4.30	4.98	5.25	4.85	4.71±0.13
Leucine	5.52	6.57	5.92	6.01	5.05	5.51	6.15	5.12	6.30	5.97	5.81	5.70±0.19
Lysine	4.27	3.12	3.84	8.15	7.09	7.91	5.58	8.89	8.11	8.27	7.17	7.57±0.41
Arginine	4.98	3.90	4.57	4.31	4.14	4.02	3.83	3.23	4.73	4.44	4.40	4.11±0.19

[a] g/16 g non-ammonia nitrogen.
[b] Mean value for 7 samples.

duodenal concentration of α-ε-diaminopinelic acid. Calculation of bacterial crude protein using this acid showed that 71–83% of the duodenal non-ammonia nitrogen was bacterial in origin (Table 7). The rates of bacterial protein synthesis in the rumen necessary to account for this contribution are shown in Table 8 in relation both to the amount of organic matter "disappearing" in the rumen and to the amount fermented, that is allowing for the organic matter incorporated into the microbes. In sheep 515, the rate of synthesis was low but in other animals the efficiencies were relatively constant. Mean values were 14.31 g crude protein/100 g OM "disappearing" and 11.43 g crude protein/100 g OM fermented.

Table 7. Bacterial nitrogen entering the duodenum (g/day) and the ratio of bacterial nitrogen to non-ammonia nitrogen in each sheep

Animal no.	Bacterial N	Bacterial N: non-ammonia N
512	5.27	0.71
515	3.90	0.81
28	6.83	0.77
37	9.18	0.84
6	9.96	0.83
15	9.90	0.83
47	11.50	0.82
Mean with standard error		0.80±0.02

Table 8. The efficiency of synthesis of bacterial crude protein (g crude protein/100 g of organic matter either "disappearing" or fermented) in the rumen of each sheep

	Bacterial crude protein	
Animal no.	g/100 g OM "disappearing" in the rumen	g/100 g OM fermented in the rumen
512	15.12	12.00
515	10.06	8.56
28	15.06	12.00
37	15.25	12.06
6	14.18	11.44
15	14.35	11.51
47	16.19	12.44
Mean with standard error	14.31 ±0.75	11.43 ±0.49

4. Discussion

With the diet of sugar beet pulp and barley used here there were variations between animals in the composition of the mixture of short-chain fatty acids in the rumen (Table 4). The variations were less extreme than those reported by Ishaque *et al.*[1] in sheep receiving a diet of ground barley, ground hay and flaked maize but were still substantial amounting to about 11 percentage units for acetic acid, 10 percentage units for propionic acid and over 7 percentage units for butyric acid. In the work of Ishaque *et al.*[1] marked variations between animals were also observed in the extent of digestion of organic matter, energy and cellulose in the rumen but in the present experiment the importance of the rumen as a site of digestion for these constituents, and for α-linked glucose polymers, was virtually constant between sheep (Tables 2 and 3).

As far as protein synthesis in the rumen is concerned, in all animals bacterial protein accounted for about 80% of the total protein entering the duodenum. However, the efficiency with which this bacterial protein was synthesised was low, on average about 14.31 g crude protein/100 g OM "disappearing" in the rumen. This figure contrasts sharply with the value of 23 g crude protein/100 g OM "disappearing" obtained in work with forage diets[15] but is similar to that reported in other experiments with high-concentrate diets.[1,16,17] Thomas[18] has summarised many of the factors known to influence the efficiency of bacterial protein synthesis in the rumen but the reason for the low efficiency of synthesis with the present diet is not clear. It is possible that the supply of nitrogen as ammonia to the rumen organisms was deficient for maximum synthesis since to achieve 23 g crude protein/100 g OM disappearing would require an entry of endogenous nitrogen into the rumen of about 0.09 g N/kg body weight/day and it is doubtful whether this quantity of nitrogen could be recycled.[19] On the other hand, the average rumen ammonia concentration recorded was 12.8 mg/100 ml, a level considered adequate for maximum bacterial growth.[20,21]

With the exception of animal 515, in which the rumen ammonia concentration was relatively high and the efficiency of bacterial protein synthesis was especially low, both the ammonia concentration and the efficiencies of protein synthesis were fairly constant between animals. This situation contrasts with that reported by Ishaque *et al.*[1] where variations in the mixture of short-chain fatty acids in the rumen between animals were closely correlated with variations in rumen ammonia level and with the efficiency of protein synthesis. Assuming that the mixture of rumen short-chain fatty acids reflects the composition of the rumen microbial population, present results indicated that the population may change without alteration in the efficiency of protein synthesis. A corresponding argument applies if the rumen fatty acids are considered to reflect variations in the metabolism rather than the composition of the population. In either case, it is clear that the composition of the mixture of fatty acids in the rumen will act as an index of the efficiency of bacterial protein synthesis under only some dietary conditions.

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Nutritive Value to the Growing Pig of Deoiled Liquefied Herring Offal Preserved with Formic Acid (Fish Silage)

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Deoiled herring silage contained an average of 17.6% dry matter (DM) and per 100 g DM: 1.86 MJ GE, 12.1 g N, 5.5 g available lysine and 3.7 g oil. The apparent digestibility coefficients for growing pigs of gross energy (GE) and nitrogen (N) for a diet containing 25% herring silage DM and 75% barley meal DM were 0.83 and 0.91, and the efficiency of retention for digested N was 0.42. The digestible energy (DE) value for the deoiled herring silage was 17.9 MJ DE/kg DM and the digestible nitrogen (DN) value was 119 g DN/kg DM.

1. Introduction

Fish silage results from the liquefaction, by naturally occurring enzymes, of fish or fish offal. Formic acid is an appropriate preservative for the silage as it reduces the liquid mass to pH 4 which is suitable for enzyme activity, is sufficient to restrict bacterial growth, and does not require neutralisation prior to feeding to animals. An assessment of the potential of fish silage as a protein source for animal diets has been made by Tatterson and Windsor,[1,2] but no nutritive values have been reported for United Kingdom fish silages preserved with formic acid. However, as judged by growth performances and carcass characteristics, fish silage made from white fish offal can replace conventional white fish meal and soya bean meal in diets for bacon pigs.[3] A study of the acceptability of the pork from pigs fed white fish offal silage[4] indicated that there was no serious deleterious effect upon pork quality.

Herring provides a suitable raw material for the production of fish silage. In the United Kingdom in 1973, 157 000 tonnes of herring were landed, 20 000 tonnes of which were not sold for human consumption. In the course of preparing the remaining 137 000 tonnes, it may be estimated that approximately a further 50 000 tonnes of herring offal was produced. As the production of fish silage requires relatively low capital expenditure, uses simple technology and poses no pollution problems, the process is particularly appropriate for fish and fish offal produced from fish processors at ports not possessing a fish drying plant.

Herring contains greater quantities of oil than white fish; in summer, the oil content of herring offal may rise to 40% of the DM. Herring oil has been shown to cause off-flavours in poultry meat.[5] These off-flavours appear to relate to the concentration of herring oil in the diet and the presence in the oil of fatty acids with more than four double bonds. A high and seasonally varying level of fish oil therefore detracts from herring silage as an acceptable protein source for growing pigs because fat quality as measured by flavour, firmness and fatty acid composition may be reduced, and an unpredictable growth response may result from the variable energy value brought about by a variable oil concentration.

Feeding trials conducted in Denmark[6] suggested that if the oil is removed from the herring, the silage may be a much more acceptable product for supplementing the protein in diets for growing pigs. In addition, herring oil of adequate quality itself forms a saleable product, although if the oil remains for any length of time in the silage prior to extraction the free-fatty acid content of herring oil rises and the iodine value falls.[1]

This report describes the extraction by centrifugation of oil from herring fish offal silage and the determination of the nutritive value for pigs of the deoiled product by chemical analysis and digestibility study.

2. Experimental

2.1. Production of deoiled herring silage

Formic acid (BP Chemicals Ltd) was added to the guts, heads, tails and skeletons of filleted herring at the rate of 35 kg/tonne. The material was minced and circulated by intermittent pumping to aid liquefaction which was completed in 24 h. The silage was heated to 75°C and centrifuged (Westfalia De-canter, CA 220-000, (SDB 230), solid bowl, horizontal centrifuge with screw conveyor) to remove solid particles. The effluent from the De-canter was reheated to 70°C and separated into solids, effluent and oil (Westfalia Separator, SAI-03-175 (SAOWH 205) pilot scale, centrifugal, self-cleaning de-sludger). The fractions other than oil were mixed to form the deoiled herring silage, with a final pH 4.0.

2.2. Digestibility and nitrogen balance study

Three diets were prepared, barley meal, barley meal with deoiled herring silage and barley meal with fish meal.

The deoiled herring silage was produced in three separate batches. White fish meal (Norsesea) was used in the control diet. A mineral and vitamin supplement was added to all diets. The quantity of herring silage fed was adjusted with the object of providing the same dry weight as that provided by fish meal. Herring silage and fish meal provided 25.2 and 25.9 g/100 g respectively of the final diet dry matter (DM). The chemical composition of the herring silages, fish meal and barley is given in Table 1.

Table 1. Chemical composition of diet ingredients used for digestibility trials

	Deoiled herring silage			White fish meal[a]	Barley meal[b]
	Batch 1	Batch 2	Batch 3		
Dry matter (DM) (%)	19.8	17.9	15.2	91.5	85.7
Gross energy (MJ/kg DM)	17.9	19.3	18.7	18.2	18.1
Nitrogen (% in DM)	11.9	12.9	11.5	11.7	1.86
Percentage of mixed diet from silage or meal[c]					
Percentage of diet DM		25.2[d]		25.9	
Percentage of diet GE		25.8[d]		26.0	
Percentage of diet N		68.6[d]		68.7	

[a] The dried white fish meal also contained in the DM 4.83% ether extract, 4.68% P, 0.73% Na, 0.82% K, 8.24% Ca, 0.22% Mg.

[b] 10 g/kg of $CaCO_3$ and 3 g/kg of trace element and vitamin mix (Coopers 10TE) were added to the barley meal.

[c] Remaining percentage is from the barley meal.

[d] Mean values for the three batches.

For the digestibility determinations, 44 Large White × Landrace castrated male pigs of 45.2 ± 0.36 kg liveweight were used. Fourteen balances, each of 10 days' duration, were completed both with the herring silage diet (4 with batch 1 silage, 4 with batch 2 silage and 6 with batch 3 silage) and with the fish meal diet. A further 16 balances were completed with the barley meal diet. Digestibility was determined as the coefficient for apparent digestibility (the difference between intake and faecal excretion expressed as a fraction of the intake). The standard procedures used for

the digestibility study were as detailed in a previous report.[7] Amino acids were determined by the method described by D'Mello.[8]

3. Results

3.1. Production of deoiled herring silage

The chemical composition of the herring silage and of the fractions obtained in the course of deoiling is given in Table 2.

Table 2. Chemical composition of the fractions obtained in course of the production of deoiled herring silage[a]

	% of original material	Dry matter (%)	% of original dry matter	Composition of the dry matter (%)							
				EE(oil)	N	Ash	P	Na	K	Ca	Mg
Herring silage	100	27.3	100	28.2	7.73	12.5	1.76	0.55	0.77	3.48	0.17
De-canter solid[b]	11.3	41.5	17.2	3.22	6.51	47.0	7.75	0.33	0.39	13.2	0.11
De-canter effluent	86.2	27.7	87.5	33.4	7.70	8.60	1.15	0.55	0.77	2.34	0.15
Separator solid[b]	1.6	21.3	1.25	44.4	8.08	11.8	1.79	0.50	0.71	3.59	0.15
Separator effluent	74.5	19.5	53.2	2.84	11.5	12.0	1.77	0.87	1.19	3.56	0.23
Separator oil	6.9	100.0	25.3	101	0.01	0.03	0.03	0.00	0.01	0.08	0.00
Deoiled herring silage[c]	87.4	22.4	71.7	3.65	10.8	16.5	2.55	0.79	1.08	4.81	0.21

[a] These values were determined by weighing and sampling the total outputs of each fraction; they do not necessarily sum to unity.

[b] Composition of the solids was more variable than other fractions owing to some being retained in the centrifuges.

[c] Mixture of De-canter solid, Separator solid and Separator effluent.

De-canter solids comprised 11% of the total mass, but contained approximately 65% of the total ash. Only 2% of total oil (determined as ether extractives (EE)) was lost in this fraction. Oil yield was 25% of the original silage DM and 90% of the total oil was extracted by the centrifugation process. The oil fraction contained traces of N (0.01%) and mineral (0.03%) contaminants. Deoiled herring silage comprised 87.4% of the original silage and 71.7% of the original silage DM. The deoiled silage contained 22.4% DM which was low in oil (3.7% of the DM) and rich in protein (10.8% N). The amino acid composition of deoiled herring silage and fish meal is given in Table 3. The FDNB availability of lysine was 90% for deoiled herring silage and 88% for white fish meal.

Differences in chemical composition were evident between the batches of deoiled silage prepared for the digestibility trial (Table 1). DM varied from 19.8% (Batch 1) to 15.2% (Batch 3). Oil varied from 2.4% in DM (Batch 1) to 5.0% in DM (Batch 2). Nitrogen (N) in DM varied from 11.5% (Batch 3) to 12.9% (Batch 2). Differences in oil and N content were reflected in gross energy (GE) values which were 17.9 MJ/kg DM for Batch 1, 19.3 MJ/kg DM for Batch 2 and 18.7 MJ/kg DM for Batch 3.

3.2. Digestibility study

Results are presented in Table 4. As there was no interaction between diet treatment and silage batch, mean results are given.

GE and N in the herring silage diet were more digestible than the GE and N in the fish meal diet.

The GE and N concentration of the DM in the herring silage also tended to be higher than respective concentrations in fish meal. In consequence, the digestible energy (DE) and digestible N (DN) values were higher for the diet containing herring silage. Respective values were 15.2 MJ

Table 3. Amino acid composition of deoiled herring silage and fish meal

	Content of amino acids (g/100 g DM)	
	Herring silage	Fish meal
Aspartic acid	7.0	5.7
Threonine	3.0	2.5
Serine	3.2	3.1
Alanine	5.2	4.5
Valine	3.3	2.9
Cystine	0.8	0.5
Methionine	1.8	1.7
Isoleucine	2.8	2.4
Leucine	5.2	4.2
Phenylalanine	3.5	2.3
Lysine	6.2	4.4
Histidine	1.7	1.1
Arginine	5.1	4.3
Glutamic acid	9.8	9.8
Glysine	6.4	6.7
Tyrosine	1.6	2.0

Table 4. Apparent digestibility of energy and nitrogen, and nitrogen balance for barley meal fed alone and for diets of barley meal with 25.2% deoiled herring silage or 25.9% of fish meal

	Barley meal diet (±S.E.)	Deoiled herring silage diet	Fish meal diet	S.E. of treatment means for diets containing fish	Significance of difference between diets containing fish[a]
Intake of nutrients daily					
Dry matter (g DM)	1007±42.9	861	931	20.4	
Gross energy (MJ GE)	18.2± 0.78	15.6	16.8	0.37	
Nitrogen (g)	18.9± 0.74	38.7	41.4	0.78	
Digestibility coefficients[b]					
DM	0.79±0.008	0.81	0.77	0.004	***
GE	0.79±0.010	0.83	0.81	0.004	***
N	0.74±0.014	0.91	0.89	0.003	***
ME/DE ratio	0.97±0.002	0.94	0.94	0.002	n.s.
Nitrogen balance (g/day)					
Urinary N	8.8±0.29	20.6	22.9	1.09	n.s.
N retained	5.2±0.68	14.5	13.8	1.01	n.s.
Efficiency of N retention					
Of ingested N	0.27±0.030	0.38	0.33	0.025	n.s.
Of digested N	0.35±0.035	0.42	0.37	0.028	n.s.

[a] n.s. $P > 0.05$, *** $P < 0.001$.
[b] Digestibility coefficients are for apparent digestibility.

DE/kg DM and 4.08 g DN/100 g DM for the fish silage diet and 14.5 MJ DE/kg DM and 3.94 g DN/100 g for the fish meal diet.

Although loss of urinary N was slightly less, and N retention and efficiency of retention slightly

greater for pigs fed the herring silage diet, these differences between the two diets were not sufficiently great to attain significance ($P > 0.05$).

The fish component of the diets contributed 0.259 of the total diet GE and 0.687 of total diet N (Table 1). By use of the digestibility coefficients determined for barley meal (Table 4), digestibility coefficients for the herring silage and fish meal may be calculated by difference. Respective values for deoiled herring silage and white fish meal were 0.96 and 0.86 for digestibility of GE, and 0.99 and 0.96 for digestibility of N. DE and DN values for the herring silage and the fish meal were respectively 17.9 and 15.7 MJ DE/kg DM and 119 and 112 g DN/kg DM.

4. Discussion

Unprocessed herring silage contains more oil, less N and less ash than white fish silage;[1,3] but when deoiled by centrifugation the herring silage has contents of oil and N similar to white fish products. The quality of the herring silage as measured by the content of amino acids and available lysine appeared slightly superior to that of white fish meal. If herring silage of 5% oil was included in a pig diet at the rate of 15% of the DM, the oil would comprise 0.75% of the diet. A level of 1% or less has been suggested as unlikely to cause off-flavour problems in poultry meat.[9] However, possible changes in the composition of carcass fat or the presence of off-flavours in the meat were not studied in the present experiment. Should a further reduction in oil content be required, the separator solid could be discarded; this fraction comprised only 1.3% of the original DM but contributed 22% of the silage oil.

It should be noted that the herring silage, as received, is a product of low DM (15–20%), and in the present study the DM and the chemical composition of the DM differed between batches. If such differences as were experienced reoccur, they may present quality-control problems should the material be offered for general distribution.

Pigs confined to crates for the digestibility study and fed the diet containing 25% of the DM as herring silage ate slightly less than pigs fed the diet containing fish meal. However, this is unlikely to detract from the commercial acceptability of herring silage as a protein supplement for growing pig diets. Growing pigs would be most unlikely to be fed more than 15% of the diet DM as fish silage, and results from feeding trials now in progress[10] would not suggest any acceptability problem under such circumstances.

Acknowledgements

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The Temperature Coefficient of Beef Ageing

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A study has been made of the effect of holding temperature on the ageing of beef sternomandibularis muscle. Ageing rate increased exponentially up to 40°C, rose more slowly to a maximum at 60°C, and then decreased sharply to approach zero at 75°C. The temperature coefficient of ageing was 2.4 and was constant over the range 0–40°C. This high Q_{10} was reflected in the high positive enthalpy of activation, (61.5 kJ). The extent of ageing was also sensitive to holding temperature; maximal extent of ageing was achieved up to ~60°C, above this, the extent fell to a minimum at ~65°C, despite the fact that the initial ageing rate at this temperature was still quite high.

1. Introduction

Despite advances in meat science and technology over the last 50 years, ageing, which was discovered in antiquity, remains an integral part of many modern methods for tenderising meat. The appreciation of its importance has directed attention to determining its cause.[1–3] Some evidence favours the myofibrils as the muscle components most distinctly affected;[4] they lose tensile strength, apparently through *Z*-line decay,[5–7] but a strict correlation between this event and tenderising has yet to be established. Davey and Gilbert [8] observed that *Z*-line disappearance required Ca^{2+}, supposedly discharged to the myofibrils from the sarcoplasmic reticulum during rigor onset. On this basis ageing would commence, not at death, but at rigor, although the point needs to be verified. The dependence of *Z*-line disappearance upon Ca^{2+} has been confirmed by others and has led to studies of the enzyme involved.[9–11] Past investigations of the time-course of ageing are difficult to interpret, largely through lack of uniformity in tenderness, even within single samples.[12] Much of the variation has been explained by the discovery of a quantitative relationship between meat toughness and shortening.[13]

In the present study the temperature dependence of ageing has been determined and is an extension of work on whole meat cuts to small samples of uniform geometry. The advantage of such a model approach is that heat and mass transfer variables can be excluded leading to more precise analysis of the changes observed.

2. Experimental

2.1. Meat

Beef sternomandibularis muscles (neck muscles) have been used. They are available within a few minutes of slaughter, are structurally very uniform with fibres parallel to the muscle axis and they can be set unshortened in *rigor mortis*. Although their content of connective tissue is comparatively high[14] the substantial tenderising they undergo through ageing can be measured with accuracy and is sufficiently large for the purposes of the present investigation. Neck muscles of Angus bulls (2–3 years of age) were obtained within 30 min after slaughter from the normal kill of the local abattoir, and were trimmed of surrounding tissue. Only those muscles with an average cross-section > 12 cm^2 were used. In experiments to determine the change in tenderness with the development of *rigor mortis*, strips of pre-rigor muscle (6 cm along the fibre $\times 3$ cm $\times 3$ cm) were held in plastic

bags at 15°C and cooked at hourly intervals. A small strip (3 cm along the fibre × 1 cm × 1 cm) was cut from the pre-rigor muscle. It was loaded with 50 g and mounted within 90 min *post mortem* on a rigorometer operating at 15°C in moist nitrogen. The extensibility change during rigor onset and subsequent ageing was measured.[15]

For all other experiments the neck muscles had first to be set in *rigor mortis* in an unshortened state. The pre-rigor muscles were wrapped in 4 layers of thin polyethylene film, and were then restrained horizontally between metal plates pressed lightly together to achieve a close fit, without stretching the muscle. The restrained muscles were held at 15°C for 24 h, and then at 2°C for a further 24 h to achieve full rigor and those with ultimate pH values above 5.8 were discarded. The muscles used for further experimentation were therefore close to zero shortening (±1%), were fully in rigor, and were of low ultimate pH.

For studying tenderness distribution within the rigor muscles, one of the pair from an animal was cut into strips 4 cm along the fibre × 2 cm × 2 cm, and labelled so that their location within the whole muscle could be identified. The strips were cooked immediately and their tenderness determined. The other muscle of the rigor-muscle pair was aged before similar assessment of the tenderness profile. It was sprayed with aureomycin (100 parts/10^6) and chloramphenicol (100 parts/10^6) to prevent microbial spoilage,[8] and stored at 15°C in a controlled-climate chamber at 95% relative humidity for 72 h, by which time full ageing was achieved.[16] Strips were then cut from the aged muscle as before and assessed for tenderness.

For determining rate of ageing, the rigor muscles at zero shortening were cut into samples (6 cm along the fibre × 2 cm × 2 cm). Each sample was placed in a weighted plastic bag and immersed in a water-bath at the appropriate ageing temperature. The open end of the bag was above the water level so that hydrostatic pressure forced air from the bag, achieving intimate contact of film and meat. Temperature equilibration measured by thermocouple was achieved in 5–10 min. Necessary sampling intervals were determined from experience; two or three days at 0–5°C and 30 min at 30–75°C.

2.2. pH evaluation

Estimations of pH were made on homogenates of meat samples in sodium iodacetate solution 2mM, pH 7.0). Ultimate pH values were measured in muscle held for 24 h at 15°C and a further 24 h at 2°C.

2.3. Cooking and tenderness evaluation

Meat samples in weighted bags were cooked for 40 min in a water-bath at 80°C.[17] The samples were then removed from the bags and chilled. Tenderness was measured as a shearing force (SF), using the MIRINZ tenderometer described by Macfarlane and Marer.[18] In this a wedge is forced through the sample of accurately measured width (~1.0 cm) and thickness (~0.8 cm) at right angles to the fibre axis. The SF values, given in arbitrary units, were corrected to a sample cross-section of 1 cm^2,[19] and are approximately nine times those given by the Warner Bratzler tenderometer in kg/cm^2 (1.27 cm cores).

3. Results

The accuracy of the results has depended very much on the care with which neck muscles were selected and set, unshortened, in *rigor mortis*. Figure 1 shows that despite the precautions there were trends in SF values along the muscle, especially if this were unaged (Profile I). In the example shown, values were higher in the centre than at the ends. However, the extents of variation (a scatter of ±5 SF units about the means) were small, especially across the muscles. This slight problem of tenderness variability was overcome as far as possible by obtaining the required samples for ageing, at a given temperature, from the same section of muscle.

For the ageing-rate studies, neck muscles had to be fully in rigor. The exact time of entry is determined by a number of factors, not least being muscle glycogen level and post-mortem holding

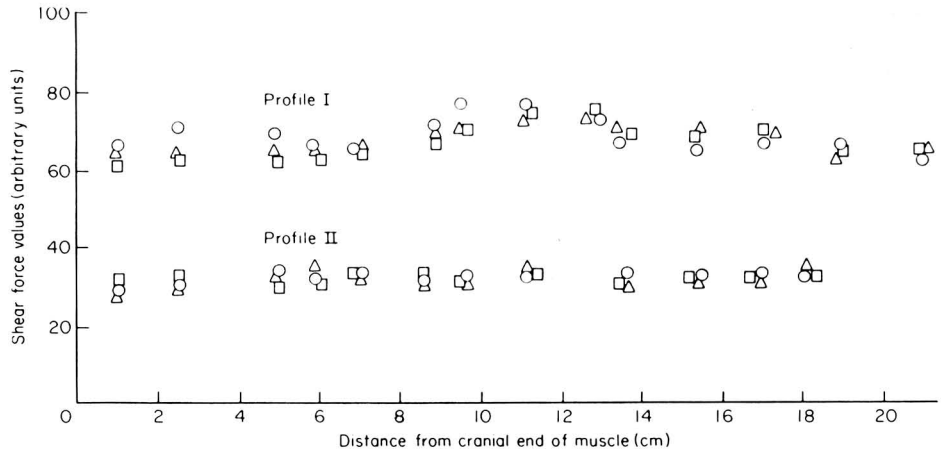

Figure 1. The shear-force profile in a pair of beef neck muscles set in *rigor mortis* in an unshortened state. The rigor muscles, oval in cross section, were cut parallel to the lesser of the cross-sectional diameters into three longitudinal strips. △, SF values of the central strip of each muscle; ○, SF values of one of the outer strips; □, SF values of the other outer strips. Profile I: unaged muscle. Profile II: aged muscle.

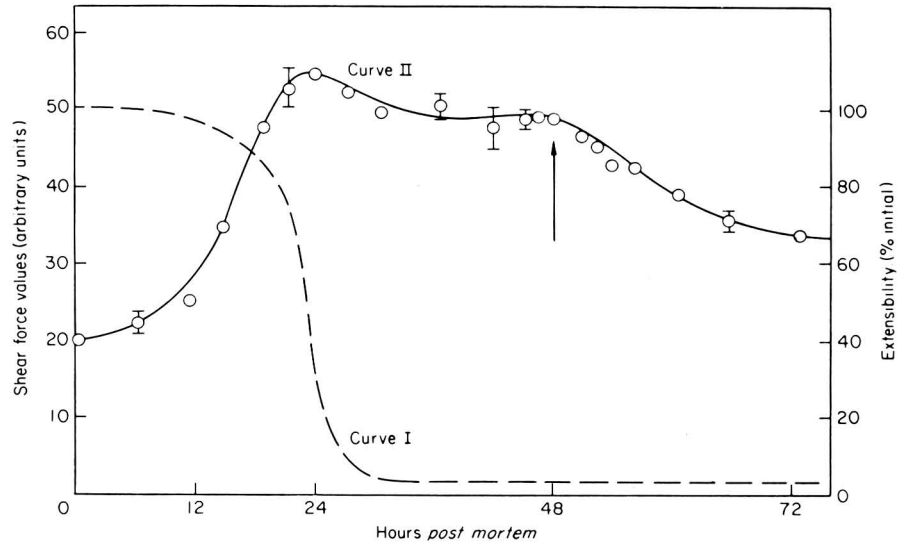

Figure 2. Changes in the extensibility and shear-force values of beef neck muscle as it progresses into *rigor mortis* at 15°C over 24 h, is stored for a further 24 h at 2°C to ensure full rigor, and is then aged at 15°C. Curve I: Extensibility as percentage initial (90 min *post mortem*). Curve II: The time-course of change in shearing force of cooked meat. The arrow (48 h) indicates the start of ageing at 15°C. The first 30% fall in SF values on ageing is essentially linear with time and has been used for determining ageing rate. Each SF value is the mean of 3 or 4 determinations on each sample. Those showing standard deviations (vertical lines through means) are the mean of 6–8 determinations.

temperature. Figure 2 illustrates the typical post-mortem time courses of extensibility and SF changes in neck muscle at 15°C. The extensibility change (Curve I) was characteristic of muscle in its progress into *rigor mortis*.[15] In the present example the delay phase lasted 16–18 h and was

followed by the rapid phase. With further holding, first at 2°C and then at 15°C, the low extensibility of rigor muscle was maintained. Although not shown in Figure 1, in confirmation of earlier studies[7, 20] the muscle strip under periodic loading stretched progressively during ageing to almost twice its unshortened length. In contrast to the changes reflecting rigor onset, SF values (Curve II) rose from death to reach their maximum (22 h) approximately half-way through the rapid phase. They usually dropped (10–20%) from their maximum over the remainder of the phase and then fell slowly with ageing. To ensure full rigor, muscles were held for 24 h at 15°C and a further 24 h at 2°C, by which time the complex earlier tenderness changes shown in Figure 2 would have been complete in all samples. Although at this point some samples could have begun to age, rates were still based upon the essentially linear, initial part of the ageing versus time-course curve commencing 48 h *post mortem*.

Figure 3 illustrates the relationship between temperature and tenderising rate, $R = \Delta SF/h$, calculated from the linear part of the curves. The rising phase of the curve, 0–40°C, is strictly

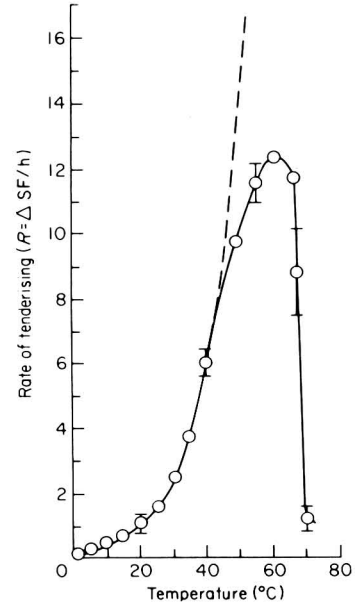

Figure 3. The relationship between rate of tenderising, (ΔSF/h) and holding temperature. Each point is the mean of 24–48 estimations of shearing force. Standard deviations are given by vertical lines through the means. For clarity not all standard deviations are shown. The dotted curve above ~40°C is the mathematical extension of the strictly exponential portion of the curve from 0 to 40°C.

exponential, having the form $R = 0.185e^{0.086t}$, where t is the Celsius temperature. The broken line above 40°C is an extension of the curve and serves to demonstrate the departure from this rate beyond 40°C. The maximum ageing rate occurred near 60°C and was still high at 65°C, then declined sharply to a low level at 75°C.

The curve describing ageing-rate rise (0–40°C) gives us an insight into the nature of the underlying reaction (Figure 4). The temperature coefficient of ageing in $Q_{10°C}$ notation can be derived from the ageing rate curve. In addition, from the theory of rate processes [21] a modified Arrhenius plot can be derived, and from it the enthalpy of activation (ΔH^*) of the ageing reaction can be determined. Up to 40°C the $Q_{10°C}$ value (2.4) was constant (Figure 4(a)), and was typical of an enzymic reaction. The value for ΔH^*, given by the slope of the plot in Figure 4(b), is 61.5 kJ. Such a high and positive enthalpy of activation is again consistent with ageing being due to a biochemical reaction.

The fall off in ageing rate above 40°C is presumably due to denaturation of the enzyme system involved. Through use of $Q_{10°C} = 2.4$, a rate at a given temperature can be calculated back to an equivalent rate at 0°C. As expected from Figure 3, such equivalent 0°C rates remain at their maxi-

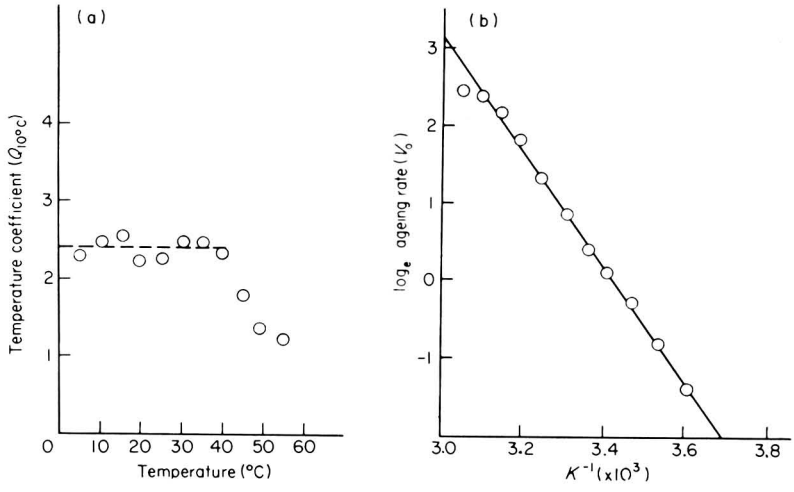

Figure 4. Analyses of the ageing-rate curve from 0 to 60°C. (a) The relationship between temperature (°C) and the temperature coefficient of ageing in $Q_{10°C}$ notation. The horizontal broken line is the mean (2.4) of $Q_{10°C}$ between 0 and 40°C, measured at 5°C intervals. (b) The Arrhenius relationship, comparing ageing temperature (K^{-1} 10^3) with ageing rate ($R = \Delta SF/h$).

mum up to ~40°C, but fall sharply from there towards zero at ~70°C. The temperature for half fall is 55°C. Such facts describe the temperature inactivation curve for the ageing enzyme.

As far as practicable the above analyses have been concerned with rates calculated from the initial, linear parts of the ageing curves. While such information is useful in elucidating the ageing mechanism, of more practical significance are the time-course curves at different temperatures. These will indicate the degree of tenderising it is possible to achieve at any ageing temperature. Since initial rates have been shown to be very large at temperatures in the region of 40–60°C, high-temperature ageing, either during processing or as a preliminary to cooking, becomes a possibility. In this respect a fall of 20–30 SF units is all that occurs to fully age unshortened beef neck or longissimus muscle.[16, 22]

Figure 5 shows the effect of a wide range of temperatures on the time courses of ageing in beef neck. In confirmation of the ageing-rate studies (see Figure 3), initial rates increased with temperature to reach a high maximum in the region 60–65°C. Thereafter they fell drastically and at 70°C were no more than that of meat at 10°C. The maximal extent to which the meat aged was also dependent upon storage temperature. Up to 60°C at least, the samples aged to the same extent, albeit at differing rates, with SF values halving to ~30. In contrast, at 65°C, despite a rapid initial rate, the extent to which the meat aged was much less. With a further 5°C rise to 70°C, values had not reached their lowest level even after 4 days. This tenderising above ~65°C is due not to ageing but to melting of interstitial connective tissue. Details of such cooking tenderising will be reported in a later study.

4. Discussion

In this study the tenderising of beef neck muscle during ageing has been shown to have a high $Q_{10°C}$ and this is reflected in a high positive enthalpy of activation. This supports the view that ageing is due to a biochemical reaction of considerable temperature sensitivity. In as much as *Z*-line decay is due to enzymic attack it would appear to be the most plausible of presently held reasons for the increase in tenderness during ageing.

The ageing enzyme has been shown to retain activity at temperatures (~60°C) which denature a large proportion of the muscle proteins. Heat treatment to 60°C may therefore be useful in

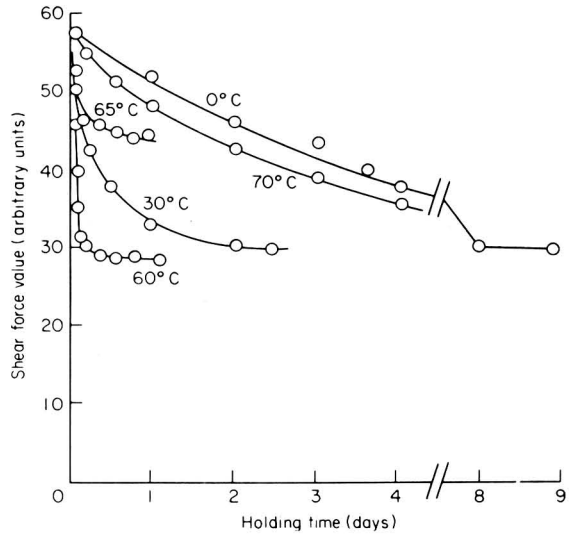

Figure 5. The time-course of tenderising of beef neck held at different temperatures from 0 to 70°C. The holding temperatures are given in the figure beside their appropriate curve. Each point is the mean of at least 12 separate estimations of shearing force.

isolating this enzyme. Its relative heat stability calls to mind a protease similar in many respects to cathepsin C, which has recently been identified in muscle.[23]

The present study emphasises the need to determine temperature-dependent tenderness changes in meat which has been fully set in *rigor mortis*. A full 24 h at 15°C followed by a similar period at 2°C has been used by us as standard practice. Only then, we consider, has all meat entered the ageing phase. Indeed experience has shown, in many cases, that in the second 24 h it is refractory and does not age significantly. The implication from this is that the onset of ageing occurs only after the full achievement of *rigor mortis*. This supports the view that onset of ageing coincides with the rigor-induced discharge of Ca^{2+} from the sarcoplasmic reticulum.[8]

No account has been taken of the possible effect on ageing rate of the comparatively high connective tissue content in the muscle used.[14, 24] Since only one ageing mechanism is presumed to exist, its temperature coefficient amongst muscles will be equal to that determined in this study on neck muscle. In contrast the extents to which muscles tenderise will depend very much on their connective tissue content. The degree to which this is so has yet to be tested.

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Effects of Ageing and Cooking on the Tenderness of Beef Muscle

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When beef sternomandibularis muscle was subjected to prolonged cooking, toughness, measured by shearing force across the grain, was reduced by about 50%. The shortened state of the muscle determined the final shear-force value attained as in normal cooking, being considerably higher in meat at 40% shortening, than in either unshortened meat or in meat shortened by 60%. The tenderising effect of ageing was additional to that from long cooking. Ageing reduced the tensile strength of the myofibrils as measured by resistance to the shearing stresses of homogenisation. Cooking tenderising resulted from a breakdown in the collagen of the interstitial connective tissue. In cooked meat distinct linkages were shown to exist between *Z*-lines of adjacent myofibrils. The mechanical strength of cooked meat is ascribed to the tensile strength of the fibrous components of muscle and these lateral linkages.

1. Introduction

In the last 10 years the much clearer understanding of muscle structure and function has advanced our knowledge of the mechanism of meat ageing. According to Davey and Gilbert,[1] ageing is due to a loss of myofibrillar tensile strength through decay of the *Z*-line structures of the sarcomere, precipitated by the release of calcium ions from the sarcoplasmic reticulum with rigor onset.[2] This point has been taken up by Busch *et al.*[3] and Penny *et al.*[4] in their attempts to isolate and characterise the mediating enzyme. However, in their studies no direct correlation of the changes in structure and tenderness was achieved. In fact the decay of the *Z*-line to the point of disappearance is much slower than tenderisation.[5] It may be that *Z*-lines first undergo an unobservable weakening affecting mechanical strength and that further decay is merely an extension into the observable range. The major load-bearing structures of raw muscle are the myofibrils and connective tissue; prolonged cooking destroys the collagen of the latter[6] and the fibres are then held only loosely together and the force needed to shear cooked meat across the grain is due largely to them.

In this study ageing and prolonged cooking of beef have been studied as a means of determining the contributions of connective tissue and myofibrils to meat toughness.

2. Experimental

2.1. Meat

Beef sternomandibularis muscles (neck muscles) were used in this project. They are ideal for the present study since they are structurally very uniform with the fibres parallel to the muscle axis and they undergo distinct cooking tenderising having a high connective tissue content. Neck muscles from young Angus bulls (2–3 years of age) were excised within 30 min after slaughter and cut into pieces (100–150 g) with the largest dimension (approximately 10 cm) along the fibre direction. The number of pieces obtained from a pair of neck muscles from one animal ranged from 8 to 16. The samples were set in *rigor mortis* at different degrees of shortening (S) ranging from zero to S, 0.65, as described by Davey and Gilbert.[8] Only those muscles with ultimate pH values below 5.8 were

used. For ageing a number of samples at zero, 0.4 and 0.6 S were sprayed with a mixture of aureomycin (100 parts/10^6) and chloroamphenicol (100 parts/10^6) and stored at 15°C, 100% relative humidity for 3 days. In this time the samples had become fully aged.[8]

2.2. Cooking and tenderness evaluation

Each sample was placed in a weighted polyethylene bag and cooked in a water-bath at 80°C.[8] At intervals of cooking, samples were removed and chilled for estimation of tenderness. The briefest cooking period was 40 min by which time maximum cooking toughening had been achieved. Tenderness was measured using the MIRINZ tenderometer described by Macfarlane and Marer.[9] The tenderometer wedge is forced through the sample at right angles to the fibre axis, the sections for biting being 1.5 cm wide by 0.7 cm thick. Shear force (SF) values are given in arbitrary units which are approximately 9 times those given as kg/cm^2 by the Warner Bratzler tenderometer (1.27 cm cores).

2.3. Cooked meat dispersal and homogenisation

For dispersal of the meat into individual fibres, 1 cm cubes of the samples cooked for 24 h were teased coarsely into 12 ml, 0.16 M KCl in MacCarteney bottles containing 10 glass beads (diameter, 0.5 cm) and hand shaken for 3 min. For further breakdown into myofibrils,[2] the dispersed fibres in 12 ml were homogenised for 1 min in a blade homogeniser[10] at 11 000 rev/min, the cup being immersed in an ice bath to maintain the temperature below 2°C. The blade was inserted well beneath the surface of the suspension to limit frothing.

2.4. Microscopy

Light micrographs of fibres were obtained using a planachromat objective (magnification, 2.5; NA, 0.08) which ensured uniform focus through the depth of the field. Myofibrils were examined by phase-contrast microscopy. For electron microscopy of meat cooked for 24 h the fixing, embedding and staining procedures described by Davey and Dickson[5] were followed.

2.5. Ultimate pH

One of the muscle portions from each animal was used after it had been held for the 48 h post-mortem period to enter *rigor mortis*. Estimations of pH were made on homogenates of 1–2 g muscle in 10–15 ml neutralised sodium iodo-acetate solution (2mM).

3. Results

Typical changes in the tenderness of beef neck muscle with cooking are shown in Figure 1. For unaged meat at zero shortening (Curve Ia) the SF value was 45 after 40 min cooking. Tenderness increased with further cooking, and in 12 h, SF values had dropped by more than half. By 24 h the curve had flattened with values approaching the ultimate of ~18 SF units. For aged neck muscle the relationship is that shown by Curve Ib. A similar considerable drop in SF value occurred with cooking. Compared with unaged meat the values were lower by 15 units, a difference preserved throughout the prolonged cooking period.

Meat shortened to S, 0.4 and therefore close to the peak of toughness followed a somewhat different time course (Curve IIa). SF values fell from their high, initial values (~120), to ~65 units in 24 h after which there was little further decline. Although the cooking tenderising of 50% was considerable, shortened neck muscle was still quite tough and remained so on long cooking (100 h). If the meat at S, 0.40 had been aged before cooking then Curve IIb was obtained. Confirming earlier studies, the aged neck muscle cooked for 40 min was essentially as tough as the unaged meat. However, with continued cooking (24 h), its SF value had fallen 15 units more than its unaged counterpart.

Neck muscle shortened to S, 0.60 and therefore well beyond the peak of toughening was about as tender as unshortened meat. Curve IIIa, Figure 1, shows that the toughness of such meat was

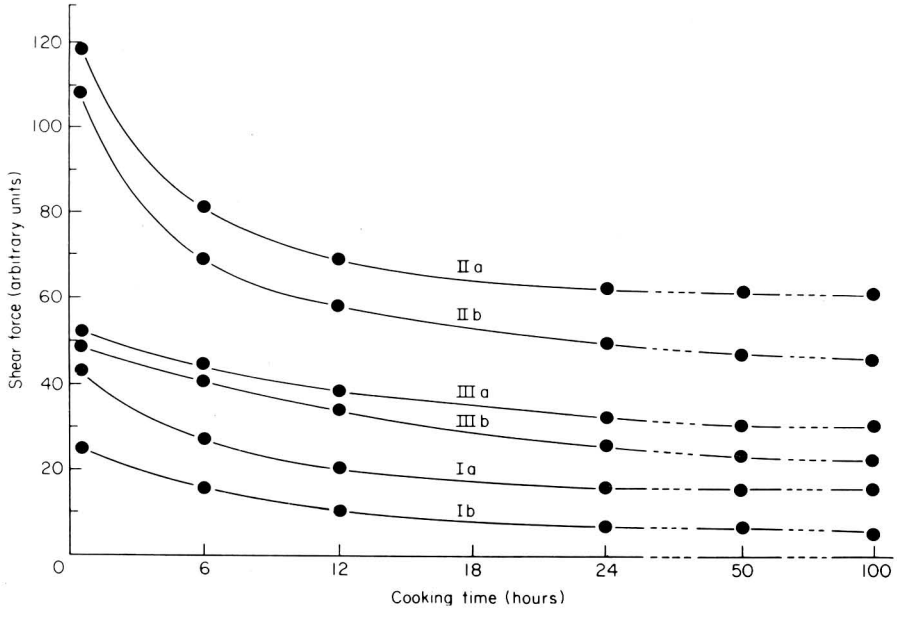

Figure 1. The relationship between cooking times and SF values for unaged and aged beef neck at different degrees of shortening. Curve I, unshortened meat. Curve II, meat shortened to S, 0.4. Curve III, meat shortened to S, 0.6. The a series of curves refer to unaged samples, the b series to samples aged 3 days at 15°C.

more resistant to cooking, and underwent a slower decline in SF values amounting to 30% in 24 h and 40% in 100 h. If such highly shortened meat was aged, then Curve IIIb was obtained and was only slightly different from the unaged curve.

While the intention of Figure 1 was to demonstrate the effect of ageing on SF-cooking curves, of equal interest is the relationship suggested from the figure between shortening and tenderness in meat cooked for a long time. It is implied from Figure 1 that meat cooked for 24 h gives a diminished form of the peaked shortening–toughening relationship.[11] This is illustrated fully in Figure 2. For unaged neck muscle cooked for 40 min the relationship is that given by the open circles and shows the expected rise in SF values to S, 0.4 with subsequent fall at higher shortenings. If cooking was extended to 24 h the relationship was that given by the closed circles. At all shortenings the maximum drop in SF values from long cooking was ~50% being a little more than this at low shortenings and a little less at high shortenings.

The curve for aged meat cooked for 40 min has been reported previously and for clarity is not shown here. At low shortenings ($S < 0.25$), SF values (~20) were uniformly low. They rose with further shortening to the same high value of unaged meat at S, 0.4 and declined again at higher shortenings. The curve for aged meat cooked for 24 h is shown in Figure 2 (open squares) and still clearly demonstrated the existence of a characteristic but much flatter shortening–toughening relationship.

Structural changes on prolonged cooking have been followed histologically (Figure 3). Meat cooked for 24 h, whether unaged or aged, disintegrated to constituent fibres on gentle agitation. These preserved their basic integrity and presumably retained intact sarcolemmae. The unaged fibres (Plate 1a) were 36.8 μm (s.d., 8.9) in diameter and were generally somewhat crimped. Fields of uniform contrast were impossible to achieve, probably through varied light refractance from the fibre surfaces. Fibres from aged muscles (Plate 1b) were more uniform, showed little crimping and gave more even contrast. Their diameter was 43.8 μm (s.d., 9.5), significantly greater than the unaged diameter ($P < 0.01$).

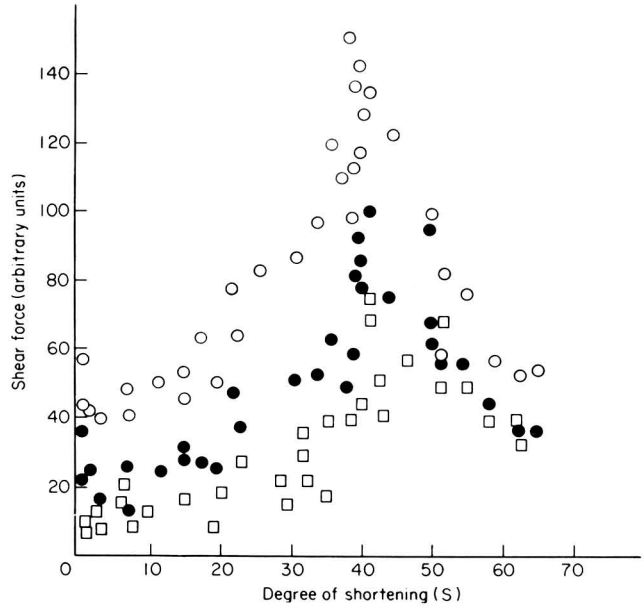

Figure 2. The relationship between degree of shortening (S) and SF values for beef neck muscle; ○, unaged meat cooked 40 min, 80°C; ●, unaged meat cooked 24 h, 80°C; □, meat aged 3 days at 15°C then cooked 24 h, 80°C.

A clearer distinction between the unaged and aged meat appeared at the level of the myofibril. With a period of vigorous but standardised homogenising unaged fibres broke down to the ordered and clearly striated myofibrillar pieces shown in Plate 2a. In contrast, aged fibres disintegrated into much shorter filaments (Plate 2b). The aged, cooked myofibrils were therefore more fragile than their unaged counterparts.

The basic sarcomere structure is known to survive brief cooking.[12] Figure 3 examines the degree to which fine structure is also preserved on prolonged cooking (24 h). Contrary to expectation the highly organised striated appearance of muscle survived. In both unaged and aged meat (Plates 3a, b) clearly defined detail can be seen in the sarcomere bounded by prominent *Z*-lines. The fine longitudinal striations of the *A*-band and the *M*-line have virtually disappeared; although not shown, they can still be identified clearly after a 40 min cook. The *A*-band shrank longitudinally by 27% to 1.1 μm. They also stained heavily probably through adsorption of denatured sacroplasmic proteins which would also mask underlying detail. The *I*-bands were clearly defined, with parallel filaments running through them. Their coarseness and much reduced number would suggest that thin filaments have aggregated laterally into bundles. The *N*-lines were quite pronounced again probably through protein adsorption. The empty membranous sacs between the myofibrils are presumably the remnants of the reticulum and mitochondria. The *T*-system at the *A*–*I* junctions[13] disappeared entirely.

An important new feature is exposed by cooking. These are the lateral and quite dense bridges linking adjacent myofibrils at the *Z*-bands, especially evident in the seemingly more open structured unaged material. Such links were proposed earlier[2,5] and it was supposed that in common with the *Z*-lines they weakened during ageing. Their intensity and rather regular width tells against them being merely protein condensed at these positions. The nature of these connections and their possible relevance to ageing is being further studied.

4. Discussion

Meat is a complex tissue and the relative contribution to toughness of its various structural components has yet to be established. The present study has examined the contribution of the two

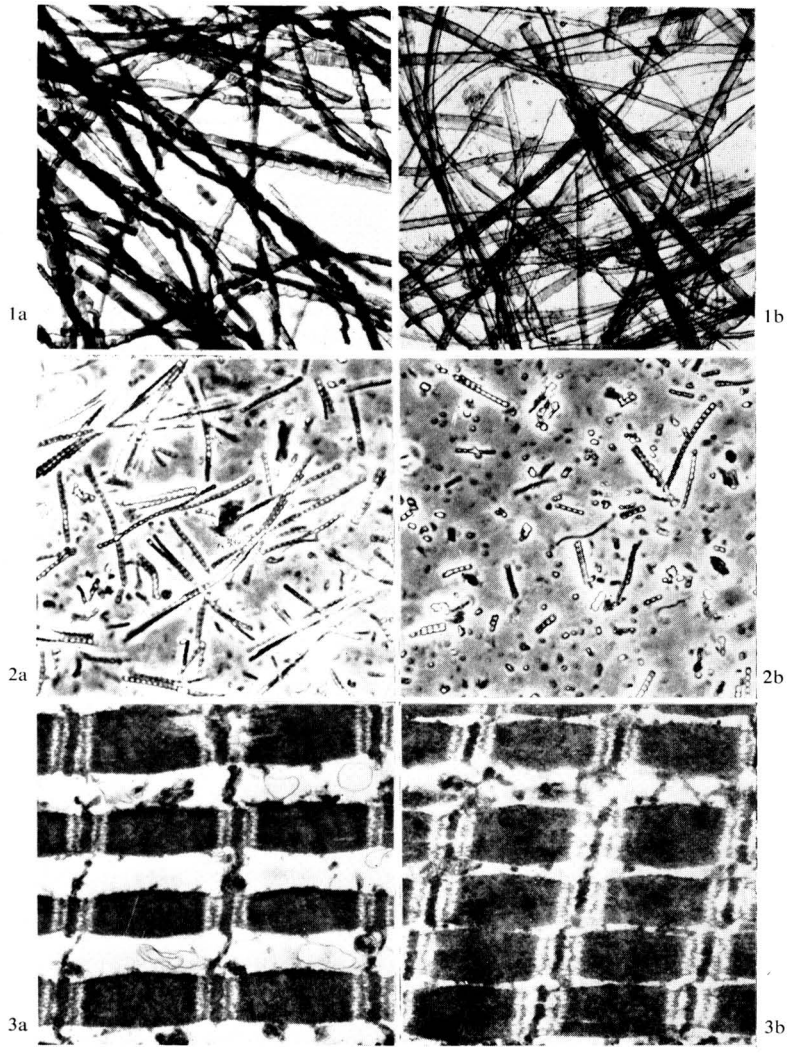

Figure 3. Micrographs of unaged and aged meat cooked 24 h, 80°C. The a series of plates refer to unaged meat, the b series to meat aged 3 days, 15°C. 1, Light micrograph of meat fibres (× 32). 2, Phase contrast micrographs of myofibrils (× 480). 3, Electron micrographs of sectioned meat (× 12 000)

Plates 1a, 1b (magnification, 32): Fibres obtained by gentle disintegration. The unaged fibres (1a) are thinner, more crimped, and often more dense than their aged counterparts (1b).

Plates 2a, 2b (magnification, 480): Myofibrils produced by controlled homogenisation. The unaged myofibrils (2a) on average are much longer than their aged counterparts (2b). Striations can be seen in both preparations.

Plates 3a, 3b (magnification, 12 000): Electron micrographs of sections of the cooked meat. In both the unaged (3a) and aged (3b) meat, characteristic fine sarcomere structure exists, with few gross changes from protracted cooking. In both, *Z*-lines bounding the sarcomeres are particularly prominent. Material links the *Z*-lines of adjacent sarcomeres.

major components, the myofibrils and connective tissue. Prolonged cooking destroys collagen and reduces inter-fibre adhesion virtually to zero. With such treatment SF values of neck muscle fell by half, independent of variations in initial tenderness induced by shortening. This does not mean that decreased values are due solely to lost adhesion since the tensile strength of the myofibrils

may also have been reduced. However, certain evidence would suggest otherwise. Thus there is no loss in myofibrillar tensile strength after 12 h and this minimum SF value is independent of cooking temperature between 70 and 100°C;[14] in addition, the increased tenderness of aged meat persists even with the halving of SF values through loss of collagen.

We consider that myofibrils and connective tissue interact in their contribution to tenderness. After long cooking with loss of adhesion between fibres, the peaked shortening–toughening relationship[11] persists and, although reduced to lower SF values, the maximum toughness remains at ~S, 0.4. Myofibrillar shortening is therefore its sole cause. The evidence therefore supports an earlier theory of tenderness based on myofibrillar and connective tissue interaction.[5, 15]

The tenderness of highly shortened meat (S, 0.6) is affected quite differently, neither ageing nor prolonged cooking produced such marked changes in SF values. Since highly shortened meat is in a disorganised and ruptured state with zones of contraction creating adjacent zones of stretch and even myofibrillar fracture,[16] it might be expected that few of the cooking or ageing characteristics of unshortened meat would be displayed.

The results have shown for the first time that ageing induces a clearly observable change in the structure of cooked meat; firstly, aged fibres become less crimped and somewhat more swollen; secondly, the myofibrils are much more fragile in the cooked state than their unaged counterparts. As in raw meat,[2] ageing produces loss of myofibrillar tensile strength. In view of past studies it is surprising that the *Z*-lines of aged meat after cooking remain organised and intense. To reconcile this seeming conflict it must be assumed as in the past, that *Z*-lines weaken to cause ageing but that the event is not identified unless ageing is carried beyond the time of maximum tenderness development.

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Localised Occurrence of *N*-nitrosopyrrolidine in Fried Bacon

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Experiments have been carried out in which bacon rashers were fried to a final temperature of 140°C either whole or after division into lean, fat and rind. Where rashers were fried whole, similar levels of *N*-nitrosopyrrolidine were found in the lean, fat, rind and exuded fat of individual bacon sides, although there was up to a ten-fold difference between bacon from different sides. When the lean, fat and rind were fried separately, the *N*-nitrosopyrrolidine was found almost exclusively in the residual fatty tissue and the exuded fat, with only just detectable quantities being found in the lean and rind of a few samples.

1. Introduction

N-nitrosamines, derived from a reaction of nitrite with nitrogen-containing meat components, have been found in cured meat products,[1–9] and, in particular, *N*-nitrosopyrrolidine (NPYR) has been reported consistently in fried bacon, generally at concentrations in the range 5–20 μg/kg, but occasionally at considerably higher levels (108 μg/kg).[7,10] Bacon containing a high proportion of fat appears to produce more NPYR on frying than leaner cuts,[7] and the fat exuded during cooking has been found on occasions to contain at least as much NPYR as the bacon itself. Cooking temperature appears to influence the amount of NPYR formed; below 100°C, Pensabene *et al.*[8] detected no NPYR whereas concentrations increased at higher cooking temperatures. Recently, it has been reported that the quantity of NPYR formed in frying is proportional to the amount of nitrite used in the manufacture of the bacon.[9]

Various procedures are used in bacon curing to disperse salt, nitrite and sometimes nitrate evenly through the meat. Wiltshire curing is a relatively slow process in which sides of pigmeat are first injected with, and then immersed in, brine for several days followed by maturation, to allow diffusion of the curing salts. The maximum final concentration of nitrite permitted in cured meats is 200 mg/kg (parts/10^6), a concentration resulting, in Wiltshire-cured bacon, from the use of brines containing about 2000 mg/litre (0.2%) nitrite. During experiments with such nitrate-free brines, it was observed that the distribution of nitrite in the bacon after immersion (5 days) and maturation (7 days) was irregular, and that high concentrations were present on the exposed surfaces of the side, especially in the rind.

Experiments were carried out to determine whether these high levels of nitrite resulted in the formation during simulated domestic frying of high concentrations of NPYR in the rind and whether they enhanced NPYR formation in the fat.

2. Experimental

2.1. Materials

Bacon sides were prepared in a factory by a Wiltshire process using injection and immersion brines containing 2000 mg/litre sodium nitrite and 26% sodium chloride; weight gain from injection was approximately 10%. After maturation, backs were sliced to provide rashers for cooking and NPYR

analysis, and also samples for analysis of salt, nitrite, pH and water content. In sides 1–4, slices were taken at 4 in intervals along the back and bulked to provide a representative "whole bacon" sample. The rind was then trimmed closely from the fat of the bacon remaining and the resulting rind-free sections sliced (3 mm). For sides 5–10, the backs were sliced, and sample rashers taken at random for curing salt analyses; the remainder were used for cooking and nitrosamine analysis.

2.2. Cooking procedures

Rashers were either fried whole (sides 5–10) or were separated into lean, fat and rind before frying the individual tissues (sides 1–4). Frying was carried out in a thermostatically controlled electric frying pan for 10 min after the temperature of the rashers, recorded by thermocouple, had risen to 95–100°C; a final temperature in each rasher component of 140 ± 5°C was obtained by this procedure.[11] The rashers had the appearance of being "well done" but were not as crisp as bacon is commonly fried in the United States. The procedure was repeated until sufficient fried material was accumulated for NPYR analysis. Fat exuded during cooking of each batch was poured off and stored. Rashers fried whole were separated into lean, fat and rind before analysis.

2.3. Analysis procedures

2.3.1. Raw bacon

Lean, fat, rind and whole bacon from each side were bulked separately and minced. A minced sample (3 g) was homogenised in 10 ml distilled water and the pH measured with a Pye Universal pH meter. Residual nitrite was estimated in 10 g sample by the method of Follett and Ratcliffe.[12] Sodium chloride was determined on the same extract by the method of Volhard. Dry weights were obtained by drying minced samples at 100°C for 24 h.

2.3.2. Cooked bacon

N-nitrosopyrrolidine: Analysis of samples for NPYR was carried out by the method of Crosby *et al.*[4] using the following quantities: 250 g minced sample, 100 g sodium chloride, 250 ml distilled water, 2 antifoam tablets (antifoam S, Thompson and Capper, Liverpool) and *N*-nitrosodipropylamine as internal standard (250 μl of mg/litre solution) were steam distilled and 400 ml distillate collected in an ice/salt cooled receiver; 50 g sodium chloride and 4 ml 10 N sulphuric acid were dissolved in the distillate which was extracted with 4 × 40 ml redistilled dichloromethane. The organic layers were combined and extracted with 70 ml 1.5 N sodium hydroxide. After separation and drying with anhydrous sodium sulphate, the extract was filtered through Whatman No. 54 paper and the volume reduced by evaporation at 47°C to 2.5 ml, 0.8 ml hexane was added and evaporation continued at 50°C until 0.25 ml remained.

Extracts from sides 1–4 were analysed for NPYR by the Laboratory of the Government Chemist using their standard gas chromatographic–high resolution mass spectrometric procedure.[13] Extracts 5–10 were examined initially by gas chromatography using a 6.15 m × 3 mm stainless steel column packed with 15% FFAP on Chromosorb W (80–100 mesh) at 130°C and 25 ml/min argon carrier gas flow; qualitative and quantitative data were obtained by coupling the chromatograph via a heated (240°C) transfer line to a Coulson electrolytic conductivity detector operated in the nitrogen-specific mode. The identity of NPYR was confirmed subsequently by combined gas chromatography–high resolution mass spectrometry.

3. Results and discussion

The concentrations of NPYR and residual nitrite found in the bacon are shown in Table 1, and the pH, salt and moisture contents in Table 2. NPYR was confirmed by mass spectrometry in all but four extracts (indicated by superscript "*a*" in Table 1) which had given positive g.c. responses at the retention time of authentic NPYR. No NPYR was detected in any of the uncooked bacon rashers containing between 64 and 192 mg/kg residual nitrite. When the lean, fat and rind were fried separately (samples 1–4), the highest NPYR concentrations (8–19 μg/kg) were found in the

Table 1. *N*-nitrosopyrrolidine (μg/kg) in fried bacon. Residual nitrite values (mg/kg) determined before cooking are shown in parenthesis

Side no.	Uncooked whole rashers	Fried			
		Lean	Fat	Rind	Exuded fat
(a) *Rasher components separated and fried independently*					
1	ND (135)	ND (94)	8 (35)	ND (497)	8 (—)
2	ND (163)	ND (94)	13 (60)	0.5 (570)	3 (—)
3	ND (64)	ND (42)	13 (39)	1 (225)	5 (—)
4	ND (98)	ND (76)	19 (34)	1 (336)	11 (—)
(b) *Rashers fried whole*					
5	ND (125)	7 (92)	9 (38)	9 (420)	15 (—)
6	ND (143)	ND (67)	3 (17)	3 (479)	3 (—)
7	ND (129)	2 (164)	2 (34)	3 (471)	5 (—)
8	ND (135)	ND (93)	0.5 (23)	ND (277)	1 (—)
9	ND (125)	ND (93)	1.5[a] (54)	1[a] (443)	2 (—)
10	ND (192)	1.5[a] (172)	4[a] (52)	2 (583)	4 (—)

ND = None detected (detection limit 0.5 or 1.0 μg/kg depending upon instrument variation).
(—) = Not determined.
[a] NPYR not confirmed by mass spectrometry.

Table 2. Mean values for pH, salt and moisture content of uncooked bacon components (standard deviations in parenthesis)

	Whole rasher	Lean	Fat	Rind
Sides 1–4:				
pH	5.85 (0.15)	5.64 (0.10)	6.21 (0.13)	6.39 (0.14)
NaCl (%)	2.9 (0.2)	2.8 (0.3)	0.9 (0.1)	8.1 (2.0)
Moisture (%)	45.7 (3.4)	71.4 (0.3)	9.9 (1.2)	54.6 (3.2)
Sides 5–10:				
pH	5.96 (0.17)	5.76 (0.14)	6.38 (0.20)	6.49 (0.10)
NaCl (%)	3.6 (0.4)	3.8 (0.7)	0.9 (0.1)	7.8 (0.9)
Moisture (%)	51.3 (4.7)	71.8 (0.9)	9.6 (1.8)	55.0 (4.0)

fried fat and the fat exuded during frying. None was found in the fried lean but three samples of rind contained NPYR at concentrations at the detection limit of the procedure (0.5 or 1.0 μg/kg, depending upon instrument variation). Thus the high concentrations of nitrite (225–570 mg/kg) present in the rind at the time of frying did not result in high levels of NPYR being formed.

In samples 5–10, where rashers were fried whole, similar levels of NPYR were found in the lean, fat, rind and exuded fat of individual bacon sides, although there was up to a ten-fold difference between sides. Concentrations of NPYR were smaller in the fried fat but larger in the rind than in samples 1–4; NPYR was also detected in the fried lean of these samples. These differences probably result simply from the spread of exuded fat containing NPYR over the surfaces during frying. The results indicate that a high level of nitrite in rind adjacent to fat does not influence NPYR formation in the fat during frying, suggesting that there is no diffusion of active precursors from the rind to the fat or vice versa.

The higher quantities of NPYR found in the fat were formed presumably because either higher concentrations of the necessary precursors were present or the physicochemical conditions in the fat phase were advantageous. For example, high temperature may be achieved more quickly in the

fat than in the lean; alternatively, the non-aqueous nature of hot frying fat may promote formation of NPYR by mechanisms other than the ionic reactions of aqueous systems.

Considering the gross composition of the lean, fat and rind, if the water (~10%) and lipid (~88%) of the adipose tissue are discounted as contributing directly to NPYR formation, the remaining 2% consists of structural components over 90% of which are comprised of collagen. Recently, 0.07 mg NPYR/g collagen were found when collagen and nitrite were heated at 140°C in a simulated frying system.[14] Adipose tissue contains approximately 2.5 g,[15] lean 0.3 g and rind 20 g collagen/100 g tissue. On a water, collagen and nitrite basis only, i.e. ignoring other lipid and solid components, the aqueous phase of adipose tissue contains 20% collagen, the lean 0.42% and the rind 26%; similarly, for nitrite, the percentages are 0.03, 0.01 and 0.06 respectively. Therefore if the concentration of collagen was an important factor, the rind should have yielded more NPYR than fat since it contains 1.3 and 2.0 times more collagen and nitrite respectively. However, the results of this experiment do not support this thesis and the explanation of the higher levels of NPYR in the fat may depend upon the existence of particular physicochemical conditions which favour NPYR formation.

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Pectinesterase in New Zealand Grapefruit Juice

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Samples of juice from both early and later season New Zealand grapefruit have been analysed for the presence of three enzymes that have been reported in some overseas citrus varieties. While both early and late season juice contained pectinesterase, neither polygalacturonase nor ascorbic acid oxidase was detected in juice extracted from early or late season fruit.

1. Introduction

New Zealand grapefruit is the most important citrus fruit grown in New Zealand, accounting for over 38% of the total citrus crop.[1] It is not a true grapefruit but is a natural hybrid of obscure origin with tangelo characteristics.[2] That it originated in the Orient is suggested by Bowman's statement[3] that it was brought to Australia (presumably the fruit) from Shanghai early in the 19th Century. It was taken to New Zealand and the name "New Zealand Grapefruit" (hereafter referred to as NZGF) was later given to thin-skinned high quality strains.

Because NZGF is not a true grapefruit, the present investigation was carried out to see if certain enzymes which had been reported in overseas citrus fruits were present in NZGF juice. Juice rather than whole fruit was analysed because it is the juice that is of prime commercial interest in New Zealand.

The enzymes of interest were: (a) pectic enzymes, which if present could lead to cloud destabilisation; (b) ascorbic acid oxidase, which if present could reduce the ascorbic acid content of the juice.

1.1. Pectic enzymes

The most extensive investigations on enzyme activity in citrus fruits have been concerned with the occurrence and activity of pectic enzymes, particularly as these effect the undesirable changes in the appearance of citrus juices. Pectic enzymes may be divided conveniently into two main groups, the pectinesterases, and the depolymerising pectic enzymes or polygalacturanases.[4]

1.1.1. Pectinesterase (PE)

MacDonnell *et al.*[5] first reported on the isolation and characterisation of orange PE and Rouse[6] using Florida oranges showed all the PE to be completely adsorbed on water-insoluble cell tissue. In 1954 Rouse *et al.*[7] reported a straight line relationship between PE activity and the pulp content of orange juice.

PE activity is usually estimated by measuring the amount of alkali required to keep a synthetic pectin substrate at a constant pH (near neutrality) when the enzyme-containing extract is added to it. This gives a measure of the carboxyl groups freed by enzyme action per unit of time.

The method used in this study was based on such a titrimetric procedure developed at the University of Florida Citrus Experimental Station.[8]

1.1.2. Polygalacturonase (PG)

The thermal destruction times for pectin and pectic acid depolymerising enzymes were reported

in 1953 but owing to problems with analytical methods, it was not possible to differentiate between PG and PMG (polymethylgalacturonase). Of the 14 samples of grapefruit juice tested, only 4 showed any depolymerising enzyme activity.

The presence of PG in oranges was considered to be unlikely by Sinclair and Jolliffe[10] who were unable to find free galacturonic acid in oranges ripened in storage, and in juice allowed to stand until the cloud precipitated. Nevertheless, Primo *et al.*[11] reported the presence of galacturonic acid in Spanish Valencia orange juice.

No PG activity could be detected in the flesh of the tangerine[12] or in oranges, mandarins, and lemons,[13] but slight activity has been detected in some samples of Marsh Seedless grapefruit.[13]

The method in common use for determining PG activity in citrus juice is a modification of the Willstalter–Schudel hypoiodite method, involving the iodometric determination of the increase in the concentration of reducing groups due to PG activity.[14] Mannheim and Siv[13] used a variation of this method and their technique was followed in this study.

1.2. Ascorbic acid oxidase

The presence of this enzyme in citrus peel was reported by Huelin and Stephen[15] but they found negligible amounts in orange juice. However, this work was based on the ability of the juice extract to oxidise ascorbic acid, and many compounds are capable of this in the absence of ascorbic acid oxidase. The presence of the enzyme in oranges was confirmed by Vines and Oberbacher[16] who found it concentrated in the flavedo layer of the peel. It commenced its action as soon as the peel was subdivided.

The same workers[17] surveyed ascorbic acid oxidase activity in several citrus fruits and found the order of decreasing activity to be Marsh grapefruit, Pineapple and Valencia oranges, Thompson grapefruit, Persian limes, and Villafranca lemons. On a fresh weight basis, the ascorbic acid oxidase content was highest in the immature citrus fruit and decreased as the fruit matured.

2. Experimental

2.1. Raw material

The NZGF samples used in this study were of the Morrison's Seedless variety grown on trifoliata root stock. The trees formed part of a commercial orchard at Te Puke, Bay of Plenty, and were 4 years old.

Twelve representative trees were selected from the orchard, and early in the season (July) and late in the season (December) one fruit from each of the 12 trees was picked, packed in a wooden box, and sent by rail and bus to Palmerston North. The journey occupied 3 days, and the analyses were performed on the fourth day after picking. The fruit remained at ambient temperatures (in the range 10 to 18°C) from the time of picking to the time of analysis.

The fruit was halved and the juice removed using the reamer attachment on a domestic Kenwood mixer operating at speed one. The juice was then passed through a double layer of cheese-cloth to remove gross suspended matter to give an insoluble solids content in the juice of approximately 10%, as determined by centrifugation.[18]

2.2. Pectinesterase determination

To a 1% solution of pectin (Sigma Chemical Co., USA Grade 1 Citrus Pectin) in 0.1 M sodium chloride (20 ml), was added sufficient 0.1 N sodium hydroxide to bring the pH to 7.5. The solution was then placed in a constant temperature water bath at 30°C. After the addition of the juice (10 ml), the pH was readjusted to 7.5, again with 0.1 N sodium hydroxide. The pH was then maintained at 7.5 for 30 min by the addition of 0.02 N sodium hydroxide through an automatic titrimeter (Radiometer TTT1c).

Pectinesterase activity was expressed by the symbol (PE.u) g soluble solids which represents the milliequivalents of ester hydrolysed per minute per millilitre of juice per gram of soluble solids

(Brix) as determined by a refractometer and corrected for the presence of acid. The formula used to compute PE activity was:

$$\text{(PE.u) g soluble solids} = \frac{\text{ml sodium hydroxide} \times \text{normality}}{\text{weight of sample} \times 30 \text{ min} \times {}^{\circ}\text{Brix}/100}$$

For simplicity, the activity of the enzyme was multiplied by 10^4.

Samples of juice from both early and late season NZGF were analysed for the presence of PE activity.

2.3. Polygalacturonase determination

To freshly extracted NZGF juice (99.5 ml) was added the sodium salt of polygalacturonic acid (0.5 g) (sodium polypectate Grade II, Sigma Chemical Co., USA) and the mixture stirred until the acid had dissolved. At the same time, a blank was prepared by adding an equal quantity of the sodium salt of polygalacturonic acid to juice (99.5 ml) which had been heated to 100°C, held at that temperature for 5 min, and then cooled.

The samples were incubated at 40°C for up to 96 h and checked periodically for enzyme activity. The activity was determined by adding to the sample (10 ml) in a 250 ml conical flask, 1 M sodium carbonate solution (1.8 ml) and standardised 0.1 N iodine solution (10 ml). The mixture was set aside in the dark for 30 min, acidified by the addition of 2 M sulphuric acid, and then immediately titrated with 0.05 N sodium thiosulphate solution using starch as the indicator. The difference in thiosulphate consumption between sample and blank yielded the increase in the concentration of reducing groups due to enzyme activity, after taking into account the differences in normality of the iodine and thiosulphate solutions; 1 ml of 0.1 N iodine is equivalent to 0.05 mEq of galacturonic acid.

Samples of juice from both early and late season NZGF were analysed in duplicate for the presence of PG activity.

2.4. Ascorbic acid oxidase determination

The method used was based on the manometric method described by Vines and Oberbacher.[16] Standard Warburg manometers were used to measure oxygen uptake at 25°C.

The reaction mixture contained ascorbic acid (30 μmol), potassium phosphate at pH 5.6 (33 mmol), and varying quantities of freshly extracted NZGF juice (1, 2 and 5 ml). A control manometer containing juice which had been heated for 5 min, and a thermobarometer, were employed in all experimental runs. A 60 min reaction period was used.

Samples of juice from both immature and mature NZGF were tested.

3. Results

3.1. Pectinesterase

The results are presented in Table 1.

Table 1. Pectinesterase activity in NZGF juice

Samples	pH	Acidity (ml 0.02 N-NaOH)	PE activity (PE.u) g soluble solids ($\times 10^4$)
Early season (July)	2.95	6.55	38.0
Late season (December)	3.40	16.7	83.0

3.2. Polygalacturonase

The results are presented in Tables 2 and 3.

Table 2. Polygalacturonase activity in early season (July) NZGF juice

Sample	Incubation time (h)	Thiosulphate consumption (ml 0.05 N solution) x	Δx	PG activity (mEq GA per ml of juice)
(a) Juice	24	8.9	+0.1	0.000025
Blank	24	9.0		
(b) Juice	48	9.1	0.0	Nil
Blank	48	9.1		
(c) Juice	72	9.6	−0.6	Nil
Blank	72	9.0		
(d) Juice	96	9.1	−0.1	Nil
Blank	96	9.0		

Table 3. Polygalacturonase activity in late season (December) NZGF juice

Sample	Incubation time (h)	Thiosulphate consumption (ml 0.05 N solution) x	Δx	PG activity (mEq GA per ml of juice)
(a) Juice	24	10.9	+0.2	0.00005
Blank	24	11.1		
(b) Juice	48	11.3	−0.9	Nil
Blank	48	10.4		
(c) Juice	72	11.9	−0.9	Nil
Blank	72	11.0		
(d) Juice	96	8.9	+0.1	0.000025
Blank	96	9.0		

3.3. Ascorbic acid oxidase

In no run was there any significant or reproducible uptake of oxygen.

4. Discussion

4.1. Pectinesterase

The levels of PE detected in juice from both early and late season NZGF are comparable with those reported by Rouse[19] for juice from the fruit of 6-year-old orange trees. If the trend found by him for oranges is followed, it is expected that juice from older NZGF trees would contain higher levels of PE.

4.2. Polygalacturonase

In the early season juice, extremely low PG activity was detected after 24 h, but this was not confirmed after 48 h incubation. The analyses performed after 72 and 96 h showed that the blanks had more reducing groups than the samples of juice. Thus the experimental variations are greater than the detected levels of PG in the juice.

The late season juice showed a similar pattern, with extremely low PG activity being detected after 24 and 96 h incubation. The blanks had more reducing groups than the samples of juice after 48 and 72 h incubation.

Mannheim and Siv[13] using the same method, found low levels of PG activity (0.0008 mEq GA per ml of enzyme extract) in some but not all of their extracts of Marsh Seedless grapefruit. They found no PG activity in extracts of Shamouti and Valencia oranges, mandarins and lemons. However, using the same method, they did find relatively high levels of PG activity in all tomato extracts, a fruit renowned for its PG content.

Pratt and Powers[9] reported the presence of PG activity in 4 out of 14 samples of grapefruit juice tested, but they used different assay techniques from those used in this study and the one by Mannheim and Siv.[13] Only one of their assay methods (a viscometric technique) indicated the presence of PG activity, the other method (determining the amount of non-degraded pectin after incubation) giving negative results for all 14 samples. They were unable to account for the difference in sensitivity of the two assay methods.

4.3. Ascorbic acid oxidase

In no juice sample was there any significant or reproducible uptake of oxygen.

These results are not altogether surprising as reported results for overseas varieties of citrus show that not all juice contains ascorbic acid oxidase.

However, as only juice was examined, these results do not preclude the presence of the enzyme in whole NZGF.

5. Conclusion

Pectinesterase: the presence of PE activity in both early and late season NZGF juice has been established. The activity was twice as great in the late season juice as in the early season juice. Polygalacturonase: no PG activity either in early or late season NZGF juice has been detected. Ascorbic acid oxidase: this enzyme was not detected in either early or late season NZGF juice.

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Distribution and Identity of Labelled Products Following Autumn Application of ^{15}N-labelled Urea or Potassium Nitrate Fertilisers to Apple Trees

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Young apple trees growing in pots of soil were treated in October with ^{15}N-labelled urea on the foliage or with $K^{15}NO_3$ via the soil. Whole trees were sampled in the following February and May and the tissues extracted to determine the concentrations of the major nitrogenous constituents and their ^{15}N enrichments. At the February samplings following both treatments half the ^{15}N was in the insoluble fraction, and the soluble part consisted almost entirely of arginine and asparagine. But the two treatments gave quite different distributions of ^{15}N between the two amino acids. Urea applied to the leaves resulted in over four times as much ^{15}N in arginine as in asparagine; in contrast, from the soil nitrate treatment the distribution was almost equal. When sampled in May the new leaves and blossoms contained a large proportion of the total ^{15}N in the tree. Comparison of these results with those from the February sampling leads to the conclusion that the nitrogen for this new growth had come from the insoluble N and arginine reserves and that the asparagine reserve had made little contribution to growth up to this time.

1. Introduction

The nitrogenous constituents of apple trees have been studied extensively, with particular reference to seasonal variations and the effects of added fertiliser.[1,2] There is general agreement that at all times and in all tissues, except possibly the leaves, most of the soluble N is present as arginine or asparagine. Both these compounds are thought to act as reserve forms of N for use during periods of rapid growth,[3,4] but the biosynthetic route of N into these amino acids has not been determined, nor is it known whether they have similar or separate functions.

We have already reported an experiment in which small amounts of ^{15}N-labelled fertilisers were applied to young apple trees, at different times of year either as a soil treatment or a foliar spray, and the resultant distribution of total ^{15}N in the different tree tissues was determined.[5] We have since extracted material from the trees to which fertilisers were applied in the autumn and have determined the ^{15}N content of the separate major constituents in all the tissues with the results reported below. Labelled arginine and asparagine, extracted from one of the tissues, have also been hydrolysed and the ^{15}N enrichments of the products determined separately, thus giving the relative labelling of the different N atoms in the original amino acid.

2. Experimental

Full details of the management of the experiment have been published previously,[5] but a brief outline is repeated below.

Young apple trees, cv. Cox's Orange Pippin on MM 106 rootstocks, growing in pots of soil were given ^{15}N-labelled potassium nitrate or urea, as a soil treatment or foliar spray respectively,

in mid-October 1972. Whole trees were sampled for analysis when dormant in mid-February and at about full-bloom in mid-May 1973. At harvest the trees were divided into their constituent tissues and, after weighing, representative samples of each were freeze-dried and milled.[6]

2.1. Methods of extraction and analysis

All tissues were extracted with ethanol/water/N HCl (75:25:2) except the leaves for which ethanol/chloroform/water (12:5:3) was used. The extractions were carried out at −15°C over 24 h; full details of the procedure have been published previously.[6]

Total nitrogen determinations were carried out after Kjeldahl digestion using sulphuric acid and selenium, followed by distillation in a Markham still, and titration with standard acid.

The nitrogen-15 content was determined by emission spectroscopy using a separate aliquot of the Kjeldahl digest and precipitation of the ammonia with a Nessler reagent.[7, 8]

The amino acids in the extracts were separated by ion-exchange chromatography using an 80 × 0.7 cm column of Technicon Chromobead A resin and the system of lithium buffers described by Perry *et al.*[9] The column eluate was split into two streams, in one of which the concentration of the amino acids was determined with ninhydrin, while the other was taken to a fraction collector. Samples of the fractions containing asparagine or arginine were subjected to Kjeldahl digestion so that their individual ^{15}N concentrations could be determined as above.

2.2. Hydrolysis of asparagine and arginine

Certain samples of these amino acids, collected after chromatographic separation, were hydrolysed to determine the relative labelling of the different N atoms in the molecule.

Asparagine was hydrolysed with alkali to give aspartic acid and ammonia. The reaction was carried out in a Cavett micro-diffusion flask, using column eluate (2 ml) and 10 N NaOH (1 ml) in the base of the flask and N H_2SO_4 (1 ml) in the cup. Preliminary work had indicated that, by leaving the flasks overnight at 30°C, both the hydrolysis of the amide and the absorption of ammonia by the sulphuric acid were quantitative. Separate ^{15}N determinations were made on the solution in the flask base and on that in the cup after overnight hydrolysis.

Arginine was first hydrolysed to ornithine and urea with arginase using the normal reaction condition for this enzyme[10] and then, after a pH adjustment, the resultant urea was further hydrolysed to ammonia with the enzyme urease.[11] Finally the ammonia was separated from the reactants by micro-diffusion in a Cavett flask by adding saturated potassium carbonate solution to the flask base and using N H_2SO_4 as absorbent at 30°C overnight as before. The ^{15}N enrichment of this ammonia is representative of the mean value of that of the 2 N atoms of the guanidino group in the parent arginine molecule. The isotope content of the ornithine moiety cannot be measured directly on the hydrolysate as protein N was added during the procedure and its value must be inferred from calculations.

3. Results

The weights of all the tree tissues, their total N contents and ^{15}N atom per cent excess values are given in Table 1. It is apparent that at each sampling the trees were of very similar size and total N content. However, the isotopic analyses show considerable differences resulting from the two methods of fertiliser application. For example, following urea treatment of the leaves the atom per cent excess values of the total N in the different tissues at the February sampling have a range of less than two, with the lowest values in the roots. In contrast, after soil application of nitrate there was an eight-fold range between the enrichments of the different tissues, with the highest values occurring in the roots.

The results of the analysis of the extracts are given in Table 2 as the total soluble N and the totals in the two major soluble constituents, asparagine and arginine, together with the atom per cent excess values. These results show that the enrichments of the two major soluble constituents in any one tissue were often very different. Following application of labelled urea the enrichment

Table 1. Analysis of tissues from apple trees treated with ^{15}N-labelled urea or potassium nitrate on 13 October 1972 and sampled in February or May 1973. Urea, approximately 75 mg N at 47 atom per cent excess ^{15}N, applied to foliage or KNO_3, 172 mg N at 20 atom per cent excess ^{15}N, applied to soil

	Dry weight (g)				Total nitrogen (mg) (and ^{15}N atom per cent excess)			
	14 February		14 May		14 February		14 May	
Tissue	Urea	KNO_3	Urea	KNO_3	Urea	KNO_3	Urea	KNO_3
Leaves			9.5	6.3			325 (2.22)	240 (0.63)
Scion bark	5.4	4.0	6.0	5.8	90 (3.39)	71 (0.24)	96 (2.19)	86 (0.33)
Scion wood	10.8	6.5	9.9	8.6	91 (3.11)	66 (0.28)	81 (2.04)	67 (0.31)
Stock bark	7.8	8.2	7.3	5.8	119 (2.93)	114 (0.57)	71 (1.42)	65 (0.43)
Stock wood	20.4	28.0	21.6	18.1	133 (2.45)	180 (0.65)	128 (1.25)	116 (0.35)
Old roots	8.5	7.9	4.0	9.0	163 (2.20)	149 (1.52)	86 (1.70)	197 (0.87)
Young roots	3.6	3.3	2.8	1.4	74 (2.39)	65 (1.83)	58 (0.85)	27 (0.99)
Total	56.5	57.9	61.1	55.0	670	645	845	799

Table 2. The soluble nitrogen and ^{15}N enrichment in the tissue extracts from apple trees treated with labelled fertilisers in October 1972 and sampled in February or May 1973. Values are given for the total soluble N and after sub-division into the two major constituents, asparagine and arginine

		Treatment: October 1972					
		Urea applied to leaves			KNO_3 applied to soil		
		Soluble nitrogen (mg) (and ^{15}N atom per cent excess)			Soluble nitrogen (mg) (and ^{15}N atom per cent excess)		
Sampling date	Tissue	Total	Asparagine	Arginine	Total	Asparagine	Arginine
14 Feb. 1973	Scion bark	11.9 (4.17)	2.2 (3.43)	7.4 (5.05)	8.3 (0.59)	3.5 (0.52)	3 2 (0.71)
	Scion wood	41.5 (3.58)	11.8 (1.85)	29.4 (3.87)	35.5 (0.25)	14.3 (0.15)	21.1 (0.36)
	Stock bark	29.4 (4.54)	8.8 (2.95)	18.2 (5.52)	21.4 (1.06)	9.5 (1.18)	8.9 (0.85)
	Stock wood	70.0 (3.01)	5.8 (2.81)	63.2 (3.03)	83.5 (0.83)	12.6 (1.43)	69.8 (0.69)
	Old roots	83.8 (2.60)	30.8 (1.49)	50.5 (3.06)	67.4 (1.78)	28.7 (1.99)	37.8 (1.56)
	Young roots	32.9 (2.95)	10.8 (2.36)	20.7 (3.29)	24.6 (2.35)	9.9 (2.90)	14.1 (1.90)
	Total	269.4	70.2	189.4	240.7	78.5	154.9
14 May 1973	Leaves	21.2 (2.06)	9.7 (1.90)	1.7 (3.20)	14.5 (0.68)	6.1 (0.52)	0.9 (0.25)
	Scion bark	32.1 (2.46)	15.5 (1.90)	12.6 (3.20)	22.0 (0.38)	8.9 (0.52)	9.9 (0.25)
	Scion wood	32.5 (2.17)	12.9 (1.66)	15.6 (2.74)	22.6 (0.37)	9.5 (0.50)	10.5 (0.25)
	Stock bark	22.3 (1.85)	12.5 (1.61)	7.2 (2.54)	23.8 (0.60)	11.5 (0.73)	9.9 (0.47)
	Stock wood	75.3 (1.36)	19.1 (1.50)	54.1 (1.30)	64.7 (0.41)	11.9 (0.75)	50.6 (0.34)
	Old roots	52.9 (1.95)	21.7 (1.36)	29.5 (2.38)	112.9 (0.99)	52.3 (1.17)	56.8 (0.78)
	Young roots	22.4 (1.08)	7.4 (0.70)	13.7 (1.32)	7.8 (1.12)	2.1 (1.30)	5.1 (1.05)
	Total	258.7	98.8	134.4	268.3	102.3	143.7

of arginine was, with one minor exception, always greater than that of asparagine in all parts of the tree. In contrast, following $K^{15}NO_3$ treatment, the asparagine in the roots and stock tissues was more highly enriched than the arginine.

The N derived from the fertiliser has been calculated from the ^{15}N enrichment of the applied fertiliser, the total N content of a fraction and its atom per cent excess value. It was not possible to determine the enrichments of the soluble asparagine and arginine fractions in the leaves as the

quantities present were too small, but in order to complete the totals for the whole tree the values for the leaves have been assumed to be equal to those of the scion bark on the same tree.

3.1. February sampling

There was little difference between treatments in the proportion of absorbed fertiliser N recovered in the soluble fraction of the tree tissues, but there was a pronounced treatment effect on the form in which the fertiliser N was present. Thus in February the trees previously treated with urea had over four times as much fertiliser N in arginine as in asparagine, while after nitrate treatment the fertiliser N was almost equally distributed between the two amino acids (see Figure 1).

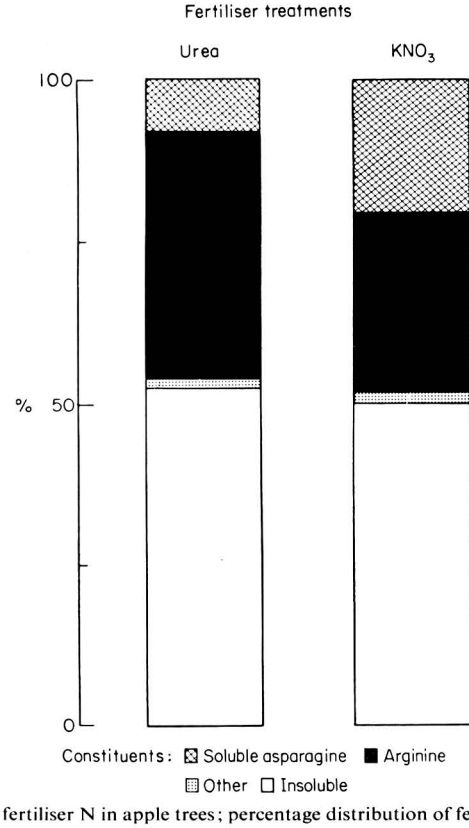

Figure 1. Chemical form of fertiliser N in apple trees; percentage distribution of fertiliser N in February following treatment the previous October with ^{15}N-labelled urea on the foliage or $K^{15}NO_3$ to the soil.

3.2. May sampling

The changes in total N content of the whole trees between February and May are shown in Figure 2A, with the totals in the newly developed leaves shown separately from that in the remainder of the tree.

The total N in the trees in May was greater than that in February by about 160 mg. Some 30–40% of the total N in May was found in the new leaves; in the remainder of the tree the insoluble fraction showed the greatest decrease from the February values.

The corresponding distribution of N derived from the fertiliser in the trees and new leaves is

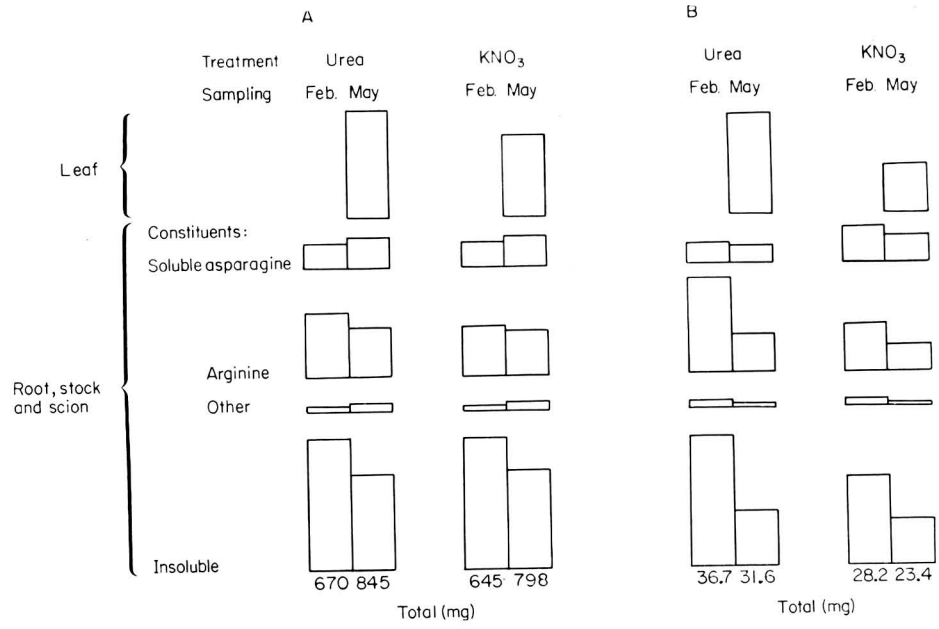

Figure 2. Chemical form of total N, Figure 2A, and of fertiliser N, Figure 2B, in apple trees sampled in February and May; distribution following treatment the previous October with ^{15}N-urea on the foliage or $K^{15}NO_3$ to the soil.

shown in Figure 2B. It can be seen that there had been a reduction of about 15% in the total fertiliser N recovered between the February and May samplings and that a large proportion of the May total was in the new leaves. In the remainder of the tree a comparison of the distribution of fertiliser N in February with that in May shows a reduction in the insoluble fraction to about one half, a similar marked reduction in labelled arginine, but very little change in the amount present in asparagine.

3.3. Hydrolysis of asparagine and arginine in the old roots

The asparagine and arginine present in all the old root extracts were separated chromatographically and subjected to controlled hydrolysis, after which the ^{15}N enrichments of the products were determined.

The results show that from all the samples of asparagine the atom per cent excess ^{15}N found in the ammonia was higher than that in the aspartic acid. The ratios of

$$\frac{\text{atom \% excess } ^{15}N \text{ in ammonia}}{\text{atom \% excess } ^{15}N \text{ in aspartic acid}}$$

were:

	Fertiliser treatments	
	Urea	KNO_3
February sampling	1.5	1.4
May sampling	1.2	1.2

Arginine was hydrolysed to ornithine and urea, and the latter was further hydrolysed to ammonia. The enrichments of the ammonia fractions were found to be almost equal to those of the original arginine samples at both times of sampling and for both fertiliser treatments.

4. Discussion

The total N and fertiliser N found in these trees have been discussed previously.[5] It was a feature of the experiment that the amounts of total N taken up from the applied fertiliser were small in relation to the total in the whole tree; hence the results reflect normal behaviour. This paper deals with the analysis of only one of each of the replicate trees but the experimental material was considered to be sufficiently uniform for valid conclusions to be drawn from the results.

The soluble nitrogenous constituents found in the trees at both sampling times were very similar to those in earlier reports,[2,4] namely a preponderance of arginine and asparagine over all other amino acids. However, the demonstration that a foliar urea application led to the overwinter accumulation of arginine while absorption of nitrate through the roots favoured asparagine (Figure 1) is a novel and unexpected result. There has been no previous suggestion that different N sources or different methods of application could affect the resultant plant constituents. Both these N sources are thought to be metabolised in plants through a common intermediate, ammonia, urea being hydrolysed with urease and nitrate being reduced with the nitrate and nitrite reductases. However, the biosynthesis of amino acids in the shoot could differ from that in the root if a different spectrum of organic acids was present in the two tissues. The four month interval between treatment and the first analysis is also relevant. Dilley and Walker[12] using detached apple leaves found that the ^{15}N from labelled urea taken up through the petioles was incorporated into a wide range of amino acids within a few hours. If this incorporation also takes place with a urea foliar spray the arginine we found in the other tree tissues in February must have been formed by export of these amino acids before leaf-fall and their further metabolism.

Comparing the amounts of total N in the trees in May with the corresponding February values (Figure 2A) shows that with both treatments there has been a marked increase over this period, presumably by further root absorption from the soil. In contrast there was a decrease in the total fertiliser N contents of the trees over this period (Figure 2B). Losses of fertiliser N at successive samplings have been noted earlier[5] and presumed to be caused by the continual death and renewal of the youngest roots. Demonstration of this turnover of nitrogen between roots and soil implies that the total root absorption is greater than the simple difference between the February and May values in Figure 2A.

Figure 2A also shows that the insoluble N and arginine decreased from February to May and that the N was incorporated into the new growth, mainly as leaf protein. The insoluble N is not a homogeneous fraction, being composed of both a wide range of enzymes and also some protein apparently serving as storage N. Nitrogen-15 from autumn-applied fertilisers would be expected to accumulate preferentially in the storage proteins, and hydrolysis of these reserves for use in spring growth would account for the disproportionate reduction in insoluble fertiliser N in Figure 2B compared with the corresponding changes in total insoluble N in Figure 2A.

In contrast, it is believed that there is a homogeneous pool of soluble arginine within apple trees, for in our previous work with ^{14}C-labelled arginine it was shown that arginine was constantly degraded and re-formed throughout the year.[13] The hydrolysis results reported above are also consistent with a constant turnover for in all the samples examined the arginine molecules were found to be equally labelled with ^{15}N in both the urea and the ornithine moieties. However, with a homogeneous pool the decrease of labelled arginine from February to May should be in proportion to the total decrease of this amino acid. But it will be seen from Figure 2B that the fertiliser N present as arginine was down by about 50% compared with a much smaller decrease in the total amount of arginine (Figure 2A). One explanation would be that the insoluble fraction, which is less highly enriched than the soluble fraction, was hydrolysed to arginine in early spring. Thus considerable isotopic dilution of the soluble arginine pool would take place before much of its N was used for new shoot growth. This suggestion would be in accord with results published by Tromp and Ovaa[14] who found an arginine-rich storage protein in apple shoot bark.

Degradation of the asparagine extracted from tissues harvested 4 months after fertiliser application showed that the two N atoms in the molecule still had quite different enrichments. This result would suggest that asparagine was metabolically relatively inert at this time and was not being

turned over continuously. The small increase in the total amount of asparagine between February and May while the amount of ^{15}N in this compound remained almost constant shows that asparagine made little contribution to the initial growth in these trees up to full bloom. Asparagine could well act as a second reserve providing the N for shoot extension growth, indeed Sahulka[15] has shown that asparagine is the dominant soluble constituent of young apple shoots.

Acknowledgement

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The Measurement of Cation Exchange Capacity of Soils

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The implications of the different methods available for measuring the cation exchange capacity (CEC) of soils are examined in the light of cation exchange mechanisms, and the issues involved in selecting a suitable method are discussed.

1. Introduction

Cation-exchange capacity, CEC, is extensively used in characterising soils for survey purposes and in assessing their ability to supply cation nutrients to plants. Since the many methods that have been used to measure CEC often give different values for the same soil, it seems useful to make an appraisal of the methods available, of the factors to be considered when choosing a method, and of the interpretation of the results. A simplified account is included of the relevant aspects of cation exchange mechanisms, but experimental details of standard methods are readily obtainable[1–3] and are not repeated here.

2. Cation exchange mechanisms

2.1. Soil negative charge

Cation exchange is a consequence of the negative electric charges on the colloidal clay and humus particles of the soil matrix. These charges can be clearly demonstrated by microelectrophoresis experiments.

There are two main components of negative charge, a constant or permanent charge and a variable pH-dependent charge.

The permanent charge is generated by partial isomorphous substitution within the lattices of clay-size layer silicates, and in particular the substitution of Al^{III} for Si^{IV} in the tetrahedral sheet and of Mg^{II} or Fe^{II} for Al^{III} in the octahedral sheet. The consequent deficiency in positive valency results in a crystal with an excess negative charge.

The variable charge is generated by the pH-dependent dissociation of hydroxyl groups, which may occur in silanol groups, located at surfaces of alumino-silicate gels or the edges of layer silicate crystals, or in phenols, or in carboxylic acids. Where appreciable amounts of organic matter are present, such as in surface soils, the major part of the variable charge arises from proton dissociation from carboxyls and phenols. A further possible mechanism contributing to pH-dependent charge is the "blocking" of negative charge by strongly adsorbed hydroxy-aluminium cations. As the pH rises these cations are precipitated as aluminium hydroxide and the negatively charged sites are then free to participate in cation-exchange reactions.

Thus, the negative charge depends not only on the nature and amount of silicate clay minerals, a relatively permanent feature of a soil, but also on the pH and organic matter content, both of of which are altered to some extent by agricultural practice.

2.2. Adsorbed cations

The adsorption of positively charged counter ions (mainly Ca^{2+}, but also K^+, Na^+, NH_4^+, Mg^{2+}, Mn^{2+} and Al^{3+}, depending on circumstances) by soil particles ensures their overall electrical

neutrality. When a soil is suspended in a salt solution, the cations of the salt desorb and replace the freely diffusible counter ions of the soil—the phenomenon of cation exchange.

Cation exchange reactions can thus be considered as a competition between different cations for the negatively charged surfaces to which they are electrostatically attracted. The distribution of each cation between the surface and the outer solution depends on (a) the charge, size and hydration state of the cations; (b) the surface charge density of the soil particles; (c) the ionic strength of the solution; and (d) any specific interactions that may occur, such as a size-preference for K^+ by some minerals or organic-complexing of some cations.

Cation exchange capacity is, therefore, a measure of the ability of a soil to adsorb cations in such a form that they can be readily desorbed by competing ions. It can be considered as equivalent to the negative charge of the soil, expressed in milliequivalents per unit mass (mEq/kg soil). In CEC determinations it is assumed that a large excess of one saturating salt will carry the reaction to completion and displace all the "native" cations, and the CEC is then found by determining either the amount of displaced cations or the amount of adsorbed saturating cation (section 3).

2.3. Charge of the adsorbed cations

An estimate of CEC from analytically determined amounts of cations presupposes that the charge on these cations is known. Many polyvalent cations form hydroxy ions, and Carlson and Overstreet[4] showed that montmorillonite adsorbed appreciable amounts of $CaOH^+$ and $MgOH^+$; their results indicate that the CEC at pH 7 would be overestimated by about 5% if it were assumed that the adsorbed ions were wholly Ca^{2+} and Mg^{2+}. Errors of a similar type but greater magnitude occur for Al^{3+} at lower pH values, but for Ba^{2+} they are very small provided the pH is below 9. The common monovalent cations are wholly adsorbed as NH_4^+, Na^+, etc., but soils saturated with these may hydrolyse at low electrolyte concentrations resulting in loss of cation and underestimation of CEC.

2.4. Positive charges in soils

Further complications can arise if positive as well as negative charges are present, as with iron-rich acid clay subsoils. The two types of charge can be measured separately in a strong electrolyte solution, where the electrical double layers are compressed, but at low concentrations the double layers expand and may overlap to such an extent that the positive and negative charges neutralise each other. This may occur during washing procedures in CEC measurements.[5] However, a low electrolyte concentration is likely to be the normal situation in strongly leached soils, and the charge neutralisation referred to will result in a more realistic measure.[6] The CEC then equals the *net* charge, which may be considerably lower than the negative charge.

Positive charges originate from the specific adsorption of protons (H^+) on oxide/hydroxide surfaces, and the magnitude of this charge therefore depends more critically on the ionic strength of the solution than does the negative charge which is mainly due to lattice substitutions. Positive charge is strongly pH-dependent and is substantially neutralised by OH^- at pH 7.

These considerations indicate that for CEC measurements where the adsorption of potential-determining ions may contribute to either the positive or negative charge, low electrolyte concentrations should be used for the final equilibration that determines CEC.

2.5. Acid soils

The situation in acid soils needs further consideration. The proportion of charge not satisfied by $Ca^{2+}+Mg^{2+}+K^++Na^+$ has been traditionally attributed to exchangeable hydrogen and constitutes the degree of unsaturation of the exchange complex. It is now known that truly exchangeable hydrogen, meaning diffusible H_3O^+ ions electrostatically attracted to charged surfaces, is very small for most soils.[7]

Two types of reaction occur when bases react with acid soils: (a) aluminium ions neutralising

negative charges on the clay surface react with hydroxyl ions of the base, precipitating aluminium hydroxide, thus:

$$Al^{3+} + 3OH^- \rightarrow Al(OH)_3$$

The surface aluminium ions are replaced by the cations of the base and the amount of charge is unaffected; (b) ionisation of acidic –OH groups, which may occur in a number of soil components as mentioned previously:

$$ROH + H_2O = RO^- + H_3O^+$$

Reactions of type (b) cause an increase in the negative charge which is then neutralised electrostatically by the added basic cations. This is analogous to the dissociation of a simple weak acid but the acid groups involved occur on the surfaces of the solid soil matrix. The fraction of charge known as "exchangeable hydrogen" is either exchangeable (or soluble) aluminium as in (a) above, or the increase in charge brought about by ionisation during the CEC determination as in (b). "Base unsaturation" is the proton-donating ability of the soil when brought from its natural acidity to the pH of the determination (i.e. the *total* acidity of the soil as distinct from pH which is a measure of the *degree* of acidity). Clearly the size of this fraction for a given soil depends on the pH and buffering power of the saturating salts used for its determination.

2.6. Saline soils

The presence of excess soluble salt clearly confuses the determination of exchangeable cations, and therefore the use of the summation methods for CEC (3.2 below) would require the prior leaching of soluble salts, or their separate determination.[2] However, CEC methods based on the saturation of soil with an index cation should automatically ensure the prior removal of soluble salts.

3. Experimental methods

3.1

A large number of different experimental methods have been proposed. It is unnecessary to describe them all, but most of them are examples of one or other of the following four different approaches to the problem.

3.2. Summation methods

The exchangeable cations can be displaced with a saturating salt, and the CEC is taken as equivalent to the sum of exchangeable cations in the extract.

1. If an unbuffered salt is used (e.g. 1.0 M NH_4Cl or KCl) it is usual to determine Al^{3+}, Ca^{2+}, Mg^{2+}, K^+, Na^+ and perhaps also Mn^{2+} in the extract, and the sum of their charges is taken as the exchange capacity at the natural pH of the soil.

2. If a buffered salt (e.g. ammonium acetate at pH 7.0, barium acetate at pH 7.0 or 8.2, or $BaCl_2$–triethanolamine at pH 8.0–8.2) is used, only Ca, Mg, Na and K can be determined in the extract. Their sum must be added to a separate estimate of total acidity[8] at the pH of the buffer solution. The acidity in an acetate extract is estimated by titrating it with alkali to its initial pH. The acidity in a triethanolamine extract is found by titrating equal volumes of the original extractant and the soil extract with acid to the same pH (usually 5.1) and taking the difference of the titrations as the estimate of acidity.

The variant (**1**) measures the fraction of soil acidity due to exchangeable Al whereas the total acidity measurement in (**2**) includes the extra fraction due to pH-dependent charge, and any other reactions that may consume base (such as anion displacement[9]) although the magnitude of these is probably low. The more acid the soil, the greater is the difference between the values for (**1**) and (**2**). If calcium carbonate is present, it is sufficiently soluble to give inflated results for Ca^{2+} at pH 7.0 but not at pH 8.2.

3.3. Direct displacement of the saturating salt

After a salt solution (e.g. NH_4Cl) has been used to displace the exchangeable cations and to saturate the negative charges with an index cation, the adsorbed cation and the small amount of solution entrained by the soil after centrifuging can be displaced directly by another salt solution (e.g. KNO_3) without further treatment of the soil. The cations (NH_4^+) and anions (Cl^-) are then determined in the resulting KNO_3 extract, and the difference between them equals the CEC of the soil.[10] The positive charge, if any, can also be found if the moist soil is weighed in the centrifuge tube prior to extraction with KNO_3 so that the amounts of NH_4^+ and Cl^- in the entrained solution can be calculated; the adsorbed Cl^-, equal to the positive charge, is then given by the difference between the Cl^- determined in the KNO_3 extract and that in the entrained NH_4Cl solution.

The method has been used with unbuffered salts,[10] and with NH_4Cl buffered at pH 8.5.[3]

3.4. Displacement of index cation after washing out excess salt

When the exchange sites have been saturated with an index cation, the soil can be washed free of excess saturating salt and the amount of the index cation adsorbed by the soil can then be displaced and determined. There are a number of variations, of which two are of particular interest.

1. The ammonium acetate method, probably the most widely used CEC method in the past. Experimental details are given by Chapman.[1] The soil is saturated with 1.0 M ammonium acetate at pH 7.0 and then washed free of excess salt. Alcohol is used for washing to minimise hydrolysis,[5] isopropanol being preferred because NH_4-humates are less soluble in this than in ethanol.[11] Then either (a) the adsorbed NH_4^+ is displaced directly from the soil by an air stream after treatment with 5% Na_2CO_3 solution and the ammonia gas passed into standard acid and titrated, or (b) the soil is washed with acidified NaCl and the NH_4^+ in the NaCl extract is determined by steam distillation and titration. The method has much to commend it, particularly if the exchangeable cations are needed as well as CEC. However, the washing procedure to remove excess ammonium acetate is rather tedious, counting against it as a rapid procedure. This step also introduces a number of uncertainties which militate against its value for research purposes.[11,12]

2. The buffered $BaCl_2$–$MgSO_4$ method, developed by Bascomb.[13] The soil is saturated with Ba^{2+} by treatment with 0.5 M $BaCl_2$ solution buffered at pH 8.1 with triethanolamine/triethanolamine hydrochloride. It is then washed once with water to remove excess $BaCl_2$ and the adsorbed Ba^{2+} displaced with 0.025 M $MgSO_4$ solution. The excess (i.e. non-adsorbed) Mg is then determined in the solution by EDTA titration. A wide soil:solution ratio (5 g to 200 ml) means that only one saturating treatment need be used. The small amount of $BaCl_2$ remaining after the water wash does not affect the result because an equivalent amount of Mg^{2+} remains in solution. The efficiency of displacement of Ba^{2+} by Mg^{2+} in the $MgSO_4$ treatment is increased by the precipitation of Ba^{2+} as $BaSO_4$ ("compulsive exchange"). Although this uses the same saturating reagent as for total acidity (i.e. $BaCl_2$–triethanolamine), it determines permanent charge as well as acidity to give the total CEC at the pH of the buffer.

Clay soils are sometimes difficult to disperse in Bascomb's reagent, and require an initial dispersion in water for the method to be effective (A. S. de Endredy, personal communication, 1972).

3.5. Radioactive tracer methods

These have some obvious advantages, for, once having saturated a soil with an index cation, it is not necessary either to wash out all excess electrolyte or to displace the index cation, but only to reduce the electrolyte concentration to an appropriate level before labelling the suspension with a radioactive isotope. The concentration of the index cation in the solution is then determined, and the distribution of isotope (and hence of the total cation) between the two phases is given by radiation measurements. Tracer methods are more often used for research then for routine purposes, probably because extensive counting facilities are not often available. ^{45}Ca in a Ca^{2+}-saturated system has often been used, following Borland and Reitemeier[14] but ^{133}Ba in a Ba^{2+}-saturated system also gives excellent results.[15]

4. Factors to consider when choosing a CEC method

4.1

The main questions arising here are the quantities that should be measured and the experimental methods that will most conveniently measure them.

4.2. What quantity should be measured?

It will be appreciated from the account of exchange mechanisms given above that there is no single unique value for the CEC of a soil.

The CEC of a laboratory sample depends critically on the pH at which it is determined and for some soils it depends also on the ionic strength of the solutions used. It might seem therefore that it should be determined at its natural field reaction using unbuffered solutions. However, the CEC of a field soil also varies because its pH and organic matter content may be affected by agricultural practice.

The CEC of subsoils is much less variable than that of topsoils. In the first place the charge variation with pH is less in subsoils because of their lower organic matter contents. Then the pH variation itself is less in subsoils, both because their pH is often higher than that of the corresponding natural surface soil (at least in cool temperate regions) and because surface dressings of lime do not usually penetrate into subsoils. Therefore the CEC of subsoils may be more useful for characterising soil series, although that of topsoils is clearly more important agriculturally. These considerations are illustrated in Figure 1, which shows some typical variations of CEC with pH for some contrasting arable soils.

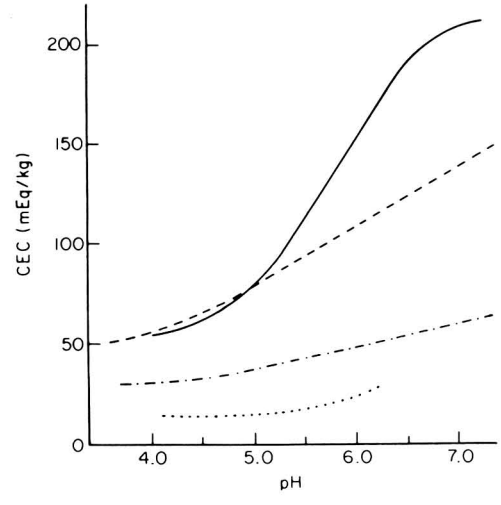

Figure 1. Effect of soil reaction (pH in 10^{-2} M $CaCl_2$) on CEC, for some contrasting soils adjusted to different reactions in the field. ————, Craibstone (Aberdeenshire) topsoil, 13% clay, 3.1% C. ·····, Craibstone (Aberdeenshire) subsoil 10% clay, 0.3% C. — — —, Shardlow (Derbyshire) topsoil, 24% clay, 1.7% C. —·—·—, Kennington (Oxfordshire) topsoil, 9% clay, 0.5% C.

The more extensive results given in Table 1 for several Scottish soil profiles compare different CEC methods run at the same pH, and at different pH values. The major variations are caused by the pH of the determination, and this is more pronounced for soils that are naturally strongly acid and have large amounts of organic matter, such as the surface horizons of the Linhope and Balrownie series. There is also a large difference with depth of sample for soils with high organic matter contents in their surface horizons. The pH effect is relatively small for subsoil horizons which contain little organic matter, but the Balrownie series is an exception and may contain considerable mineral sources of pH-dependent charge.

The possibilities then are to measure CEC at approximately the soil's natural acidity, using unbuffered solutions, or to measure it with buffered solutions at some standard pH. If the aim

Table 1. A comparison of different methods of measuring cation exchange capacity (mEq/kg) for some typical Scottish soil profiles

Association, series, site and map ref.	Parent material	Major group	Depth of sample (in)	Horizon	pH ($CaCl_2$)	Clay (%)	Carbon (%)	CEC unbuffered			CEC at pH 7			CEC at pH 8.1
								Ba/Mg	Ba isotope	Sum ex cation	Ba/Mg	Ammonium acetate	Ex cation + acidity	Ba/Mg
Ettrick association	Till derived	Non-calcareous	2–5	S	4.9	25	3.0	87	91	89	123	156	163	167
Ettrick series	from Ordovician	gley	10–14	B_2g	5.3	38	0.6	114	119	112	124	135	142	145
Falahill	and Silurian		19–23	B_3g	5.9	21	n.d.	130	117	144	131	150	169	147
NT 391 564	graywacke and shale		30–34	Cg	6.1	20	n.d.	135	135	153	145	152	178	155
Ettrick association	Till derived from	Brown forest	0–2	A	3.5	14	9.6	107	130	101	206	286	292	313
Linhope series	Ordovician and	soil	5–9	B_2	3.9	33	1.8	61	62	78	92	144	153	151
Greenwood	Silurian graywacke		15–17	B_3	4.0	27	n.d.	44	44	59	50	103	93	63
NT 841 642	and shale		24–30	C	4.1	28	n.d.	77	67	74	91	123	111	93
Kilmarnock association	Till derived from	Brown forest	3–7	S	5.1	23	2.1	73	96	96	120	137	141	131
Kilmarnock series	lower carboniferous	soil with	15–19	B_2g	6.1	24	0.6	91	82	96	98	99	111	99
Abbey Mains	sediments and	gleyed B and	30–34	B_3g	6.7	27	n.d.	126	111	131	126	112	140	116
NT 539 763	lavas	C horizons	40–46	C	7.2	30	n.d.	126	126	166	125	121	161	115
Balrownie association	Till derived from	Podzol	0–2	A_1	3.7	10	11.5	117	140	132	252	359	336	456
Balrownie series	Old Red Sandstone		3–7	A_2	4.0	27	2.6	52	69	74	135	173	177	186
Muirhead			8–12	B_2	4.4	15	n.d.	14	20	27	70	101	104	120
NN 843126			16–20	B_3	4.4	12	n.d.	6	13	23	38	76	73	86
Stirling association	Estuarine silt	Warp gley	2–6	A_2	5.9	18	8.1	280	287	285	295	325	349	359
Stirling series	and clay		9–12	A_2g	5.7	35	1.4	145	146	153	150	189	185	170
Palwhilly			16–19	B_2g	6.4	28	n.d.	122	116	132	134	135	147	130
NX 448607			25–29	Cg	7.2	19	n.d.	114	109	177	109	120	177	107

n.d., not determined.

is to compare soils as an aid to series classification there are obviously strong grounds for doing all measurements at a standard pH; otherwise CEC values for natural soils and limed soils from the same series may differ greatly (by a factor of three for some topsoils in Scotland, for example) whereas they will differ little if measured at the same pH. If this is done, however, it should be realised that CEC of acid soils in the field may be very different from the values given by the laboratory measurement, and that this difference will be greater the higher the organic matter content and the more acid the soil; in such circumstances there may even be a case for running two determinations, one at the standard pH and one at the field pH. If, on the other hand, the aim is to study soil reactions that may be affected by CEC, the measurement should obviously be made at the natural acidity of the field soil.

If all measurements are to be made at a standard pH, its value must be decided. There appear to be good arguments in favour of both the commonly used pH values of 7.0 and 8.2.

The pH of fully base-saturated soil in equilibrium with excess $CaCO_3$ and a CO_2 partial pressure equal to that in the atmosphere (0.0003) is 8.2.[16] This was the basis for using 8.2 as a standard pH for measuring CEC. There is also less experimental uncertainty at pH 8.2 because of the lower solubility of $CaCO_3$ at this pH.

On the other hand, the partial pressure of CO_2 in biologically active soils, such as organic surface horizons, is much higher than that of the atmosphere, and the pH of the HCO_3^-/CO_3^{2-} buffer system is then nearer to 7.0 than to 8.2. For example, 1% CO_2 in equilibrium with $CaCO_3$ gives pH 7.3.[17] Unless a majority of the soils being investigated have pH values (in 10^{-2} M $CaCl_2$ solution) nearer 8 than 7, which is only likely for some saline and alkali soils, CEC measured at pH 7 will also be nearer to the natural pH than that measured at pH 8.2.

4.3. What experimental methods should be used?

The factors to consider in choosing an experimental method are the accuracy and precision required and the amount of work involved. The writer has used most of the procedures described in section 3 above, and considers that to obtain unambiguous results for research purposes as accurate as any other method and with less work than most, the barium isotope method[15] is preferable, but it requires the use of sophisticated automated counting equipment if a large number of samples have to be processed. For most routine survey and advisory purposes high accuracy is not required, and indeed field heterogeneity of soils makes it pointless to strive for it; reproducibility between field samples of $\pm 20\%$ is as good as can be expected, and for all the experimental methods duplicate determinations on the same sub-samples are well within these limits.

Of the methods described above, the $BaCl_2/MgSO_4$ method of Bascomb[13] in section 3.4 (**2**) appears to be outstanding. It requires less work than the others, i.e. only three shaking-centrifuging steps because the "compulsive exchange" principle on which it is based ensures an efficient displacement of the saturating cation Ba^{2+} by the displacing salt $MgSO_4$. The method as described by Bascomb is carried out at pH 8.1. It can be adapted to pH 7, where triethanolamine/triethanolamine hydrochloride is still a better buffer than ammonium acetate, but the concentration of $(Et)_3NH^+$ ions is then appreciable and may compete with Ba^{2+} for the charged sites on the soil. The method may also be used with unbuffered $BaCl_2$ if required. The slight solubility of calcium carbonate at pH 7 may introduce two complications: (a) saturation of the soil with Ba^{2+} may be less efficient; (b) the Ca^{2+} released can interfere with the determination of Mg by EDTA titration, but this is no problem if atomic adsorption spectroscopy were to be used for Mg determination.

Both the barium isotope method and the $BaCl_2/MgSO_4$ method avoid uncertainties over the charge of the displacing cation (Ba^{2+} accounts for all the adsorbed barium), the nature of the total acidity (because its separate determination is not required) and the problems associated with the removal of excess ammonium acetate.

There appears to be relatively little difference between the results in Table 1 for the three methods used in the unbuffered systems. At pH 7 the $BaCl_2/MgSO_4$ method generally gives lower results than the other two, particularly for soils that are strongly acid; the presence of $(Et)_3NH^+$ ions in the saturating reagent, referred to above, may partly account for these low values. It is impossible

to decide which of these results is nearest to the true value, and how important these differences are likely to be will depend on what deductions, if any, are subsequently made from the results.

4.4. The problem of continuity

There is always resistance to a proposed change in methods on the grounds that results from new methods may be different from those previously used, and thus comparisons between future work and past work may be difficult. Two factors must be weighed here: the degree of superiority of a proposed method over one already in use, and the magnitude of the differences in results given by the two methods. However, it is always possible to run two methods together for a selected number of contrasting soils, and to use these results as a basis for correcting the values given by the previous method.

4.5. The need for extra determinations

In some of these methods, estimations of exchangeable cations or of total acidity may also be provided as a bonus during the CEC method, and a requirement for either of these values may affect the ultimate choice of a CEC method. Thus the traditional ammonium acetate method at pH 7 is very straightforward for desorbing exchangeable Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}, but the need to remove all excess ammonium for the CEC estimation introduces errors. A $BaCl_2$–triethanolamine extract can be used for total acidity determination as well as CEC if the concentration of reagents is suitably adjusted, and exchangeable Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} could also be determined in the extract by spectrochemical methods if required. It seems preferable to the writer, however, to determine exchangeable cations in a separate extraction using unbuffered salts, particularly for acid soils because Al and Mn are then included, and to choose the most acceptable method for CEC according to the requirements of the work in hand.

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Effect of Nitrogen Source on Oxalate Accumulation in *Setaria sphacelata* (cv. Kazungula)

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Setaria sphacelata (cv. Kazungula) was grown under controlled environment conditions on sand provided with mineral nutrient solutions containing nitrate, ammonium, urea, or nitrate plus ammonium as sole nitrogen sources. Growth in urea or ammonium resulted in small but significant decreases in oxalate concentrations of foliage compared with growth in nitrate, but nitrogen source had little effect on plant growth rates. Similar levels of potassium and total nitrogen were found in foliage from all treatments. Nitrate concentration was highest in leaves of plants supplied nitrate plus ammonium and lowest in leaves of plants supplied only ammonium as the nitrogen source. Nitrate reductase activity was virtually absent from new fully expanded leaves from all treatments, but readily detectable in young regrowth when highest activities were associated with the nitrate plus ammonium treatment and lowest with ammonium alone.

Leaves continued to accumulate oxalate after reaching maturity and it is considered that anions other than nitrate must be responsible for the cation excess which prompts the synthesis of this oxalate.

No diurnal variation in oxalate content of controlled environment or field grown *Setaria sphacelata* was observed.

1. Introduction

Certain grasses of tropical origin produce high dry matter yields when grown over the New Zealand warm season. Among the most promising are selections of *Setaria sphacelata* which in small scale plot trials produced more dry matter during a 5-month growing season than did temperate grasses over the whole year (Slack, C. R., unpublished information). An undesirable feature in varieties of *Setaria*, however, is the accumulation of oxalate up to levels which may prove fatal to ruminants. A study of factors affecting oxalate concentrations in *S. sphacelata* in the field[2] suggested that high rates of nitrogen application increased oxalate content of the forage, but no well defined relationship was established. In container trials, however, applying high levels of nitrate and potassium resulted in large increases in the oxalate content of *S. sphacelata*.[3]

A theory has been advanced that organic acids accumulate as a result of excess cation concentration in the leaves. This is a consequence of nitrate transport from roots to shoots and subsequent reduction of nitrate to organic nitrogen in leaves.[4–7] Cations which originally accompanied the nitrate from the roots are then balanced by the production of organic acids within the leaf and some (e.g. Ca-oxalate) may be immobilised by precipitation in the vacuole in the form of insoluble salts.[4] If this were the only reason for oxalate accumulation in leaves it seemed reasonable to expect that plants growing in solutions containing no nitrate nitrogen would not accumulate significant amounts of oxalate.

This paper explores the relationship between the type of nitrogen supplied to, and oxalate concentrations in the leaves of *S. sphacelata* grown in sand culture. Oxalate concentrations in leaves of different ages were measured and the proposed diurnal variation in oxalate content of *S.sphacelata*[2] was re-examined.

2. Methods

Seedlings of *S. sphacelata* (cv. Kazungula) were transplanted into 4.5 litre pots containing washed sand and were maintained in a glasshouse (25°C maximum, 15°C minimum) on a complete (NCSU—Table 1) mineral nutrient solution for 45 days. Plants were then cut back to a 2.5 cm stubble and pots were leached thoroughly with water prior to random division into groups of 15 and transfer to a controlled environment room (DSIR Climate Laboratory, Palmerston North, New Zealand). Conditions throughout the experiments were daylength 12 h; temperatures 25°C day, 20°C night; light intensity 156 W m^{-2} PAR, relative humidity 72% day, 90% night. All pots received 150 ml of appropriate nutrient and 2 × 100 ml of water each day. Nutrient solutions were composed of macroelements as shown in Table 1, and microelements consisting of (in parts/10^6) boron 1.44,

Table 1. Macroelement composition of mineral nutrient solutions containing different forms of nitrogen

Nutrient type	Concentration (mM) in working strength solution							
	N	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	$H_2PO_4^-$	Na^+	Cl^-
Nitrate	7.5	3.0	2.5	1.0	1.0	0.5	0.5	0.5
Low potassium	7.0	0.5	2.5	1.0	2.0	0.5	0.5	0.5
Urea	7.4	3.0	2.5	1.0	2.25	0.5	0.5	5.5
Ammonium	7.4	3.0	2.5	1.0	5.95	0.5	0.5	5.5
Nitrate + ammonium[a]	5.9[b]	1.56	1.37	0.23	0.85	0.25	0.6	—

[a] NCSU = a "balanced" nutrient solution originally developed at the North Carolina State University, and subsequently modified in this laboratory.
[b] 3.9 mM NO_3^- + 2.0 mM NH_4^+.

manganese 1.02, zinc 0.11, copper 0.04 and molybdenum 0.0075. Ferric iron was supplied at 2.5 parts/10^6 as Sequestrene (Geigy).

At 19- to 21-day intervals, all treatments were harvested (cut to leave a 2.5 cm stubble) and material from individual plants was dried in a vacuum oven at 40°C, weighed and ground to pass a 2 mm screen prior to chemical analyses. Three such harvests were made. When checking for diurnal variations in oxalate content, whole tillers were harvested from plants within the nitrate treatment at the times stated and were dried at 100°C prior to grinding for oxalate analyses. Similarly, tillers harvested from plants within the nitrate treatment were dissected into sub-samples of leaves of various ages and into leaf sheaths. These were also oven dried prior to analysis.

Two separate experiments were conducted. In the first, comparisons were made of high and low nitrate, urea, low potassium and nitrate plus ammonium. In the second experiment the comparisons were between the nitrate, urea, ammonium and nitrate plus ammonium treatments. The low nitrate treatment (0.5 mM nitrate as the sole source of nitrogen) was included in the first experiment as a control but was discontinued when plant growth in this treatment ceased between the first and second harvests.

Oxalate was measured by gas–liquid chromatography as previously described[8] and is expressed as anhydrous oxalic acid. A single, vacuum-dried preparation of *S. sphacelata* foliage was repeatedly sampled and analysed to test the reproducibility of the gas–liquid chromatographic assay for oxalic acid content. Ten analyses resulted in a mean value of 6.51%, with a standard error of 0.07%. Total nitrogen was determined on Kjeldahl digests of the plant material by a phenol/hypochlorite colorimetric method[9] and nitrate was measured in water extracts of the dry powder by a phenoldisulphonic acid procedure.[10] Potassium was measured by atomic absorption spectroscopy after ashing sample material at 500°C for 2 h. Chemical analyses were performed on bulked materials prepared by combining equal weights (200 mg) from each individual within a treatment at the three harvests.

For measuring the activity of nitrate reductase, 1.5 g of regrowth tissue was homogenised at 0°C in a mortar with 1.5 ml of 0.1 M Bicine/NaOH, pH 8.0 containing 2 mM EDTA and 10 mM dithiothreitol, squeezed through Miracloth (Chicopee Mills Inc., NY) and 0.5 ml of the filtrate immediately passed through a 5 ml Sephadex G-25 column equilibrated in the above buffer diluted ×4. The protein eluate was collected in a final volume of 1 ml of which 0.2 ml was incubated at 30°C with 50 mM potassium phosphate pH 7.5, 10 mM potassium nitrate and 0.25 mM NADH in a final volume of 1 ml. Aliquots (0.2 ml) of the reaction mixture were removed at 0, 30 and 60 min for determination of nitrite.[11]

3. Results

3.1. Plant growth

Plants grew vigorously in all treatments, differences in dry weight yields between treatments being either not significant or not consistently significant. In the second experiment, total yields were a little lower compared with the first experiment as plants were harvested 2 or 3 days earlier. However, differences in dry weight yield between treatments were again small.

Table 2. Dry weight yields of *Setaria sphacelata* (cv. Kazungula) growing on sand and provided with different nitrogen sources

	Mean dry weight at harvest (g/plant)				
	Nitrate	Urea	Ammonium	Low potassium	Nitrate+ ammonium
Expt 1	13.27a[a]	12.33b	—	10.50b	11.87b
Expt 2	10.16a	10.67a	10.18a	—	9.58a

[a] Yields possessing different suffices are significantly different at the 5% significance level (Fisher l.s.d.).

3.2. Oxalate content

In the first experiment, the urea treatment resulted in a small, but statistically insignificant, decrease in oxalate concentration compared with the nitrate, low potassium and nitrate plus ammonium treatments. In the second experiment, both the urea and ammonium treatments resulted in significantly lower oxalate concentrations than either the nitrate or nitrate plus ammonium treatments.

Table 3. Influence of nitrogen supply on oxalic acid content of *Setaria sphacelata*

	Mean % dry weight oxalic acid at harvest[a]				
	Nitrate	Urea	Ammonium	Low potassium	Nitrate+ ammonium
Expt 1	6.6a	5.9a	—	6.4a	6.4a
Expt 2	7.5a	6.1b	6.3b	—	7.1a

[a] Bulked material from each treatment at each of the three harvests was analysed in triplicate and the values for each harvest averaged. Concentrations possessing different suffices are significantly different at the 5% level (Fisher l.s.d.).

Although oxalate concentration increased with leaf age, even very young leaves contained relatively high oxalate levels (Table 4). The concentration of oxalate in the leaf sheaths was also high.

Table 4. Variations in oxalic acid concentration with leaf age in *Setaria sphacelata*

Leaf position[a]	1	2	3	4	5	6	Leaf sheaths
% Oxalic acid	3.4	3.8	5.0	7.0	9.1	8.6	5.3

[a] Numbers 1 to 6 denote increasing leaf age, with position 3 being equivalent to the youngest fully expanded leaf. Nitrate treated plants, experiment 2.

There was no diurnal variation in oxalate content of non-flowering tillers of *S. sphacelata* (Table 5), nor was there any diurnal variation in oxalate content in youngest mature leaves of *S. sphacelata* or *Digitaria setivalva* growing in the field (unpublished results).

Table 5. Diurnal variations in oxalic acid concentration of *Setaria sphacelata* tillers[a]

Sampling sequence (h)	0000	0600	1200	1800	2400
% Oxalic acid	6.9	6.5	6.6	6.7	6.4

[a] 17–22 g fresh weight of tillers harvested from nitrate-grown plants at 6 h intervals over 24 h; photoperiod 0000–1200 h.

3.3. Nitrate, total nitrogen and potassium content of foliage

Nitrate was ten times higher in leaves of nitrate and nitrate plus ammonium treated plants compared with urea and ammonium treated plants (Table 6). Leaves from nitrate plus ammonium

Table 6. Influence of nutrient type on oxalic acid, nitrate, potassium and total nitrogen content in *Setaria sphacelata*

Nitrogen	Oxalic acid (μmol/g dry wt)	Potassium (μmol/g dry wt)	Nitrate (μmol/g dry wt)	Total nitrogen (μmol/g dry wt)
Nitrate	834	710	7.8	1830
Urea	734	745	0.8	1950
Ammonium	768	770	0.7	2010
Nitrate + ammonium	812	620	8.9	2014

Bulked materials from third harvest, experiment 2.

treated plants contained higher nitrate concentrations than did nitrate-grown leaves even though the latter nutrient contained almost twice the nitrate concentration of the former (Table 1). Total nitrogen levels in the foliage were similar in all treatments as might be expected since growth rates

were also fairly similar. High potassium levels were recorded in harvested material from all treatments and were probably sufficient to neutralise endogenous oxalic acid to physiologically tolerable pH.

3.4. Nitrate reductase activities in foliage

Nitrate reductase activity was present in high amounts only in very young emerging leaf tissue. Nitrate- and nitrate plus ammonium-grown plants contained 2–4 times the nitrate reductase activity of the urea- and ammonium-grown plants (Table 7). The trend for nitrate reductase activity in

Table 7. Nitrate reductase activity in regrowth and in mature leaves of *Setaria sphacelata* provided with different nitrogen sources

	Nitrate reductase (μmol NO_2/g fresh wt/h)			
	Nitrate	Urea	Ammonium	Nitrate + ammonium
Expt 1				
5-day regrowth (very immature, emerging leaves)	7.3	5.3	—	12.6
7-day regrowth (immature, emerged leaves)	6.0	6.0	—	9.4
Yougest mature leaves (fully emerged leaves)	<0.001	<0.001	—	0.002
Expt 2				
5-day regrowth (very immature, emerging leaves)	11.5	4.0	2.5	14.0

regrowth tissue of the different treatments was the same as the trend for nitrate concentration in whole harvested tops (Tables 6 and 7). Enzyme activity was either very low or absent from mature leaves (Table 7).

4. Discussion

The data obtained in this study do not support the theory that cation excesses, and hence oxalate accumulation, in leaves of *S. sphacelata* are a consequence of potassium nitrate uptake into the leaves and subsequent reduction there of nitrate to organic nitrogen.[4,7] Plants cultured on sand grew equally well and accumulated similar concentrations of oxalic acid whether ammonium, urea or nitrate was the sole source of nitrogen and there was no evidence of a large conversion of ammonium or urea to nitrate prior to uptake by the roots. Had there been significant nitrification[12] of the urea and ammonium in their respective treatments, then the nitrate so formed would probably have been transported to the leaves and resulted in higher foliar levels of nitrate and nitrate reductase. In this study the nitrate plus ammonium treatment resulted in higher foliar levels of nitrate and nitrate reductase compared with the nitrate-only treatment even though the latter contained twice the nitrate concentration of the former. A plausible explanation of this effect is that assimilation (reduction) of nitrate within roots was suppressed by the presence of high concentrations of ammonium ions when the two forms of nitrogen were supplied simultaneously. In corn, nitrate uptake was apparently unaffected by equimolar concentrations of ammonium ion, but assimilation of this nitrate was decreased and more nitrate accumulated relative to the treatments containing no ammonium-N.[13] Other workers have interpreted suppressed assimilation of nitrate in the presence of ammonium as being a direct inhibition of nitrate reductase.[14,15] High foliar levels of total nitrogen and low levels of nitrate reductase activity in maturing leaves of *S. sphacelata* (particularly relevant in the case of nitrate-grown plants) implied that nitrogen reached these leaves in an already reduced form, possibly as amino acids.[16]

The continuing accumulation of oxalate in ageing leaves of *S. sphacelata* is not consistent with the concept of a cation excess resulting from nitrate reduction in the leaves. Total nitrogen is generally considered to decrease slightly as leaves age after reaching full expansion (e.g. ref. 13) and, although not measured in this study, our unpublished results have confirmed a decrease in total nitrogen per unit dry weight of leaf with increasing leaf age in *S. sphacelata*. Hence cations were accumulating even though nitrogen was not and it follows that these cations must have been accompanying some anion(s) other than nitrate.

A three- to four-fold higher plant growth rate with nitrate compared with ammonium-N was reported for *S. sphacelata* growing in sand and irrigated with nutrients containing single sources of nitrogen.[17] However, much lower concentrations of nitrogen (0.145–0.29 mM nitrate and 0.725–1.45 mM ammonium) were employed in that study and it seems probable from our experience that growth rate could have been limited by insufficient nitrogen. Equivalent growth rates have been reported for corn grown in liquid culture containing either ammonium- (7 mM) or nitrate- (7 mM) nitrogen[13] and in other species supplied either nitrate or ammonium as sole nitrogen source.[18,19]

Our inability to demonstrate a diurnal variation in oxalate concentration of *S. sphacelata* foliage is in direct contrast to the findings of Jones and Forde (1972) but is consistent with reports from other sources[20–22] of extremely low turnovers of accumulated organic acids in leaves.

Results in this paper suggest that no appreciable reduction in oxalate concentrations of *S. sphacelata* foliage should be expected when ammonium sulphate or urea fertilisation is substituted for nitrate fertilisation of pastures. Nor should it be assumed that potential oxalate toxicity can be avoided by allowing animals to graze pastures only at certain times of the day.

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Some Effects of Nitrogen Fertiliser on Winter Wheat

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Between 1966 and 1969, 44 experiments on winter wheat tested five rates of N applied as spring top dressings of NH_4NO_3. There was a marked seasonal variation in the response of grain yield to N with a large response in 1969 (mean optimum 97.5 kg/ha N) and a small response in 1968 (mean optimum 49.2 kg/ha N); the mean optimum N rate for all experiments was 73.1 kg/ha N. The response curves for N and grain yield were classified into 4 types; there were 22 sites with positive curvilinear relationships, 7 were indeterminate, 4 with positive linear and 11 with negative linear relationships. At several of the latter sites substantial yield losses occurred when N was applied.

The rate of N that would have been recommended using ADAS prediction methods based on previous cropping and manuring at each site was calculated. At 18 sites the rate of N recommended by prediction methods was within 40 kg/ha of the actual optimum obtained by experiment; at 29 sites it was within 50 kg/ha N but at 8 sites it differed by 75 kg/ha or more. The optimum grain yield obtained by experiment was compared with that obtained when the "recommended" rate of N was given; on 21 sites the difference in yield was less than 300 kg/ha but at 7 sites it was over 900 kg/ha. The results show that the ADAS prediction method accurately assessed the N status at almost half the sites overall. At 11 sites (25%) yield differences between the optimum and recommended N rate were greater than 600 kg/ha but if the results for 1969 were excluded because of the unusually high response to N, at only 5 sites (15%) were yield differences greater than 600 kg/ha.

Samples of soil from 17 sites were analysed by 8 methods to assess available N; in 1966 several methods showed promise but in 1967 no method was of value, when correlated with N response.

Tiller numbers were counted in 1968 and 1969; there was a strong tendency for more tillers to survive at high N rates. The effects of N on grain size were clear and consistent each year, the highest proportion of the large grain was obtained at low rates of N; each increment of N decreased grain size, irrespective of the optimal N rate.

1. Introduction

When crop rotations were followed with little variation in each locality "standard" rates of fertiliser could be applied to each crop. With the removal of former technical and statutory restraints imposed on crop rotations the sequence of agricultural crops may now be very varied and consequently large variations in the amount of N mineralised from soil reserves may be expected. In order to deal with the residual effects of a wide range of crops the Agricultural Development and Advisory Service (ADAS) of England and Wales developed a system using an "N Index" based on previous cropping because methods of analysis for "available" soil N were unreliable.[1,2]

The N Index is a five point scale 0–4 (0, low N residues; 4 high N residues) and incorporates the results of much investigational work coupled with advisory experience.[1,2] For example for grassland, the residual effects have been shown to be related to the period under grass, the grass species,

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the legume content of the sward, N manuring and whether cut or grazed.[9–12] To determine the N Index the first step is to distinguish between arable, grass/arable and ploughed out permanent pasture; for each of these three cropping systems the N Index based on previous cropping and manuring can be determined (pages 10–12 MAFF Bulletin 209, 1973).[2] For example, for arable cropping systems where the previous crop is the second or subsequent cereal, N Index 0 is given, after a one year clover crop, N Index 2. The rate of N fertiliser recommended is then obtained from a series of tables for each crop which takes into account summer and winter rainfall, soil texture and soil depth. For winter wheat grown in areas where the summer rainfall is less than 400 mm the recommended N rate varies from nil for N Index 4 to 175 kg/ha for N Index 0 on sandy loam soils less than 23 cm deep.

The objectives of these experiments were to investigate the response of winter wheat to N fertiliser, to relate the results to the soil and field conditions at each site and to assess the validity of the N index system as a basis for N recommendations.

2. Experimental

2.1. Sites

Forty-four experiments were done on arable and grass-arable farms in central southern England, 12 in 1966, 11 in 1967, 11 in 1968, and 10 in 1969. On each farm, one field of winter wheat was chosen, an even area of crop selected during winter or early spring and the soil profile carefully examined to locate the experimental site on as uniform a soil as possible. Occasionally two or three experiments were sited on the same farm or estate.[3–6]

The soils at most sites were derived from Cretaceous or Eocene Deposits; surface soils ranged from loamy coarse sand to clay in texture, with silty loam and silt loam predominating. All but three sites had a pH value of 6.5 or higher. The level of "available" nutrients (extracted with 0.5 M $NaHCO_3$ for P and M NH_4NO_3 for K)[2] was determined and information obtained on the amount of P and K fertiliser applied by the farmer the previous autumn; at no site were yields expected to be limited by inadequate supplies of these elements.

The variety grown was Cappelle–Desprez on all sites in 1966, five sites in 1967, seven in 1968 and five in 1969. Maris Widgeon was grown on four sites in 1967, two in 1968; Champlein on two sites in 1967 and one in 1968; and Joss Cambier on one site in 1968 and five in 1969.

2.2. Design

Five levels of nitrogen were fully randomised in each of four blocks. The rates applied to each site were related to the soil nitrogen reserves predicted from previous cropping and soil data.[1] Where low nitrogen reserves were predicted a higher range of N rates was applied, e.g. 75, 100, 125, 150, 175 kg/ha N, where the soil reserves were expected to be high, lower rates were applied, e.g. 0, 25, 50, 75, 125 kg/ha N. This technique gave a narrower range of treatments around the predicted optimum, with possibly greater sensitivity, compared to selecting uniform rates for all sites. Each plot was 22.8 m long by 4.6 m wide in 1966 and 1967 and 4.9 m wide in 1968 and 1969.

2.3. Treatments

In 1966 and 1967 a granular ammonium nitrate–lime fertiliser (21% N) and in 1968, and 1969 prilled ammonium nitrate (34.5% N) was applied by hand in spring to wheat drilled the previous autumn. No allowance was made for nitrogen fertiliser applied by the farmer in the autumn seedbed; on average this amounted to 19 kg/ha N. The treatment fertiliser was applied as a single application in late April or early May except where the crop was considered to be at risk from take-all disease or where high rates were to be used, in which cases part of the application, 50 kg/ha N, was applied in March over the whole site and the rest in late April or early May.

2.4. Records

At each site two samples of soil, each made up of 20–25 cores, were taken at 0–15 cm and 15 to 30 cm depth in winter or early spring prior to the application of treatments. Records were made

each season of soil temperature, rainfall, incidence of pests and diseases and in 1968 and 1969 only, of tiller numbers; the central part of each plot was harvested by combine harvester and the weight of grain recorded. Sub-samples of grain from each plot were taken for determination of moisture content and for sieving and other tests. Climatic, soil and cropping data from each site and the N rate and crop records for each treatment are recorded in the South-eastern Regional Annual Reports on Experiments.[3-6]

3. Results

3.1. Weather

In 1966, spring was wet and soil temperature, although above average in March, was below average in mid-April; crop growth was retarded. In 1967, early spring was mild, followed by cooler conditions in late spring. In 1968, spring was wet and soil temperatures were about average. In 1969, soil temperatures in March and April were as much as 2.5°C below average and crop growth much retarded.

The harvest period was slightly wetter than average in 1966; in 1967 the early harvest period was dry but the later part wet. Severe lodging was widespread in 1968 and many crops were laid by heavy rain in late June and early July. At site 5 all plots were virtually flattened to within a few inches of the ground by late June. At other sites the degree of lodging was directly related to the level of nitrogen applied and by the end of July plots that had received the highest rate of nitrogen were badly laid except at sites 3 and 12 where the crop on all plots was standing well, even at harvest. In 1968 the weather during harvest was very unsettled with fine spells of a few days' duration between periods of heavy rain; in 1969 the harvest period was relatively dry and fine.

3.2. Weeds, crop diseases and crop pests

On several sites each year, take-all disease *Gaeumannomyces graminis* was seen but the effects on the crop were not severe; no relationships between the incidence of the disease and nitrogen treatment were noted, though detailed examination of the crop roots was not possible. Several foliar diseases, including mildew, *Erysiphe graminis*, glume blotch, *Septoria nodorum* and yellow rust, *Puccinia striiformis* were seen each year but usually only at a low level of infection. The incidence of yellow rust was higher in 1967 and observations showed that apart from sites where the attack was slight, the incidence was more severe at high rates of nitrogen. On several sites in 1966, 1968 and 1969 yellow rust was seen but the level of infection was low and bore no apparent relation to the level of nitrogen applied. Slight attacks of mildew were seen at a few sites each year and on these sites where it occurred in 1969, the incidence was greater on plots receiving higher rates of nitrogen. Loose smut, *Ustilago nuda*, was widespread in 1969 and was recorded on all but three sites; no relation was observed between nitrogen rate and the proportion of heads affected.

Significant pest attacks were few; aphids were seen on two sites in 1968 with a high number on site 12; in spring 1969 there was a slight attack by leatherjackets *Tipula paludosa* at site 2.

3.3. Tiller numbers

In 1968 and 1969 the number of tillers per unit length of drill or per unit area were counted on four occasions.[5,6] In 1968, the first count was made in early June about four weeks after the nitrogen treatments had been applied, the second in late June, the third in late July or early August, and the last in mid-August, just before harvest. At the time of the first tiller count, plots receiving the highest rate of N had on average, 6.8 more tillers per 30 cm length of drill than plots receiving the lowest rate of N. These figures refer only to crops with drills 18 cm apart; broadcast or narrow drilled crops were excluded from these comparisons. The effect of nitrogen was maintained during the season and at harvest there were on average 33% more tillers on plots receiving the highest rate of N than on those receiving the lowest rate.

In 1969, tillers were counted when the N treatments were applied and afterwards at intervals of 3–4 weeks, the last at the end of July/early August. At the first count, three lengths of drill 30 cm were marked with locating canes and subsequent counts made in the same positions. The

effects of nitrogen were similar to the previous year; just before harvest there were on average over the ten sites, 23.5 tillers per 30 cm of drill on the low N plots, and 31.6 tillers on the high N plots.

3.4. Yields (t/ha at 15% moisture content)

The maximum yield obtained at each site (Table 1) showed considerable variation; there were also considerable differences in the mean yield per year. The highest mean maximum yield, 7.17 t/ha, was obtained in 1969 which was the sunniest, driest summer (Tables 2(a) and 2(b)) the lowest being 4.52 t/ha in 1968 which had least summer sunshine.

Some of the differences in yield between sites could be due to the management of the crop because the experiments were mostly done on different farms each year. This influence was not expected to be large because sites were selected on uniform areas of crop and soil, where careful examination did not reveal any adverse features such as soil compaction or poor drainage. The greater part of the variation in yield between sites was thus likely to be due to the inherent characteristics of the site and soil.

Table 1. Maximum yield (t/ha), optimum N rate (kg/ha) and type of response curve (obtained at each site)

	1966		1967		1968		1969	
Site	Yield	N rate	Yield	N rate	Yield	N rate	Yield	N rate
1	5.56	63 (A)	3.84	118 (B)	4.69	0 (D)	7.03	115 (A)
2	—	—	5.94	80 (A)	4.97	0 (D)	6.75	73 (A)
3	4.83	0 (D)	5.45	56 (A)	4.93	92 (A)	7.87	38 (A)
4	—	—	6.12	0 (D)	4.57	94 (B)	5.84	102 (A)
5	4.99	100 (A)	4.71	0 (D)	4.28	0 (B)	7.07	134 (A)
6	4.94	55 (A)	7.08[a]	0[a](D)	—	—	5.85	176 (C)
7	5.27	33 (D)	5.91	108 (A)	4.61	0 (D)	8.92[a]	167[a](C)
8	4.52	125 (B)	4.30	75 (A)	4.73	74 (A)	8.48	63 (A)
9	5.16	0 (B)	5.97	133 (A)	4.43	127 (A)	8.53	133 (A)
10	4.97[a]	195[a](C)	5.41	102 (A)	4.07	0 (D)	5.32	108 (B)
11	4.38	72 (A)	5.91	120 (A)	3.83[a]	45[a](D)	—	—
12	5.55[a]	0[a](D)	—	—	4.58	109 (A)	—	—
13	4.76	146 (C)	—	—	—	—	—	—
14	6.02	117 (B)	—	—	—	—	—	—
Mean	5.08	75.5	5.51	72.0	4.52	49.2	7.17	97.5

[a] Indicates extrapolated values obtained outside the range of the experimental treatments.

The figures were obtained by quadratic calculation by the Statistics Department, Rothamsted Experimental Station and checked by graphical presentation; the values for sites 1, 8 and 9 in 1966 and sites 3 and 6 in 1969 were obtained graphically.

Type of response curve: A, normal, positive curvilinear; B, indeterminate; C, positive linear; D, negative linear.

Table 2(a). Weather: 1 May to 31 July 1966–69 (Central Hampshire)

	Sunshine (h)	Rainfall (mm)	Potential evapotranspiration (mm)	Soil moisture deficit (mm)
1966	630.2	213	262	49
1967	627.2	221	270	49
1968	494.5	220	248	28
1969	697.9	183	284	101

Table 2(b). Weather: Previous winter rainfall Oct.–March 1965/66–1968/69, and summer rainfall April–Sept. 1966–69 (mm)

	Oxford		Southampton	
	Previous winter	Summer	Previous winter	Summer
1966	271	404	501	411
1967	367	382	515	408
1968	319	520	457	514
1969	348	308	489	287
20 year average	335	317	461	342

3.5. Effect of nitrogen treatments

The effect of N on grain yield was variable and at many sites substantial, ranging from strongly positive to strongly negative effects. From the response curve obtained at each site the optimum rate of N and the maximum yield were obtained (Table 1). The N optima ranged from nil at 11 sites to 125 kg/ha N or more at 9 sites. The response curves were grouped into 4 types (Table 1) illustrated in Figure 1: A, "normal" positive curvilinear with a distinct positive effect at lower rates of

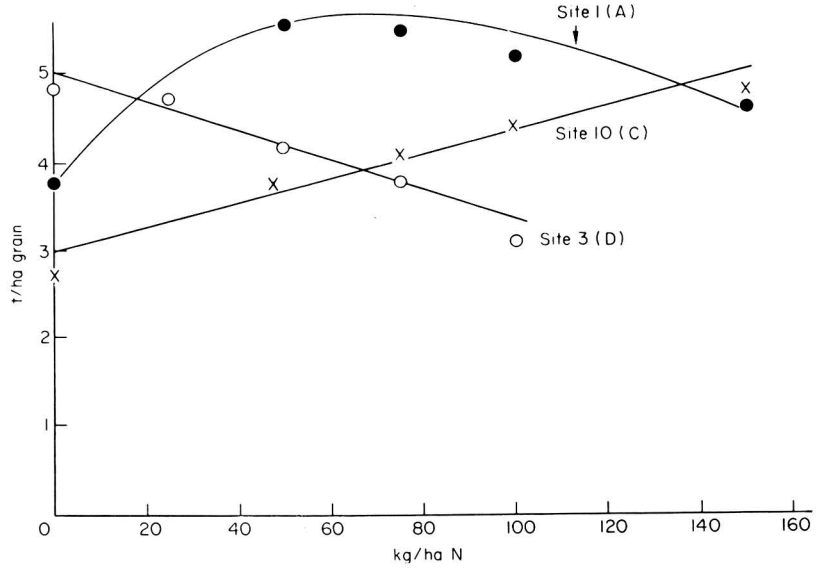

Figure 1. Effect of N on yields of winter wheat 1966. Type of response curve: A, positive curvilinear; C, positive linear; D, negative linear.

application either with a plateau or decline in yield at higher rates; B, an indeterminate relationship or with a shallow response curve; C, a positive linear relationship and D, a negative linear relationship.

There were 22 sites with "normal" response curves; at these sites the optimum N rate ranged from 38 to 134 kg/ha, the mean optima for the 4 years 1966–69 were 73, 97, 100 and 94 kg/ha N

respectively and the overall optimum was 92 kg/ha N. There were 4 sites showing a positive linear relationship with a mean optimum rate of 171 kg/ha N; on 3 of these sites the optimum was at or beyond the highest rate tested. At 11 sites with linear negative relationships, the optimum N rate was nil on 9 sites and at the other 2 sites the mean optimum rate was 39 kg/ha N. In the latter group substantial losses occurred at several sites when N was applied. On the 9 sites where the optimum was nil, the average loss in yield was 0.95 t/ha when 100 kg/ha N were applied; at the 3 sites where the losses were greatest, the average loss was 1.5 t/ha grain.

3.6. Comparison of predicted and actual optimum N rate

The N index for each site was assessed (see Introduction) and the N recommended for winter wheat i.e. the predicted optimum rate, was estimated (page 30 MAFF Bulletin 209).[2] No allowance was made for the effects of rainfall the previous winter because there was not a large or consistent variation from average (Table 2(b)). The N index, recommended N rate and the yield obtained at the recommended N rate are given in Table 3. The N indices ranged from 0 to 3, the rates of N recommended, from 0 to 125 kg/ha N with a mean of 68 kg/ha.

Table 3. N Index, N recommended (kg/ha) and yield at recommended N rate (t/ha)

	1966			1967			1968			1969		
Site	N Index	N Rec.	Yield	N Index	N Rec.	Yield	N Index	N Rec.	Yield	N Index	N Rec.	Yield
1	2	63	5.56	1	75	3.84	3	38	4.17	2	63	6.31
2	—	—	—	3	38	5.65	2	50	4.42	3	0	5.43
3	3	38	4.46	2	63	5.36	2	63	4.78	3	38	7.87
4	—	—	—	3	38	5.66	2	50	4.43	1	63	5.61
5	2	50	4.64	2	50	4.17	1½	75	4.22	1	63	6.29
6	1	88	4.64	1	75	5.56	—	—	—	0	125	5.65
7	2	38	5.20	2	50	5.53	2	63	3.89	1½	75	7.59
8	0	100	4.28	0	125	4.04	2	75	4.73	3	0	7.91
9	3	38	5.07	1	63	5.05	0	125	4.43	2	50	7.30
10	0	100	4.41	1	63	5.15	0	125	2.59	3	38	4.39
11	2	63	4.34	1	75	5.31	0	125	3.53			
12	0	100	5.15				2	63	4.32			
13	0	125	4.57									
14	0	125	6.00									

In the following discussion, the expressions "predicted optimum" or "recommended N rate" refer to the N rate recommended using the N Index system based on previous cropping etc.; the "optimum" N rate or yield refers to that obtained by experiment from the apogee of the response curve and does not take into account the cost of the fertiliser or the value of the crop.

The differences between the optimum N rate obtained in each experiment and the rate of N recommended by prediction methods were obtained from Table 1 and Table 3. The recommended (predicted) rate of N was within 40 kg/ha (+ or −) of the actual optimum obtained at 18 of the 44 sites (9 out of 12 sites in 1966) and within 50 kg/ha (+ or −) at 29 sites (66%). The average underestimation of the predicted optimum N rate (i.e. soil N residues overestimated) and the average overestimation of the predicted optimum are given below for the four years:

	(kg/ha N)			
	1966	1967	1968	1969
Underestimation	−40 (5 sites)	−49 (7 sites)	−30 (4 sites)	−67 (9 sites)
Overestimation	+38 (6 sites)	+43 (4 sites)	+61 (7 sites)	—
No difference	1 site	—	—	1 site

There was a considerable underestimation of the optimum N in 1969; this appeared to be due to the growing conditions within the season, conducive to high yields, rather than to the leaching of N residues from the soil the previous winter, since the rainfall was only slightly greater (+5%) than average (Table 2(b)). The marked seasonal variation in the response to N illustrates the dangers of drawing long term conclusions from N experiments of one or two years' duration.

The above comparisons tend to exaggerate the difference between the actual and predicted optimum because they take no account of the shape of the response curve between the two points. The difference between the optimum yield and the yield at the recommended N rate was calculated for each site from the data in Table 1 and Table 3; the results are presented in Table 4. On 21 sites (50%)

Table 4. Comparison of yields obtained at recommended N rate with the optimum yield

	Range of variation (kg/ha)							
	0–300		301–600		601–900		Over 900	
	No. of sites	Mean yield	No. of sites	Mean yield	No. of sites	Mean yield	No. of sites	Mean yield
1966	7	93	5	399	0	—	0	—
1967	5	181	3	460	1	602	2	1223
1968	6	92	3	460	1	715	1	1481
1969	3	142	1	577	2	747	4	1202
Overall	21	121	12	444	4	703	7	1248

the difference in grain yield was less than 300 kg/ha, at 33 sites it was less than 600 kg/ha, but at 7 sites the difference was over 900 kg/ha and at 5 sites over 1200 kg/ha. The larger differences occurred in 1969 which, as already discussed, produced high yields. Prediction of the recommended N was most successful in 1966 and least successful in 1967 and 1969. In order to test the effectiveness of varying the N rate by prediction methods, the yield obtained by applying a standard rate of 75 or 100 kg/ha N at each site was calculated from the response curve and compared with the optimum yield. The mean difference in yield for each year (Table 5) showed that prediction methods gave

Table 5. Comparison of yields obtained at the recommended (predicted, N rate, and at standard rates of 75 and 100 kg/ha N with the optimum yield

	Average yield difference (kg/ha) between optimum and:		
	Recommended N rate	75 kg/ha N	100 kg/ha N
1966	221	307	528
1967	533	435	437
1968	413	448	458
1969	730	434	261
Mean	462	402	429

higher yields in 1966 and 1968, but a standard rate of 100 kg/ha N would have given higher mean yields of 97 kg/ha in 1967 and 469 kg/ha in 1969.

To assess the effectiveness of the N index as a guide to N responsiveness, the average optima

for each N index were calculated from the figures in Table 3. The average optimum N rate for sites assessed as Index 0 was 101 kg/ha; for those Index 1 (and 1½), 93.1 kg/ha; Index 2, 69.9 kg/ha and Index 3, 40.2 kg/ha. Clearly on average, the N index assessment has given an effective separation of sites into different classes of responsiveness to N, but inspection of the data for individual sites shows that there are several anomalous situations. At several sites the N index assessment underestimated the amount of N available for the crop. At two sites assessed as Index 0, site 12 in 1966 and site 10 in 1968, there was no response to N and the losses in yield when the recommended rate was applied were 402 and 1481 kg/ha respectively. At site 12 in 1966 the response curve showed a negative linear relationship; the slope was not steep with the lowest rate of N tested, 50 kg/ha producing 5.36 t/ha grain and the highest rate 175 kg/ha producing 4.62 t/ha. The crops grown in the previous 3 years were barley, winter wheat and barley and soil analysis did not reveal any unusual feature. At site 10 in 1968 other data confirm the relatively high amount of N available to the crop—the nitrate content of the soil, the high degree of lodging of the crop at harvest, the high proportion of small grains, and the high nitrogen content of the grain.[5] However, the crops grown in the 4 years prior to the wheat, 2 years Italian ryegrass grown for seed and 2 years barley do not account for the high reserves of available N.

In 1967 site 6 (N Index 1) and site 5 (N Index 2) did not respond to N; at both sites previous cropping and manuring, and soil analysis, gave no indication of the high reserves of N in the soil.

The N index assessment overestimated the amount of N available at 25 sites, and at 8 of these sites more than 600 kg/ha additional grain was obtained at the optimum N rate; all but two of these sites were in 1969. For example, at site 9 in 1967, with an N Index 1, 63 kg/ha N were recommended producing a yield of 5.05 t/ha; the optimum yield of 5.97 t/ha was obtained using 133 kg/ha N showing that Index 0 would have been a more appropriate assessment (previous cropping one year ryegrass ley after 2 barley crops).

3.7. Grain size

A sample of moisture-free grain from each plot was shaken on a nest of 3 sieves with slots 20 × 3.25 mm, 20 × 2.80 mm and 20 × 2.40 mm; the weight of grain retained on each was recorded.[3–6] The effects of nitrogen on grain size were clear and consistent; the highest proportion of plump grain was obtained at almost every site by using the lowest rate of N. This effect was independent of the optimum rate of N for grain yield; each increment of N applied further decreased the size of grain. All varieties were similarly affected. Within each season the proportion of grain greater than 2.8 mm varied considerably between sites but there did not appear to be any relationship between grain size and yield, optimum N rate, variety or soil type. There were small differences between seasons; 1969 and 1967 produced the most plump grain, 1968 the smallest grain.

3.8. Prediction by soil analysis

Samples of soil from 8 sites in 1966 and 9 sites in 1967 were analysed by a number of methods as part of an extensive national investigation into the prediction of N response by soil analysis. The methods used and the correlation coefficients between the amount of available N determined by each method and the optimum N rate found at each site are given in Table 6. The figures illustrate some of the problems arising largely from variations in seasonal responses to N: for example a method showing promise in 1966, anaerobic incubation for 14 days at 30°C[7] was of little value in 1967. When data from these and other experiments in the investigation were examined the conclusions were "that none of the methods reliably predicted the response of cereals to N".[8] Nevertheless, despite the unpromising results, soil analysis may be of value in situations where residual effects of previous cropping are difficult to evaluate or where no information on previous cropping or N responsiveness are available.

4. Discussion

There was clear evidence at many sites of a decline in yield when more than the optimum amount of N was applied. At many sites where this occurred lodging of the crop was not a significant

Table 6. Predictions of N response by soil analysis

	Correlation coefficients; method *vs* optimum N rate 1966	1967
1. $(NH_4 + NO_3)$-N on air dried soil	−0.769	−0.061
2. NO_3-N on air dried soil	−0.579	−0.133
3. NH_4-N production by anaerobic incubation for 7 days at 30°C[7]	−0.816	—
4. As 3 but for 14 days at 30°C	−0.892	−0 047
5. NO_3-N production by aerobic incubation[24]	−0.201	+0.309
6. N soluble in hot water, 30 min boiling	−0.557	+0.070
7. Active-carbon determination $(Ba(OH)_2)$[25]	−0.826	+0.185
8. $(NH_4 + NO_3)$-N produced on aerobic incubation with sand for 14 days at 30°C	—	−0.048

problem. The physiological mechanism of this decline in yield without lodging, appeared to be related to the effect of N on vegetative growth. Under the prevailing climate the higher numbers of mature tillers which survive at higher N rates led to a higher number of heads which in turn produced small grains, apparently limited in size by an insufficient supply of carbohydrate possibly as a result of shading or of moisture stress. Because the sunniest summer, 1969, produced the plumpest grain, the phenomenon may be influenced partly by sunshine and its affect on photosynthesis. In Australia Dann[16] also found that N, by increasing tiller numbers, could have adverse effects on yields of wheat but under those drier conditions moisture was the limitation to growth of additional tillers leading to the "haying off" of the crop, and to the production of small grain. The significance of N manuring on tiller numbers has also been shown by experiments on barley in northern Scotland in 1969 and 1970, where there was a close relationship between tiller number in May, the yield of plots receiving no nitrogen and the yield increase due to an additional 50 kg/ha N.[17] However, seasonal variations in these relationships occur; in a comprehensive study of the growth of wheat at Cambridge, UK, it was found that while various components of yield can be affected by N, the response of each component to N was not necessarily consistent,[18] and that the response of grain yield to N dressings did not apparently bear any consistent relationship to the response of any individual components of yield. These findings are also in accord with observations in northern Scotland on barley that while the relationship between tiller number and yield was consistent within a field, this did not necessarily apply between different fields.[19] In Montana, USA, Black[20] found that in spring wheat numbers of heads per unit area accounted for 97% of the yield variance associated with N and P treatments, and were linearly related to both adventitious roots and to tillers per plant. Measurements of the effects of N treatment on plant components as well as on yields would appear to be essential if adequate interpretation of the results of N manuring experiments is to be made.

Because of the risks of yield loss occurring when more than the optimum amount of N is applied to winter wheat, any assessment of soil N status should be able to identify situations where soil N reserves are high and where a small response to N is expected. Of the 11 sites where the optimum N rate found by experimentation was nil 3 were assessed N index 0 or 1 and when the recommended N rate was applied (on average 100 kg/ha N) substantial losses averaging 1137 kg/ha occurred. Clearly at these 3 sites previous cropping was not a good guide to soil N reserves; at one, site 10, 1968 soil analysis in spring showed a relatively high level of nitrate reflecting its higher N reserves but at the other two, soil analyses did not show high levels of available N.

The effects of overestimating the N status and thereby recommending less fertiliser N than the optimum were seen frequently. This occurred at all but one site in 1969, though the average yield was high when the N recommended from the N Index system was applied. In 1969 Fiddian[21] also found a high response to N in winter wheat variety trials. Apart from 1969, the N recommended

was less than the optimum at 16 out of 34 sites but at only two sites, both in 1967, was the difference in yield greater than 600 kg/ha. At these sites, both assessed N Index 1, the difficulty was to evaluate the effects of a one year grass ley grown either 1 or 2 years prior to the winter wheat.

When using the N Index system, the effects of grassland present difficulties. After old pasture is ploughed the N effects on arable crops may last for at least 15 years.[22] It is not always possible to obtain cropping records for such a period and in the case of a farm changing ownership or tenancy, few records of past cropping may be available. Clearly accurate information on the type of grass ley and its management is required if the N Index system is to be used as a basis for estimating the N requirements of subsequent crops. In general, the N mineralised on ploughing out grassland is positively related to the amount of N that has built up but has also been shown to depend on the N content of the plant debris[13] and on its C/N ratio.[14]

Another approach used by Lessells and Webber[15] to identify N responsiveness in cereals involves previous measurement of yield in nil N plots. They found that sites with a low yield potential without N were more responsive to N, and they graded sites into 3 groups: those where the yield of the nil N plots was below 3.76 t/ha, between 3.76 and 5.02 t/ha and over 5.02 t/ha. In the 4 years 1959–62 they found average responses to 75/100 kg/ha N of 1644, 753 and 25 kg/ha grain in the 3 groups respectively. The responses to N obtained in the present experiments have been grouped in the same way and the respective responses to 75 kg/ha N were 1094, 551 and 511 kg/ha. While the overall pattern was similar the averages concealed the fact that in 1969, at many sites where the yield without N was over 5.0 t/ha, there were large responses to N and also that in 1968, sites where the yield without N was 3.76–5.02 t/ha there was on average a negative response of −375 kg/ha to 75 kg/ha N. Despite the limitation that Lessells and Webber's grouping requires prior knowledge of the yield, this approach may have some merit in adjusting N recommendations on soils well known for their ability to grow high yields of wheat, e.g. the Wantage soil series.[23]

For any method to be acceptable as a sound basis for making N fertiliser recommendations two criteria must be satisfied. Firstly, variations from a uniform rate of N applied to a particular crop must be shown to be of benefit either in economy of fertiliser or in increased yields; secondly, features influencing the N recommendations must be capable of accurate identification, e.g. previous cropping, soil properties and climate. These experiments done over 4 years in south-east England have shown that the ADAS N Index system for predicting the N requirements of winter wheat has partially satisfied both these criteria. There was a strong seasonal influence on the response to N; prediction of the optimum was most successful in 1966 which had near average weather, and was least successful in 1969, the sunniest summer. However, no prediction method is ever likely to be able to take account of weather in the latter part of the growing season.

If the results of 1969 were excluded, the difference in yield between that obtained at the optimum N rate and that at the rate of N recommended using the ADAS N Index system was less than 600 kg/ha at 29 out of 34 sites.

Several anomalous situations were found where the N Index system was unsatisfactory and improvements or the incorporation of supplementary information are required if the system is to realise its potential. It is particularly important to avoid underestimating N residues because substantial losses in yield occurred at several sites where more than the optimum rate of N fertiliser was applied; these losses were greater than those at sites where N residues had been overestimated and less than the optimum amount of fertiliser N had been applied.

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Agriculture Group Symposium
Soil Physical Conditions in Relation to Crop Growth

The following are summaries of papers presented at a symposium held at the ARC Letcombe Laboratory, Wantage OX12 9JT on 14 October 1975. The reports so published are entirely the responsibility of the authors and in no way reflect the views of the Editorial Board of the Journal of the Science of Food and Agriculture.

Plant Physiological Background: Root structure in Relation to Absorption; Effects of Nutrient Gradients; Mechanical Impedance

D. T. Clarkson

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A number of features in the soil environment can modify the form of roots and the depths to which roots can penetrate. Of the chemical factors, ionic content, the gases in the soil atmosphere were selected for discussion. The local accumulation of fertiliser can result in the proliferation of roots in that zone—in certain circumstances this may encourage superficial rooting thus leaving the crop with many roots at hazard if the surface soil dries out. In wet conditions there is evidence to suggest that root extension and branching can be modified by ethylene released by microorganisms in anaerobic zones of the soil. Other aspects of waterlogging were discussed. The physical resistance offered by the soil can increase markedly when the soil is compacted by the passage of heavy implements or if the soil is cultivated in unfavourable, wet conditions. The extensive growth of cereal roots can be drastically changed by relatively small increases in the resistance of the soil matrix to radial and longitudinal expansion of cells in the growing zone of the root. Experimental approaches to the study of this question were illustrated.

The consequences of altered root form on the nutrition of the plant can be assessed completely when the chemical and physical characteristics of the soil are considered in relation to the inherent capacity of roots of different anatomical development and branching order to absorb nutrients and translocate them to the shoots. Results were presented which suggest that a far greater proportion of the total root system is active in this respect than had been thought formerly.

Drainage and Anaerobiosis: Lysimeter System to Investigate the Effects of Short-term Waterlogging on Crop Growth

R. Q. Cannell

ARC Letcombe Laboratory, Wantage, OX12 9JT

A survey of the drainage need in England and Wales showed that 26% of the total agricultural area of 11 million ha required drainage, and a further 20% would need at least partial replacement. The variation in cost between different drainage systems and rising prices have emphasised the need

for more information on crop response to drainage. In general the length of time for which the water-table remains close to the soil surface in wet periods is reduced by more intensive, and therefore more expensive drainage systems.

Most published information on the effects of depth of the water-table on crop growth relates to water-tables kept at relatively constant depth throughout the growth of the crop. There is little information on effects of transient waterlogging. Study of this in the field is difficult for several reasons, especially because seasonal variations in rainfall can complicate interpretation of results.

A lysimeter system to study effects of short-term waterlogging has been developed in conjunction with the Field Drainage Experimental Unit of ADAS. With this it is possible to impose predetermined regimes of rainfall and water-table, which are within the range experienced in southern England. Sixty-four lysimeters (80 cm diam; 135 cm deep) containing undisturbed monoliths of two contrasting soils have been installed in a concrete sub-structure, and a well ventilated mobile glasshouse fitted with traversing irrigation system enables rainfall to be simulated; in fine weather the house can be drawn off the area. Progress in a preliminary experiment was outlined and some preliminary results were discussed.

Drainage and Anaerobiosis: Physiological Aspects of the Responses of Crops to Waterlogging

M. B. Jackson

ARC Letcombe Laboratory, Wantage, OX12 9JT

A wide range of responses are induced when crop plants are subjected to anaerobic conditions resulting from waterlogging of the soil. Effects on the growth of roots and shoots can be rapid and complex. The magnitude of the damage to overall growth and yield depends on many factors and these include the duration of waterlogging, the stage of development of the crop and also soil type. The aim of current work is to investigate mechanisms by which the performance of waterlogged plants is impaired.

Many physiological changes can be attributable to the initial effects of low concentrations of oxygen on the root system. However, damage may also result from the presence of toxic substances such as ethylene and volatile fatty acids which arise as products of anaerobic metabolism by soil microorganisms.

Some of the more rapid responses are associated with interference in the production of plant hormones by the roots. These interact with hormones produced in the shoots and influence both the pattern and the extent of growth by the whole plant. Waterlogging may also rapidly reduce the ability of roots to absorb water. The extent of any subsequent water-stress in the shoots depends on hormonal and other mechanisms controlling stomatal aperture.

Recent work has confirmed reports in the literature indicating that treatments with certain plant hormones or with nitrogenous fertilisers can reduce certain kinds of damage caused by waterlogging. The implications of these observations remain to be determined.

Reduced Cultivation: Objectives and Results of Field Experiments

F. B. Ellis

ARC Letcombe Laboratory, Wantage, OX12 9JT

Emphasis will be given in the paper to direct drilling since this is the opposite end of the spectrum to ploughing with regard to the degree of soil disturbance. Obviously, there are wide variations between these two extremes and in some practical situations these may be of greater relevance.

On commercial farms more than 43 000 ha of cereals were established in 1974 by direct drilling. Particular interest is attached to the advantages of "timeliness" of operation for establishing crops and therefore greatest interest has been attached to winter wheat, oilseed rape, kale and fodder rape.

Current research at Letcombe is directed largely to investigating the range of soils to which direct drilling is applicable and whether or not it may be practised for a prolonged period in the same area of land without adverse affect upon the soil.

The technique was originally recommended for well structured and drained soils which were in a good state of husbandry and the results of the experiments carried out jointly by Letcombe and the Weed Research Organisation, together with work elsewhere, indicate that although direct drilling results in greater compaction of the soil and early root growth of plants may be restricted, the yield of grain has not usually been significantly affected. There was some evidence that where directly drilling had been repeated in successive seasons the earthworm population increased and there was an apparent improvement in soil structure. These results therefore suggested the advisability of investigating the long term consequences of direct drilling (*ca* 10 years) on crop growth especially on soils which are subject to structural weakness or excessive wetness during the winter months.

Experiments on three sites which were commenced by Letcombe during the past 18 months have not shown consistent treatment differences between direct drilling, shallow tine cultivation and ploughing. They have, however, pointed to the importance of the interaction of crop residues on the establishment of the following crop and crop response to nitrogen at different times of application.

Soil Nitrogen: Field and Lysimeter Studies

J. R. Burford

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The application of N fertiliser to cereals and pastures at high rates (up to 100 and 400 kg N/ha/year respectively) yields economic returns in the UK but the increased nitrogen content of the herbage is seldom more than three-quarters of that applied and often may be less than one-half, especially with autumn applications. The fate of the remaining nitrogen is not well known; the aim of the work at Letcombe is to obtain information on this, particularly the significance of losses by leaching and denitrification.

Studies of the composition of the soil atmosphere have shown that, in wet soils, evolution of nitrous oxide can take place to a depth of at least 90 cm and can continue for 2–6 months in winter and spring. Mean nitrous oxide concentrations often exceed 100 parts/10^6 (v/v), but in the absence of adequate information on the rate of gas diffusion, the quantities of nitrogen lost cannot yet be estimated.

Field experiments with cereals suggest that reduced cultivation, e.g. direct drilling, may sometimes increase N_2O concentrations in the soil atmosphere, and also cause soil N to be more slowly mineralised.

More detailed studies of the fate of nitrogen applied to grassland have been made using intact monoliths of a fine sandy loam (Rowland series) equipped with tension drainage systems. Nitrogen-15 labelled fertiliser is used to enable the fate (absorption by plants, leaching, denitrification, or conversion to slowly available forms in soil) to be studied with much greater precision than is possible in field experiments. Early results were presented.

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